
**Neurotrophic factor regulation of gene expression in
primary sensory neurons of the mouse**

**A thesis submitted to Cardiff University
for the degree of
Doctor of Philosophy (PhD)**

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2007**

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Abstract

In this study, the expression of several sensory neuron specific/predominant genes, and the effects of neurotrophic factors upon them were studied in embryonic, postnatal and adult mouse sensory neurons.

In the embryonic mouse, NGF/TrkA signalling was shown to be essential for the expression of mRNAs encoding substance P and the sodium channels Nav1.8 and Nav1.9 in DRG and trigeminal ganglia. Differential regulation of the two isoforms of calcitonin gene related peptide (CGRP) mRNA was apparent in the DRG with a requirement of NGF/TrkA signalling for expression of α , but not β CGRP. This was not reflected in the trigeminal ganglia.

Postnatally, experiments revealed that NGF/TrkA signalling within the DRG and trigeminal ganglia is 1) essential for expression of SP, α CGRP, β CGRP, Nav1.8, Nav1.9 mRNAs, 2) possibly required for expression of the neuropeptide galanin and the capsaicin receptor vanilloid receptor 1 (VR1) mRNAs, 3) not required for pituitary adenylate cyclase-activating peptide (PACAP) mRNA. Conversely, within the nodose ganglia, expression of Nav1.8 and Nav1.9 mRNAs did not require NGF/TrkA signalling. No regulation of all aforementioned genes by neurotrophin-3 (NT-3) was observed in trigeminal, nodose or dorsal root ganglia.

In the adult mouse, DRG cultures were utilised to study gene regulation by the neurotrophic factors NGF, artemin and macrophage stimulating protein (MSP). Expression of SP, α CGRP, β CGRP, Nav1.8, Nav1.9 and VR1 mRNAs all showed a decrease following 96 hours in culture that was inhibited by presence of MSP (50ng/ml), NGF (10ng/ml) or artemin (10ng/ml). PACAP, galanin, damage induced neuronal endopeptidase (DINE) and activating transcription factor 3 (ATF3) mRNAs increased over time, but neurotrophic factors could impede such increases. No axotomy or neurotrophic factor-induced effects were observed for P2X3, Nav1.6 or Nav1.7 mRNAs. Interestingly the additional presence of leukaemia inhibitory factor (LIF) opposed NGF, MSP and artemin-induced effects on β CGRP, SP, VR1 and galanin mRNAs, whilst enhancing effects on PACAP and DINE transcripts.

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Chapter 1

General Introduction

This thesis concerns aspects of the development of the peripheral nervous system (PNS) and the regulation of gene expression in developing and mature PNS neurons in response to secreted signalling proteins. The PNS works alongside the central nervous system (CNS) with the primary function of ensuring the organism's ultimate survival by enabling it to react to the internal and external environment, in the most appropriate or advantageous way. Not surprisingly, the most complex organ system in a vertebrate is its nervous system. The ubiquitous presence of the system throughout the body, the diversity of neuronal type, and the intricate, yet precise, connectivity all contributes to its complexity. The development, maintenance and function of the nervous system is precisely regulated by a range of endogenous signalling proteins produced from a wide variety of cells within the nervous system and other tissues, at different times and by a number of different routes.

In this introductory chapter I will briefly review salient aspects of the development of the nervous system, with particular emphasis on the PNS, and especially sensory neurons of the PNS, as these neurons have been extensively studied in this thesis. I will provide an overview of functional aspects of sensory neurons, especially in relation to pain transmission and painful states, as this is of particular relevance to the work presented in the thesis. I will provide an overview of the neurotrophic hypothesis and the roles of secreted signalling proteins like neurotrophic factors in neuronal development. Finally, I will describe relevant aspects of the developmental and functional significance of the various families of neurotrophic factors and their receptors in the nervous system.

1.1. Origins of the vertebrate nervous system

The vertebrate nervous system is divided into the central nervous system, made up of the brain and spinal cord, and the peripheral nervous system, consisting of the cranial

and spinal nerves, and the autonomic nervous system. The function of the central nervous system (CNS) is to interpret incoming nervous activity, process it and generate a response whilst the peripheral nervous system (PNS) acts as the bridge for nerve signals between the brain and spinal cord and the peripheral receptors and actuators.

The embryological development of the nervous system begins with a process known as “gastrulation” when the blastula, which is made of a single layer of cells, is transformed into a three-layered structure, consisting of endoderm, mesoderm and ectoderm. Each of these layers eventually gives rise to specific systems and tissues. The innermost layer, the endoderm, develops into the epithelial components of the respiratory system, digestive system, pancreas, liver, urinary bladder, thyroid, parathyroids, tonsils and middle ear. The middle layer, the mesoderm, gives rise to muscles, connective tissue and the vascular and urogenital systems. The outermost layer, the ectoderm, develops into the skin and nervous system. Following gastrulation, “neural induction” occurs. In this process, the dorsal aspect of the ectoderm thickens and becomes what is referred to as the “neural plate”. The lateral edges of the plate rise up whilst the midline sinks, folding the plate centrally to produce a canyon, the neural fold. The edges of the fold then grow together and fuse to produce a hollow tube, the ‘neural tube’, which runs along the dorsal midline of the developing embryo. The neural tube gives rise to the CNS, its hollow becoming the ventricular system while the epithelial walls become the neuronal and glial tissues.

The PNS is derived from a collection of “neural crest” (NC) cells. The crest (NC) develops from outer margins of the ectodermal neural tube. The NC cells detach from the dorsal aspect of the neural tube and migrate through the mesoderm, following well-defined pathways. Differences in their environment and the inbuilt “drivers” lead the NC cells to migrate to specific regions of the developing embryo. Here these cells differentiate into the various cell types of the PNS and a variety of other cell types. Those NC cells that migrate just beneath the surface of the ectoderm

form pigment cells (melanocytes) of the skin, while the sensory ganglia are formed from those that take an intermediate pathway. The autonomic sympathetic and parasympathetic ganglia form from those that take a more medial pathway. Schwann cells and satellite cells are also of neural crest origin (Jessen and Mirsky, 1998), as are the chromaffin cells of the adrenal medulla and many of the skeletal and connective tissue components of the head. The neurons of certain sensory cranial ganglia differentiate from another group of cells, the “neurogenic placodes”, which develop as thickenings of the rostral ectoderm (D’Amico-Martel and Noden 1983; Le Douarin, 1986).

In its development, there are a number of influences. These include two important classes of proteins, the bone morphogenetic proteins (BMPs) (originating in the epidermal ectoderm) and the sonic hedgehog (SHh) morphogen, which is expressed in the axial mesoderm and stimulates vascular growth (Reviewed in Le Douarin and Dupin, 2003; Kandel, principles of neuroscience 4th edition). Genes such as *Mash1* and *ngns* are important in specification of sensory and autonomic neurons. Endothelin-3 (ET3) and its receptor, the endothelin-B receptor (EDNRB) regulate the development of melanocytes, the enteric nervous system and posterior enteric nerve plexuses (Nataf et al., 1996; Reviewed in Le Douarin and Dupin, 2003).

1.2. Development of the peripheral nervous system

The PNS is divided into the somatic and autonomic nervous systems. Somatic nerves, are those which link the CNS to systems which are under direct conscious control (movement of the limbs, eyes, etc.) and convey sensory information that is generally consciously perceived, while the autonomic nerves link the CNS with systems which run (largely) without conscious control (e.g. motor activity in the gut, accommodation in the eye, etc.). The autonomic nerves may travel with or separately from the somatic nerves. In the PNS, the nerve cell bodies are gathered into groups,

known as ganglia, but may also be found within neural plexuses. The main ganglia of these systems will be discussed briefly here.

1.2.1. Sensory ganglia of the peripheral nervous system

Sensory ganglia are subdivided into cranial ganglia and dorsal root ganglia (DRG). The DRG are located on the dorsal roots of the spinal nerves. Their neurons innervate sensory receptors in the skin, muscles and joints of the trunk. Cranial sensory ganglia innervate sensory receptors predominantly in the head and are located on five of the twelve pairs of cranial nerves. The location of the twelve cranial nerves is shown in figure 1.1 and the main function of each nerve outlined in table 1.1. Neurons of the trigeminal ganglion are located on cranial nerve V and innervate the mechanoreceptors, thermoreceptors and nociceptors of the face and oral and nasal cavities. The geniculate ganglion, located upon cranial nerve VII, innervates anterior taste buds of the tongue. The vestibular and cochlear ganglia (cranial nerve VIII) are required for hearing and balance, with neurons innervating the hair cells in the cochlea, utricle, saccule and semicircular canals. The petrosal ganglion (glossopharyngeal nerve, IX) innervates posterior taste buds of the tongue and pharynx. Neurons of the jugular (superior vagal) and nodose (inferior vagal) ganglia, (vagus nerve X) innervate the oro-pharynx, oesophagus and the gut in the chest and abdomen and the respiratory tract. Finally the superior glossopharyngeal ganglion, located on nerve IX, provides somatic sensory innervation to the tongue and throat and is important in swallowing reflexes.

The only neural crest-derived cranial sensory neurons found in a CNS site are those of the trigeminal mesencephalic nucleus (TMN), which is found in the midbrain. These neurons innervate stretch receptors in the muscles of mastication.

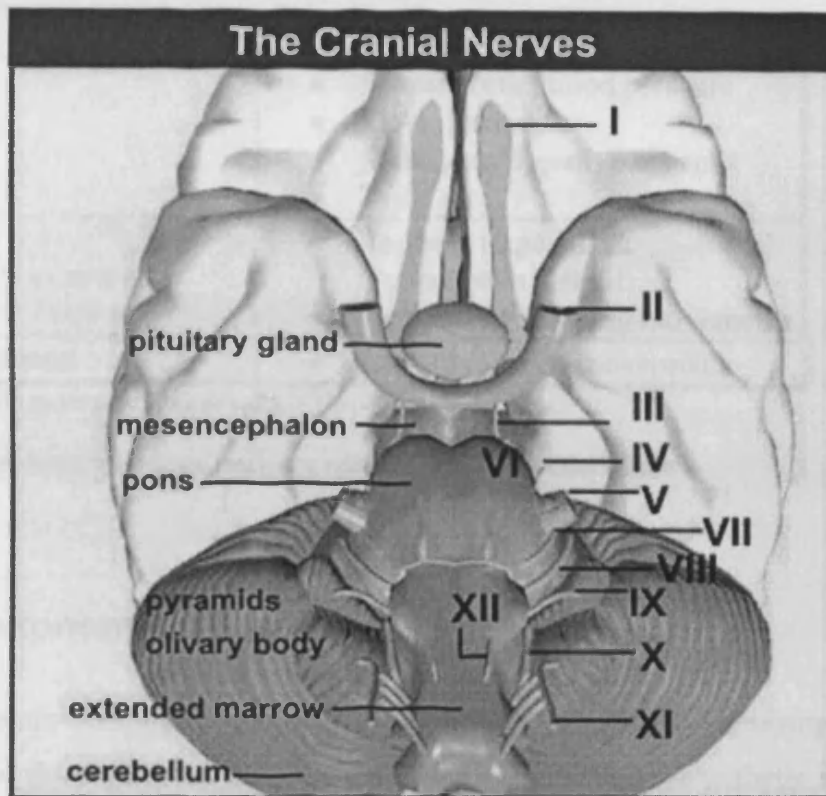


Figure 1.1. The location of the 12 cranial nerves. Taken from

http://www.becomehealthynow.com/images/organs/nervous/cranial_nerves.jpg

CRANIAL NERVE:	MAJOR FUNCTIONS:
I Olfactory	<ul style="list-style-type: none"> • Smell
II Optic	<ul style="list-style-type: none"> • Vision
III Oculomotor	<ul style="list-style-type: none"> • Eyelid and eyeball movement
IV Trochlear	<ul style="list-style-type: none"> • Innervates superior oblique • Turns eye downward and laterally
V Trigeminal	<ul style="list-style-type: none"> • Chewing • Face & mouth touch & pain
VI Abducens	<ul style="list-style-type: none"> • Turns eye laterally
VII Facial	<ul style="list-style-type: none"> • Controls most facial expressions • Secretion of tears & saliva • Taste
VIII Vestibulocochlear (auditory)	<ul style="list-style-type: none"> • Hearing • Equilibrium sensation
IX Glossopharyngeal	<ul style="list-style-type: none"> • Taste

	<ul style="list-style-type: none"> • Senses carotid blood pressure
X Vagus	<ul style="list-style-type: none"> • Senses aortic blood pressure • Slows heart rate • Stimulates digestive organs • Taste
XI Spinal Accessory	<ul style="list-style-type: none"> • Controls trapezius & sternocleidomastoid • Controls swallowing movements
XII Hypoglossal	<ul style="list-style-type: none"> • Controls tongue movements

Table 1.1. *The main functions of each of the 12 cranial nerves.*

Adapted from: <http://www.gwc.maricopa.edu/class/bio201/cn/cranial.htm>

1.2.2. Autonomic ganglia

The autonomic nervous system is divided into the sympathetic, parasympathetic systems and the enteric nervous system within the gut. The sympathetic and parasympathetic nervous systems control the gut, exocrine secretions (e.g. saliva) and the heart rate, allowing the body to react to environmental surroundings and prepare for 'fight or flight'. The sympathetic ganglia are further subdivided into paravertebral and prevertebral ganglia. Paravertebral ganglia include the superior cervical ganglion (SCG), which innervates the salivary, lacrimal and sweat glands; the middle cervical ganglion and the stellate ganglion, which innervates heart, lungs and bronchi; and the sympathetic chain ganglion, which also contributes to the innervation of the thoracic viscera, and neurons of the entire paravertebral chain innervate vascular smooth muscle throughout the body. Prevertebral sympathetic ganglia include the (abdominal) coeliac ganglion, which innervates oesophagus and stomach; aorticorenal ganglia, innervating the kidneys; the superior mesenteric ganglion, which innervates the small intestine; and the inferior mesenteric ganglion, which innervates the colon and pelvic organs. Parasympathetic ganglia include the ciliary ganglion, pterygopalatine ganglion, submandibular ganglion and otic ganglion, which innervate lachrymal, salivary and other glands. In addition, the terminal ganglion of the vagus nerve and the pelvic plexus innervate the gut and areas of the uro-genital tract. The enteric nervous system is comprised of networks of sensory, interneurons, motor neurons and astrocyte glia, which are divided into two

separate groups, the myenteric (Auerbach's) plexus and the submucosal (Meissner's) plexus. Together these neuronal plexuses regulate and co-ordinate gut movement and intestinal exocrine secretions.

1.3. Origins of the peripheral nervous system

Sensory neurons differentiate from progenitor cells that are derived from two sources: the neural crest or the neurogenic placodes. Placodes are discrete regions of thickened ectoderm in the head. Neural crest (NC) cells are generated as a result of cell-cell interaction between the epidermis and neural plate, and they separate and migrate from the epidermis as the neural plate fuses to form the neural tube (Selleck and Bronner-Fraser, 1995). NC cells migrate through the mesoderm to specific regions of the embryo where they differentiate into their destined cell type.

Detailed mapping experiments have determined the origins of avian cranial sensory ganglia. The neural crest gives rise to neurons of the dorsomedial part of the trigeminal ganglion, trigeminal mesencephalic nucleus, jugular ganglion, superior glossopharyngeal ganglion and the dorsal root ganglia (DRG), whilst neurons of the venterolateral part of the trigeminal ganglion and the vestibulo-cochlear, geniculate, petrosal and nodose ganglia are derived from neurogenic trigeminal, epibranchial and otic placodes (Noden, 1978; D'Amico-Martel, 1982; D'Amico-Martel and Noden, 1983; Le Douarin, 1986; Le Douarin, 2004).

In the autonomic nervous system, the neurons of the parasympathetic and sympathetic ganglia are derived from neural crest cells of the entire trunk, whilst enteric nervous system neurons are derived from three distinct areas of the neuronal crest: the vagal, the sacral and the truncal regions (Epstein et al., 1994; Serbedzija et al., 1991; Le Douarin et al., 2004).

All Schwann cells and satellite cells of the PNS are derived exclusively from the neural crest (D'Amico-Martel and Noden, 1983).

Transplantation experiments have revealed that both presumptive placodal ectoderm and premigratory neural crest cells are not predestined to particular cell types. Environmental cues and other external factors along their migratory track are responsible for designating a cell to a particular neuronal fate (Vogel and Davies, 1993; Le Douarin et al., 2004).

1.4. The Mature PNS

1.4.1. Anatomy of the PNS

Mature peripheral nerves are composed of myelinated and unmyelinated nerve fibres. Fibres surrounded by myelin sheaths have a greatly enhanced speed of transmission. In the PNS, the myelin sheath of the nerve fibre is formed from the encircling compressed layers of Schwann cell plasma membrane (see figure 1.2). Breaks in the myelin sheath (the node of Ranvier – see figure 1.2) occur at intervals. Unmyelinated fibres are surrounded solely by Schwann cell cytoplasm. In the CNS, oligodendrocytes, rather than Schwann cells produce myelin sheaths.

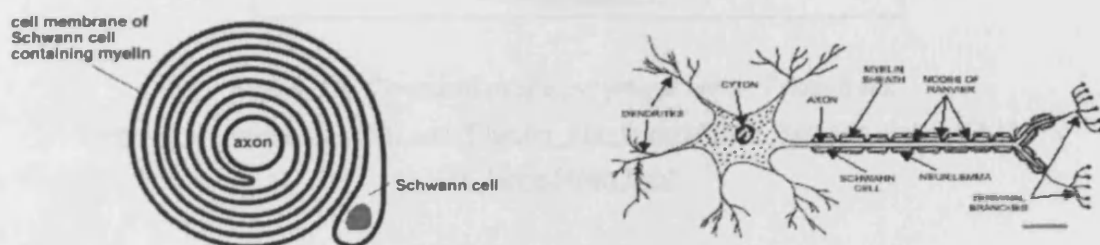


Figure 1.2. Schwann cells in the PNS

Taken from:

http://www.unis.org/UNIScienceNet/Schwann_cell_800.html

<http://www.infodotinc.com/corpsman/32.htm>

A peripheral nerve is composed of many nerve fibres. A nerve fibre bundle is known as a fascicle and is invested with connective tissue called endoneurium. Several smaller bundles of nerve fibres are enclosed in a connective tissue sheath known as

the perineurium, and the collections of these larger nerve fibre bundles that make up the complete nerve fibre are in turn invested with a loose vascular connective tissue sheath known as the epineurium. Each nerve fibre can be thought of as living inside its own protective pipe, a structure essential to assist in the accurate re-growth in the event of fibre damage (see figure 1.3 and 1.4).

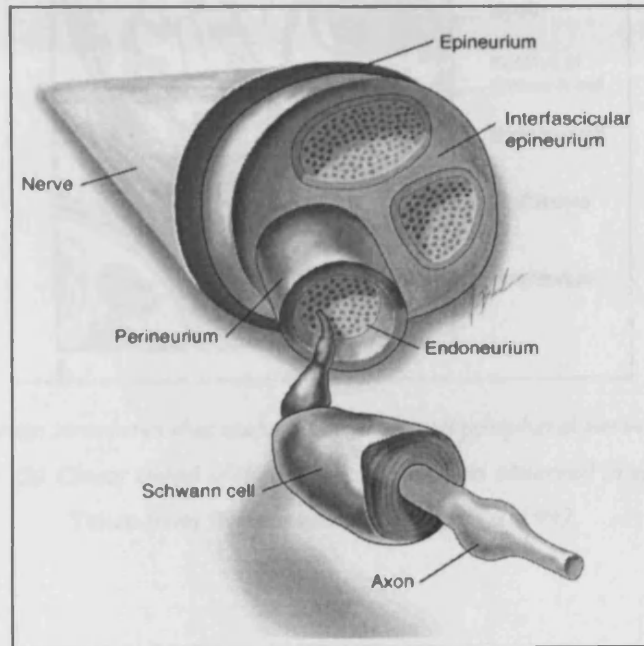


Figure 1.3. *Composition of a peripheral nerve.* Taken from:

http://www.backpain-guide.com/Chapter_Fig_folders/Ch10_Recover_Folder/Ch10-1_NerveStruct.html

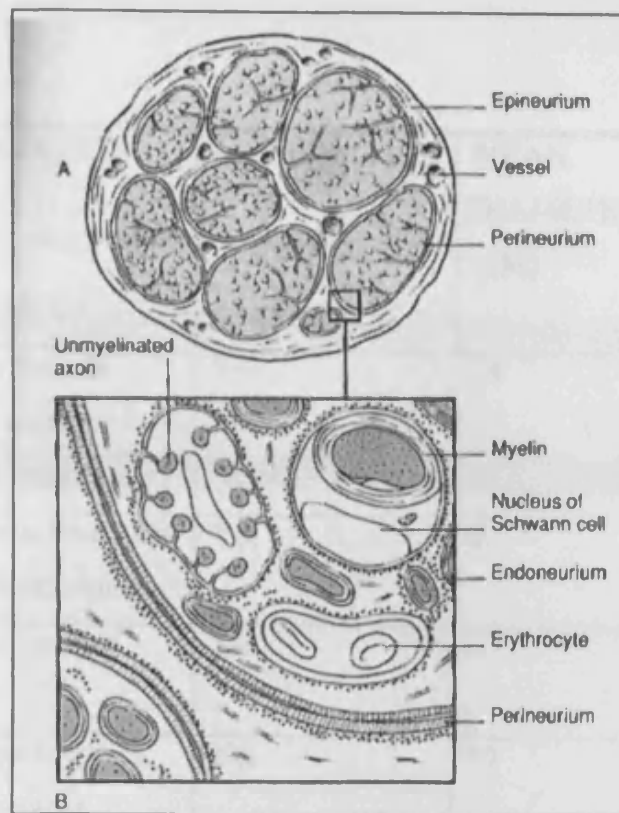


Figure 1.4. (A). *The main structures that make up a transected peripheral nerve as would be depicted by light microscopy.* (B) *Closer detail of the transected nerve as observed in electron microscopy.*

Taken from 'Neuroanatomy' Fitzgerald, 1992

1.4.2. Microanatomy of the PNS - Classification of mature peripheral neurons

Peripheral nerves can be classified on the basis of their fibres (rather than their cell bodies) with regards to myelination, diameter and conduction velocity. The Gasser and Erlanger system is one of the most commonly used classification systems. In this system, nerves are categorised as A, B and C, with the A fibres further subdivided into $A\alpha$, $A\beta$, $A\gamma$ and $A\delta$. (See table 1.2.)

FIBRE GROUP	INNERVATION	MYELINATION	MEAN DIAMETER (μM)	MEAN CONDUCTION VELOCITY (M/S)
A α	Primary muscle spindle motor to skeletal muscle	Yes	15	100
A β	Cutaneous touch and pressure afferents	Yes	8	50
A γ	Motor to muscle spindle	Yes	6	20
A δ	Mechanoreceptors, thermoceptors	Yes	<3	15
B	Sympathetic preganglionic	Yes	3	7
C	Mechanoreceptors, nociceptors, sympathetic postganglionic	No	1	1

Table 1.2. *Gasser and Erlanger classification of fibres in peripheral nerves.*

Adapted from Ferrante, Postoperative pain management, 1993.

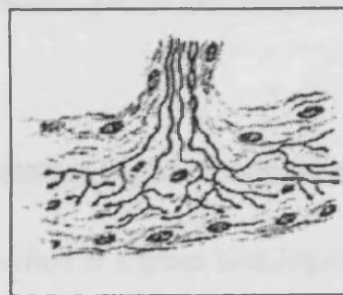
1.4.3. Sensory neurons

This thesis concerns regards sensory neurons, in particular those of the nodose ganglia, trigeminal ganglia and DRG. For this reason, the different subtypes of sensory neurons will be discussed, followed by details of the composition of the sensory ganglia of interest, and information on the proportions and biochemical properties of relevant neuronal subpopulations.

The large diameter $A\alpha$ and $A\beta$ fibres carry impulses from low threshold mechanoreceptors (LTM), and the nerve and receptor combined are often referred to as the low threshold mechanoreceptors (LTMs). They respond to low threshold non-noxious stimuli such as touch, pressure and proprioception (which enable the brain to establish its overall position in space). Many subtypes of $A\alpha$ and β LTMs exist. These include, the slowly adapting type I nerves with Merkel end organs that respond to pressure (figure 1.5A); slowly adapting type II type with Ruffini ending (figure 1.5B); G_1 and G_2 hair follicles which respond to hair movement; and rapidly adapting neurons, which respond to tapping and whose end organ is the Meissner corpuscle (figure 1.5C).



A. Merkel ending



B. Ruffini ending



C. Meissner
Capsule

Figure 1.5. *The receptor endings of LTMS*

In general non-nociceptive afferents have a lower threshold of stimulation than nociceptive. However, a subpopulation of the small diameter A δ mechanoreceptive fibres, known as the high-threshold mechanoreceptors (HTM), become more sensitive over time so that small amounts of non-damaging, but sustained, pressure cause pain.

Nociceptors (nociceptive neurons and their associated receptors) comprise small diameter A δ fibres and unmyelinated C fibres. A δ fibres are often referred to as mechano-heat fibres as respond to mechanical and thermal stimulus. They have restricted and small receptive fields. They respond to severe deformation of the skin, such as pinching. The pain sensation(s) they produce is one of sharpness and stinging. Thermoreceptive neurons are A δ fibres that sense cold and C fibres that sense heat.

C fibres are unmyelinated and of small diameter. As a result they are the slowest of the small fibres. They have a wider receptive field and respond to noxious, mechanical, thermal and chemical stimuli. As a result, they are often referred to as “C-polymodal nociceptors” (C-PMN). C-PMNs “sensitise”, developing a lower threshold of stimulation with repetitive stimulation: they can develop an ongoing, continuous discharge. It is these nerve fibres that are responsible for the more prolonged intense aching or dull pain that experienced with inflammation or following nerve damage.

Subtypes of neurons within mature DRG, trigeminal ganglia and nodose ganglia

Sensory neurons can be classified at a gross histological level into two broad groups. The first group consists of small diameter nociceptive or thermal A δ or C fibres and are often referred to as ‘dark neurons’. The second group is made up of large diameter, A α or β , mechanoreceptive ‘light’ neurons. Large light neurons bind the neurofilament antibody, RT97 (Lawson et al., 1984), whereas the majority of small, dark neurons can bind and transport the isolectin B4 (IB4) (Silvermann et al., 1988;

Kitchener et al, 1993; Wang et al., 1994, 1998). These two markers can bind the majority (approximately 80%) of DRG neurons.

Neurons can also be categorised into smaller subpopulations based upon expression of neurotrophin receptors, (the receptor tyrosine kinase of the, = Trk family, and common receptor p75) and other sensory neuron specific genes. For example, in adult rats, approximately 40% of DRG neurons express the nerve growth factor (NGF) receptor tyrosine kinase TrkA (Verge et al., 1989, 1992). Of these, the majority (approximately 92%) co-express the neuropeptides “calcitonin gene related peptide” (CGRP) and substance P (SP), but not the genes somatostatin or thiamine monophosphatase (Verge et al., 1989; Averill et al., 1995). Only 18% of these TrkA-expressing neurons bind the marker of large neurons, the neurofilament protein RT97 (Averill et al., 1995). This proportion of TrkA expressing DRG neurons are, therefore, termed small diameter “peptidergic” neurons. A proportion of adult rat DRG neurons do not express TrkA and are not labelled by neurofilament. These neurons comprise approximately 30% of DRG neurons and are unmyelinated small diameter neurons that also bind the isolectin B4 (Molliver et al., 1995; Averill et al., 1995).

Of the other neurotrophin receptors, TrkC is expressed almost exclusively by large diameter sensory afferents of the DRG (Mu et al., 1993; McMahon et al., 1994). The low affinity common neurotrophin receptor, p75, is not expressed independently of Trks and is found co-localised on the majority of TrkA and TrkB expressing neurons, but only on approximately 50% of TrkC neurons (Wright and Snider, 1995).

The “Sensory Neuron Specific” (SNS) “Tetrodotoxin Resistant” (TTX-r) sodium channels, Nav1.8 and Nav1.9, respectively, have specific expression profiles within adult rat DRG neurons. Nav1.8 is found both in small diameter non-myelinated sensory neurons (C-fibres) and in 10% of large neurons with myelinated axons corresponding to A δ nociceptors (Sangameswaren et al., 1996; Amaya et al., 2000). The second TTX-R alpha subunit, Nav1.9 (NaN/SNS2) is predominantly located in small diameter sensory neurons (Black et al., 1996; Dib-Hajj et al., 1998; Tate et al.,

1998; reviewed in Lai et al, 2004), expressed specifically within C neurons, and is not detectable in neurons with myelinated axons (Amaya et al., 2000; Fang et al., 2002).

In rat DRG, the capsaicin receptor, Vanilloid receptor 1 (VR1) is expressed by the TrkA- positive, peptidergic neurons, and the non-peptidergic IB-4 reactive population (Mantyh and Hunt, 1998; Michael and Priestly 1999; Guo et al., 1999). In contrast, in the mouse, only a small (2-3%) population of DRG neurons are both IB4 and VR1 positive (Zwick et al., 2002), the majority of functional VR1 receptors being expressed by the TrkA expressing peptidergic neurons. This observation highlights the important fact that species differences occasionally occur.

In the adult rat, only 2% of intact DRG neurons are galanin mRNA positive (Xu et al., 1996; Ma et al., 1999). These neurons are predominantly small/medium sized peptidergic neurons and also express CGRP. However some expression in large size neurons has also been observed (Xu et al., 2000; Ma et al., 1999).

The trigeminal ganglia, which convey sensory information from the facial area have many similarities with DRG. It contains similar subpopulations of neurons and shows similar patterns of expression of neurotrophin receptors and other sensory neuron specific genes (Matsumoto et al., 2001). The nodose ganglia, which are important for transmission of cardiovascular, respiratory and gastrointestinal signals, shows some clear differences from DRG and trigeminal ganglia (Zhou et al., 1997). The nodose ganglia are derived from the neurogenic placode, and during development the neurons are reliant upon neurotrophic factors that differ from those of the NC-derived DRG and trigeminal ganglia. Only approximately 5% of the nodose neurons express TrkA and 10% TrkC (Verge et al., 1992; Zhou and Helke 1996). Nevertheless, the mean density of expression of p75 has been shown to be the same or higher than that of the DRG or trigeminal ganglia (Verge et al., 1992). Expression of other genes also differs. In the nodose ganglion, TrkB, rather than TrkA is co-localised with VR1 mRNA (Michael and Priestly, 1999). This co-expression of VR1 and TrkB reflects the observation that BDNF, but not NGF, can

regulate the capsaicin sensitivity of cultured adult rat nodose neurons (Winter 1998). Galanin immunoreactivity is detected in the nodose ganglia, but in contrast to trigeminal ganglia and DRG, it is expressed predominantly in large diameter neurons (Calingasan and Ritter, 1992). Expression of other genes in the rodent, including CGRP and SP mRNA are seen in similar populations and proportions of nodose neurons to that of the DRG and trigeminal (Helke and Hill 1988; Czyzyk-Krzeska et al., 1992; Sternini and Anderson, 1991).

1.4.4. Sensory neurons, insult, injury and the inflammatory response

Because this thesis focuses on sensory neurons of the PNS a brief overview of the involvement of these neurons in pain states and diseases affecting these neurons in the adult is relevant.

1.4.4.1. Inflammation

Inflammation is characterised by four features “calor, rubor, dolor and tumor” – heat, redness, pain and swelling. Heat and redness are due to capillary dilatation; swelling is due to extravasation of protein-rich oedema fluid from the capillaries; pain occurs because of swelling (stretching of tissues) and the presence of a host of inflammatory mediators including prostaglandins. The inflammatory response has hallmarks similar to the “triple response” in which tissue injured by, for instance a scratch demonstrates the three features redness, flare and swelling.

The inflammatory stimulus is first sensed by polymodal nociceptors (C-fibres) in the deep epidermis. These receptors (Figure 1.6) subsequently transmit messages through to the CNS, and also stimulate the production of the neuropeptide Substance P (SP) from sensory nerves. SP is able to bind to receptors on arterioles allowing arterial dilation and the characteristic ‘flare response’. SP is also able to bind to mast cells, stimulating the release of histamine and 5- hydroxytryptamine (5HT), which

alters the permeability of capillaries, producing accumulation of tissue fluid and the 'wheal response'.

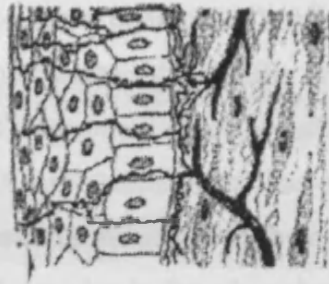


Figure 1.6. Free epidermal nerve endings found in the deep layers of the epidermis

The release of SP in response to peripheral inflammation is well characterised (Duggan et al., 1987; Oku et al., 1987; Schaible et al., 1990), and is required for the development of the whole pattern of inflammation and for the painful sensations with which it is accompanied. Intrathecal administration of SP results in hyperalgesia (Moochhala et al., 1984). The application of antagonists to the SP receptor, neurokinin 1 (NK₁), results in a loss in the increased excitability in response to noxious thermal stimulation and also in a loss of 'wind-up', (an increase in the response following repetitive stimuli of equivalent strength so that the response to the last of the stimuli is much greater than that to the first stimulus, despite being the same strength) (Thompson et al., 1995; Radhakrishnan et al., 1998). Furthermore, ablation of SP neurons results in the loss of response to capsaicin and a loss of both thermal hyperalgesia and of mechanical allodynia¹ which are normally associated with inflammatory pain (Nichols et al., 1999; Khasabov et al., 2002).

In addition to the increased expression of SP that follows inflammatory stimuli, alterations in other neuropeptides and sensory neuron specific genes are observed. These changes are also thought to be responsible for the subsequent inflammatory pain. For instance, an increase in the potent vasodilator "Calcitonin Gene Related

¹ Allodynia refers to the feeling of pain from stimuli that are not normally painful e.g. touch of feather.

Peptide" (CGRP) is also observed. CGRP potentiates the release of SP (a positive feedback), thus enhancing the transmission of nociceptive information (Oku et al., 1987; Brain et al., 1989). CGRP has also been shown to potentiate oedema formation when co-injected with histamine, a potent mediator of vascular permeability that is released in the triple response. (Brain et al., 1992). Additionally, CGRP can affect the immune response to inflammation at the cellular level, where it can directly and indirectly affect production of chemotactic compounds from macrophages e.g. cytokines². The presence of CGRP attenuates release of the cytokine interleukin-1 (IL-1), but produces an increase in production of the cytokine, interleukin- 10 (IL-10) (Torii et al., 1997). CGRP can also indirectly enhance lipopolysaccharide (LPS)³ (also known as endotoxin) induced release of interleukin-6 (IL-6) from macrophages through induction of nitric oxide (NO) (Tang et al., 1999).

Transcripts of the neuropeptide, "Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) are increased in small-medium neurons following inflammatory stimulation, suggesting a role in inflammation and the associated inflammatory pain (Zhang et al., 1998; Jongasma-Wallin et al., 2003). Furthermore, Mabuchi et al., (Mabuchi et al., 2004) showed that PACAP^{-/-} mice do not exhibit inflammatory pain induced by the injection of a carrageenan (a polysaccharide extracted from red seaweed).

An increased expression of the capsaicin receptor, VR1, is also observed in the DRG following application of the local irritant, Freund's Complete Adjuvant (CFA) (Amaya et al., 2003; Ru-Rong et al., 2002). This increase is thought to play a role in the generation of thermal hyperalgesia because mice lacking VR1 do not show this hypersensitivity effect following induction of inflammation (Caterina et al, 2000).

Following carrageenan injection (Colpaert, 1987; Butler et al., 1992; Ji et al., 1995) or administration of CFA (Calza et al., 1998), decreases in both protein and mRNA

² Cytokines are proteins produced by cells. They can effect cellular interaction/communication and can also affect cellular behaviour

³ Lipopolysaccharide (LPS) or endotoxins are structural components of gram-negative bacteria, mainly released when the bacteria are lysed. Endotoxins are pyrogens producing fever and can activate inflammation

expression of the neuropeptide galanin are observed in the DRG, despite an increase in galanin immunoreactivity in the dorsal horn (Ji et al., 1995). Calza et al., (Calza et al., 1998) further monitored its expression following the onset of inflammation and found that galanin mRNA and peptide levels recovered to normal 5 days post-injection. Interestingly, 21 days post-injection a significant increase from basal level was observed. It is thought that this increase in galanin expression could reflect neuronal damage, as discussed (above), and that the pain felt might therefore change from inflammatory to neuropathic pain (Calza et al. 1998).

One of the adenosine triphosphate (ATP) receptors, the purigenic receptor P2X3, is also affected by inflammation. A low dose of ATP injected into rats skin previously inflamed with carrageenan produces an enhanced and greater pain response than in normal, untreated rats (Hamilton et al., 2001). This suggests that P2X receptors are sensitised by inflammation, and that the enhanced sensitivity may be due to mediators released as a result of inflammatory stimulation. SP and bradykinin, which are both known to mediate pain perception, have both been shown to potentiate P2X3 and P2X2/3 heteromeric ion channels through phosphorylation. This suggests that such an action can subsequently sensitise nociceptors, producing an enhanced response to ATP (Paukert et al., 2001). CFA-induced inflammation also produces an increase in P2X3 protein in small-medium neurons of the rat DRG (Xu and Huang, 2002).

Alterations in sodium channel expression also occur following inflammation. Carrageenan injection into adult rats leads to increased expression of two of the “Tetrodotoxin sensitive” (TTX-S) sodium channels Nav1.3 and Nav1.7 in DRG, producing an increase in TTX-S currents (Black et al., 2004). It was proposed that this increased sodium conductance contributes to the neuronal hyper-excitability and onset of inflammatory pain. Additionally, the finding that application of the “Cyclo-Oxygenase (COX) inhibitors, ibuprofen or NS-398 (a COX-2 specific inhibitor), prior to CFA injection, prevents the inflammation-induced up-regulation in Nav1.7 protein observed in adult rat DRG (Gould et al., 2004). These observations suggest that components of the COX pathway are involved in the up-regulation of Nav1.7.

Alterations in the TTX-resistant (TTX-R) sodium channel Nav1.8 are also observed in inflammation. Within 48 hours of CFA-induced inflammation in adult rats, there is a dramatic increase in Nav1.8 protein in DRG neurons (Gould et al., 1998; Tanaka et al., 1998; Gould et al., 2004; Coggeshall et al., 2004). In small neurons, the inflammation-induced enhanced level of Nav1.8 persists and remains unchanged, while, as allodynia subsides, the expression of Nav1.8 in the large neurons falls back to baseline (Gould et al., 2004). It was therefore proposed that the increased expression of Nav1.8 in *large* neurons is responsible for the hyperalgesia observed in such inflammatory states, whilst the persistent up-regulation in *small* diameter neurons produces a prolonged increased sensitivity that could provide a protective role, to ensure the organism's vigilance (and self protection) during healing (Gould et al., 2004). Further evidence of Nav1.8's role in inflammatory pain has been provided by the use of antisense oligonucleotides to attenuate Nav1.8 expression. Injection of these "anti-Nav1.8" oligonucleotides into rats prevented PGE2-induced hyperalgesia and increased the threshold to mechanical nociceptive stimuli (Khasa et al., 1998).

1.4.4.2. Nerve injury and neuropathic pain

Following peripheral nerve damage or crush, in a process named Wallerian degeneration, axons degenerate distal to the lesion. During the first 48 hours following injury, lysosomal activity of Schwann cells breaks the myelin and axons into 'ellipsoids'. Monocytes enter the nerve from the bloodstream through breaks in the endoneurium and begin to clear the debris. The end result of the degenerative process is a shrunken nerve, but with intact endoneurium and a core of functioning Schwann cells.

Unlike the neuronal damage in the CNS, neuronal regeneration in the PNS can occur, provided the cell body remains intact. Following a clean cut of a nerve fibre (or bundle) the process of regeneration can begin within hours: the "clean up" process is localised and relatively quick. However, in crush or tear injuries, degeneration of

axons is more widespread and so regeneration is delayed. The process of regeneration begins with axonal sprouting from the distal nerve fibre stump, and the contact of these sprouts with the local Schwann cells. Successful regeneration relies on this contact, without which regenerating axons become trapped. In this case, continued growth leads to the development of a tangled mass and the formation of a neuroma. Neuromas are frequently painful.

Successful axonal sproutings form growth cones, which produce fine “filopodia” (fine precursors to the fibre) that can extend along the length of the Schwann cell. They anchor temporarily to the basement membrane. Actin filaments within the filopodia are now able to attach to basement membrane receptors, and once firmly bound act as a platform for onward migration of the growth cone. Schwann cells then now begin myelination of the newly regenerating nerve. Complete regeneration is likely, provided the proximal and distal ends are aligned correctly, and the appropriate endoneurial tubes have been entered: sensory axons have the ability to regenerate along former motor tubes and vice versa. For this reason, injuries in which the endoneurium is preserved (for example a nerve crush), are likely to recover better than when a nerve is cut.

Nerve injury (crush or transection) may lead to the onset of neuropathic pain. This has been found to be associated with alterations in the expression of neuropeptides and other sensory neuron specific genes. However, these changes often differ from those observed in inflammation, and may serve to protect against *or* to contribute towards the onset of neuropathic pain. Two genes, normally expressed at barely detectable levels in sensory neurons, are dramatically up-regulated following peripheral nerve injury. These are “Activating Transcription Factor-3” (ATF-3) and “Damage Induced Neuronal Endopeptidase” (DINE). ATF3 is markedly up-regulated following axotomy. In rats subject to sciatic nerve transection, 82% of L4 DRG neurons become immunoreactive for ATF3: control rats show no immunoreactivity (Averill et al., 2004). In a pattern similar to the neuropeptide galanin, DINE is also

up-regulated in specifically IB4 negative and partly TrkA positive neurons (Kato et al., 2002).

In contrast to inflammation, peripheral nerve injury results in a decrease in the neuropeptides SP and CGRP. Transection of the rat sciatic nerve results in down-regulation of SP peptide (Nielsch et al., 1987; Zhang et al., 1995; Sterne et al., 1998) and a decrease in expression of both isoforms of CGRP mRNA in DRG (Noguchi et al., 1990; Mulder et al., 1997; Sterne et al., 1998; Shi et al., 2001; Shadiack et al., 2001). This has been proposed to result from a loss of retrograde transport of neurotrophic factors. This down-regulation of SP and CGRP is not observed within the nodose ganglion following axotomy of the rat cervical vagus nerve (Helke et al., 1991), suggesting that expression of these genes in this neuronal population is regulated in a different way from that in sensory neurons of the DRG.

PACAP is up-regulated in medium to large DRG neurons following either peripheral axotomy (Zhang et al., 1995; Zhang et al., 1996; Jongsma-Wallin et al., 2001; Armstrong et al., 2003) or nerve compression (Pettersson et al., 2004) in the rat. Mabuchi et al., (Mabuchi et al., 2004) showed that PACAP^{-/-} mice do not exhibit neuropathic pain following nerve transection, implicating PACAP up-regulation in the onset of neuropathic pain.

In the rat, an increase in galanin transcripts and peptide is also observed following spinal nerve transection (Hokfelt et al., 1987; Villar et al., 1989; Noguchi et al., 1993), chronic constriction injury (Nahin et al., 1994; Ma et al., 1997; Shi et al., 1999) and spinal nerve ligation (Fukuoka et al., 1998). Following axotomy (Xu et al., 1996; Shi et al., 1997) of rat sciatic nerve, associated alterations in galanin receptors are also seen with a significant down-regulation of GAL-R1 and GAL-R2 in DRG neurons. Such an up-regulation of galanin would suggest a role in the production of neuropathic pain associated with such nerve injury. However, there are conflicting results showing both inhibitory (Wiesenfeld-Hallin et al., 1992; Hao et al., 1999; Yu et al., 1999; Liu and Hokfelt 2000) and stimulatory (Thompson et al., 1996;

Thompson et al., 1998; Murphy et al., 1999; Kerr et al., 2000) effects of galanin on pain transmission in the rodent. This is discussed in more detail in chapter 3.

A down-regulation in expression of the capsaicin receptor VR1 is observed in adult DRG neurons following axotomy or nerve transection (Michael and Priestly, 1999; Michael and Priestly 2002; Fukuoka T et al., 2002). Conversely, expression of VR1 in uninjured neurons appears to be differentially regulated. Following partial nerve injury of rat DRG, expression of VR1 protein in those neurons remaining intact was increased (Hudson et al., 2001; Fukuoka et al., 2002), and may be crucial to the development of neuropathic pain following axotomy.

Expression of the ATP receptor P2X3 is up-regulated in small-medium neurons of the DRG following chronic constriction injury to the rat sciatic nerve (Novakovic et al., 1999). In the trigeminal ganglion, an increase in P2X3 immunoreactivity was also observed following ligation/section or chronic constriction of the inferior alveolar branch of the mandibular nerve (Eriksson et al., 1998). Such an up-regulation would suggest a function following nerve damage. However other research has produced conflicting results. Fukuoka and colleagues (Fukuoka et al., 2002) found no change in P2X3 immunoreactivity in the DRG following L5 spinal nerve ligation.

Injury related decreases have also been observed. Bradbury et al., (Bradbury et al., 1998) found that following axotomy of the sciatic nerve, P2X3 immunoreactivity in the L4/L5 DR, decreased by 50%. A similar decrease was observed in lumbar DRG using a model of spinal nerve ligation (Kage et al., 2002). These discrepancies might be due to differences in the models of neuropathic pain but could also be a reflection of the differing mechanisms or types of regulation found in injured and in non-injured neurons. Tsuzuki and colleagues (Tsuzuki et al., 2001) used ATF3 as the marker of injured neurons to selectively study expression of P2X3 in injured neurons in the trigeminal ganglia and DRG following nerve transection. This study revealed a decrease in P2X3 mRNA in injured (ATF3-expressing) neurons, despite an overall increase in P2X3 mRNA expression throughout the ganglia. The increased expression of P2X3 within intact neurons might play a role in the pathophysiology of

nerve injury and the related neuropathic pain or may play a role in the regeneration of damaged neurons following injury. Further evidence supporting a role for P2X3 in injury-associated neuropathic pain was obtained using antisense oligonucleotides to downregulate P2X3 expression in a partial sciatic ligation model of neuropathic pain (Barclay et al., 2002).

Following nerve damage and axotomy of peripheral DRG neurons, an increase in excitability and an enhancement of repetitive firing has been observed. This occurs alongside a reduction in action potential threshold (Zhang et al., 1997). This pattern of response is thought to underlie the generation of the neuropathic pain associated with nerve injury. It has been attributed, at least partly, to alterations in sodium channel expression and conductance. An increased density in TTX-S channels following sciatic nerve axotomy is observed. This produces an increased sodium conductance within DRG, which is thought to contribute to the increased excitability in the axotomised neurons (Zhang et al., 1997; Black et al., 1999). This hyperexcitability has been attributed to an up-regulation of the, normally silent, voltage gated sodium channel (VGSC) Nav1.3 (Waxman et al., 1994; Cummins and Waxman, 1997, Sleeper et al., 2000). An up-regulation in Nav1.3 mRNA has also been observed in medium to large size DRG neurons following spinal nerve ligation (SNL) in the rat (Kim et al., 2001; Abe et al., 2002; Chung et al., 2004). This up-regulation is maintained for at least 7 days post-procedure (Kim et al., 2001), and is associated with a more rapidly repriming TTX-S current. These axotomy/injury-induced changes in sodium channels are thought to alter neuronal excitability and contribute to the inappropriate firing observed following nerve damage. Nav1.3 is also up-regulated in dorsal horn nociceptive neurons four weeks after rat spinal cord injury, coinciding with the onset of pain-related behaviours and neuronal hyperexcitability, implicating Nav1.3 in the development of central neuropathic pain (Hains et al., 2003).

Despite these findings, a recent paper suggested that Nav1.3 alone was not sufficient to account for the voltage-gated sodium channel dependent behavioural hypersensitivity that is associated with nerve injury (Lindia et al., 2005). It was

found that following peripheral nerve lesion, only 18% of neurons expressing the injury factor ATF3 co-expressed Nav1.3, which suggests that the up-regulation of Nav1.3 observed following nerve injury was predominantly within intact neurons. Furthermore, although the use of the voltage-gated sodium channel blocking drugs, mexiletine and lamotrigine, partially alleviated mechanical allodynia, the use of antisense oligonucleotides specific for Nav1.3 had no effect on the injury-induced mechanical and cold allodynia (Lindia et al., 2005).

Other sodium channels that may also contribute to changes in neuronal properties as a result of injury are those belonging to the family of TTX-R channels. Alongside alterations in TTX-S channels, changes in expression of the TTX-R channels are also observed following nerve damage. A decrease in expression of both Nav1.8 and Nav1.9 is observed in small diameter DRG neurons following axotomy or sciatic nerve section (Okuse et al., 1997; Cummins et al., 1997; Tate et al., 1998; Dib-hajj et al., 1998; Novakovic et al., 1998; Sleeper et al., 2000; Decosterd et al., 2002). Such a down-regulation of TTX-R channels is thought to contribute indirectly to the hyperexcitability of axotomised sensory neurons through the interaction of TTX-R currents with TTX-S sodium conductances (Zhang et al., 1997; Cummins et al., 1997; Sleeper et al., 2000). The persistent TTX-R current carried by Nav1.9 is thought to contribute a depolarising influence to the resting membrane potential of sensory axons (Cummins et al., 1999). The down-regulation of Nav1.9 following injury would therefore produce a hyperpolarizing shift in resting membrane potential. This is proposed to relieve the inactivation of TTX-S channels, producing an increase in the TTX-S currents and neuronal excitability (Sleeper et al., 2000). In a computer model, the loss of the slow inactivating TTX-R current carried by Nav1.8 has also been shown to cause a lowering of action potential threshold and spontaneous, repetitive firing even in the absence of stimulation (Elliot, 1997; Schild and Kunze, 1997). A down-regulation in expression of both Nav1.8 and Nav1.9 is therefore implicated in the neuronal hyperexcitability that contributes to neuropathic pain following nerve damage. The down-regulation of Nav1.9 mRNA has been observed in two radicular pain models and one peripheral neuropathic pain model (Abe et al., 2002). A direct link between Nav1.8 and neuropathic pain, has also been

demonstrated by Lai et al., (Lai et al., 2002), who used antisense oligonucleotides to 'knock down' the expression of Nav1.8 in DRG of rats. In these rats neuropathic pain induced by spinal nerve injury was no longer observed, although the effects of acute pain and responses to non-noxious stimuli remained (Lai et al., 2002).

A number of other alterations in gene expression occur following nerve injury. For example, an increase in the enzyme neuronal nitric oxide synthase (NOS)⁴ is also observed in rat and monkey DRG following peripheral nerve lesion (Fiallos-Estrada et al., 1993; Verge et al., 1992; Zhang et al., 1993). This increase in NOS leads to increased production of the vasodilator nitric oxide (NO). However, the purpose of this increase is not known.

1.4.4.3. Peripheral neuropathies

If the cell body remains intact, PNS neurons have the ability to regenerate. In some neuropathies only the axon is damaged ("axonopathy"), while in most, both the nerve fibre and the cell body are damaged and die. Neuropathies may be generalised or focal. Focal neuropathies are usually a result of trauma or ischemia, whilst generalised lesions are usually caused by toxic effects (e.g. heavy metals and certain drugs), alterations in metabolism such as in diabetes, the genetically transmitted porphyria vitamin deficiencies (e.g., of the B vitamins thiamine and cobalamin). Other peripheral neuropathies, such as Guillain Barré syndrome, affect just the Schwann cells and hence lead to disorders in myelination, Guillain Barré syndrome (also known as acute inflammatory demyelinating polyneuropathy or Landry's ascending paralysis) begins with a rapid onset of weakness, with numbness or paralysis of the feet and then legs, perhaps spreading to include the hands, then arms and finally face and breathing muscles. The cause is unknown. However in 50% of sufferers, it occurs following viral (cytomegalovirus, Epstein Barr) or bacterial infection e.g. Campylobacter. It is thought to be an autoimmune disorder in which

⁴ neuronal NOS is one of a family of several NOS's. This family are responsible for the synthesis of Nitric Oxide (NO). Three types of NOS exist: neuronal NOS (nNOS), endothelialNOS (eNOS) and inducibleNOS (iNOS). nNOS produces NO in neuronal tissue of both the CNS and PNS.

the body's own cellular defence mechanisms attack myelin sheaths preventing the fast transmission of peripheral information.

1.4.4.4. Post herpetic neuralgia

Herpes zoster virus causes pain and blistering of the skin supplied by a particular nerve root – its dermatome. The illness is called “shingles”. Following apparent resolution of the infection, a chronic pain, referred to as post-herpetic neuralgia (PHN) often develops. This is pain that persists after the pain associated with the viral infection has subsided. It is caused by the neuronal damage produced by the acute infection, in which inflammation and ischaemia cause necrotizing damage in the dorsal root, the DRG and the dorsal horn of the spinal cord. Predominantly large neurons are damaged leading to a relative increase in nociceptive transmission at the dorsal horn, and subsequent pain. This can be due to the reorganisation of central connections leading to enhanced spontaneous firing, but also as a result of the loss of inhibitory signals transmitted by large diameter neurons. The elderly are particularly susceptible to PHN as they already have a reduced number of large neurons.

1.4.4.5. Trigeminal neuralgia

The trigeminal nerve carries sensory information from the face, scalp, teeth and oral and nasal cavities, and is the motor nerve of the muscles involved in chewing. Trigeminal neuralgia - “tic douloureux” - is pain in one or more of the three divisions of the trigeminal nerve. It can be idiopathic (primary neuralgia), or be associated with another systemic disorder, such as compression by a tumour or central lesion, or demyelination as a result of multiple sclerosis (MS) (secondary neuralgia). Those with primary neuralgia have no abnormal physical signs, whilst those with secondary neuralgia may have sensory impairments (or other signs) as a result of the secondary disorder. Symptoms common to both types include frontal or unilateral face pain, which is abrupt in onset and can last from a few seconds to two minutes. The pain is

stereotypical to the patient and may be spontaneous or triggered by such things as chewing, talking or even a cold wind on the face. The pain felt is intense with a sharp/ stabbing quality that may be interspersed with dull aching pain.

1.5. Regulation of cell survival and neuronal number in the developing peripheral nervous system

1.5.1 The neurotrophic factor hypothesis

During the development of the vertebrate nervous system neurons are produced in excess. A large proportion are lost during a period of developmentally programmed cell death. The target fields of axons are thought to regulate this process by the production of target derived survival factors, known as “neurotrophic factors” (NTFs) (Levi-Montalcini, 1987, Farinas, 1999). These molecules are produced in limited quantities: the idea that innervating neurons compete for this limited supply is referred to as the ‘neurotrophic hypothesis’. Excess neurons and those forming inappropriate connections die by apoptosis, allowing the survival of the appropriate number of neurons.

Several lines of research led to the formulation of this hypothesis. *In vivo* experiments involving sympathetic and sensory neurons, that are shown to be dependent upon Nerve Growth Factor (NGF) *in vitro*, illustrate that administration of anti-NGF antibodies during the time of target innervation results in a death of these neurons (Levi-Montalcini, 1987). Furthermore, addition of exogenous NGF allows survival of neurons that would normally be lost, producing an excess of neurons innervating their targets (Levi-Montalcini, 1987).

The use of transgenic mice illustrates and confirms these findings. Mice with targeted null mutations for NGF or its receptor tyrosine kinase TrkA have a loss of

the same populations of peripheral neurons, whereas over-expressing NGF in target fields causes an excess of neurons to survive and innervate their target fields (Lewin and Barde, 1996). Studies of the timing, location and level of expression of NGF during development have reinforced this 'neurotrophic hypothesis'. Target fields start producing NGF at a time coinciding with target field innervation (Davies et al., 1987; Clegg et al., 1989), and the levels of NGF produced are proportional to the number of neurons innervating it (Harper and Davies, 1990). Target ablation experiments also confirm this, with increased cell death observed when specific developmental targets are removed (Caldero et al., 1998).

The neurotrophic hypothesis has become more elaborated in recent years with the recognition that survival and development of different subpopulations of peripheral neurons are dependent upon the presence of a variety of neurotrophic factors both before and after target field innervation. Sources of NTFs, other than targets, exist, with NTFs produced by cells along the route of axon extension or via autocrine pathways (Caldero et al., 1998; Maina et al., 1998; Wright et al., 1992; Robinson et al., 1996). Other work has shown that the particular NTF requirements of certain neurons change during development (Davies et al., 1994, 1997; Buchman and Davies, 1993; Ernfors et al., 1994a, 1994b; Francis et al., 1999; Zhou and Rush 1995).

1.5.2. Roles of neurotrophic factors in the developing sensory neurons

This thesis focuses upon the sensory neuronal portion of the PNS, and, in particular, upon neurons of the trigeminal ganglia, nodose ganglia and DRG. For this reason, the roles of various neurotrophic factors in the development of these sensory ganglia will be briefly discussed.

Trigeminal ganglia

Mouse trigeminal ganglia sensory neurons require several neurotrophic factors at different times through development. Initially when their axons are growing towards their targets, the neurons survive independently of neurotrophic factors. They become neurotrophin dependent following target innervation (Davies and Lumsden, 1984; Vogel and Davies, 1991). Following sensory neuron target innervation, neurons are transiently supported by Brain Derived Neurotrophic Factor (BDNF) and Neurotrophin-3 (NT-3), before becoming dependent upon NGF at approximately embryonic day 13 (E13)⁵. Survival of cultured embryonic day 10 (E10) trigeminal neurons is sustained and even increased in the presence of NT-3 or BDNF for 48 hours. However, this effect is not observed in cultured E13 neurons, which show only 5% survival after 48 hours. At this stage, NGF has become essential for survival (Buchman and Davies, 1993; Buj-Bello et al., 1994). This suggests a transitory survival response to BDNF and NT-3 and a switch in neurotrophin dependence to NGF (Buchman and Davies, 1993), which was confirmed by following the neurotrophic factor requirements of BrdU-labelled subsets of neurons in culture (Enokido et al., 1999). This change in dependence coincides with suitable alterations in Trk receptor expression, from initially high levels of TrkB (the preferred receptor for BDNF) and TrkC (the preferred receptor for NT-3), with low levels of TrkA to an increase in TrkA expression and a restriction of TrkB and TrkC in certain subpopulations (Arumae et al., 1993; Wyatt and Davies, 1993; Ninkina et al., 1996). The identity of neurotrophin production from target tissues is also altered, showing neurons respond in sequence to target-derived NTFs (Buchman and Davies, 1993; Enokido et al., 1999; Davies et al., 1987). It is of note that “late born” trigeminal neurons (those born after target innervation) do not show this switch in responsiveness, and require NGF from the offset (Enokido et al., 1999; Huang et al., 1999). The use of transgenic mice also illustrates this switch from BDNF to NGF, with TrkB^{-/-} mice showing a loss in trigeminal neurons at much earlier stages than TrkA^{-/-} (Pinon et al., 1996).

⁵ Stages of embryonic development. E1 refers to embryonic day 1, the first day a vaginal plug is found indicating embryonic conception.

This early switch from BDNF and NT-3 to NGF is not the only switch that occurs during the development of trigeminal neurons. At late fetal stages and in the neonatal period, trigeminal neurons become responsive to members of other families of neurotrophic factors, such as macrophage stimulating protein (MSP) (Forgie et al., 2003) and also the cytokine, ciliary neurotrophic factor (CNTF) (Horton et al., 1998). Additionally, it has been reported that mice with a null mutation in the gene encoding the Glial Cell Line-Derived Neurotrophic Factor (GDNF) family member neurturin (Neurturin^{-/-} mice) have a reduced number of trigeminal ganglion neurons at postnatal ages. This suggests that neurturin is responsible for the normal development of a subpopulation of trigeminal ganglia neurons at this late stage of development (Heuckeroth et al., 1999).

Nodose ganglia

Unlike trigeminal neurons, nodose neurons do not show an early developmental switch in neurotrophic factor requirements. These neurons depend mainly upon BDNF and to a lesser extent NT-3 during early embryonic development (Buj-Bello et al., 1994). A minor, subpopulation of nodose neurons are also dependent upon NGF for survival (Forgie et al., 2000).

Dorsal Root Ganglia

DRG contain subsets of neurons that respond to and require NGF, BDNF and NT-3 during embryonic development (Lindsay et al., 1985; Davies et al., 1986; Kalcheim et al., 1987). Experiments on chick sensory DRG neurons have also shown that BDNF produced by an autocrine route, is likely to allow maturation - but not survival of - early embryonic neurons prior to target innervation (Davies and Wright, 1995).

Following birth, a subpopulation of small diameter neurons, initially responsive to NGF, begin to down-regulate TrkA and become dependent upon GDNF family members *in vivo* (Tessarollo et al., 1993; Bennett et al., 1996; Molliver and Snider, 1997; Molliver et al., 1997). This subpopulation is immunoreactive for the isolectin-

B4 (I-B4) and thus is often referred to as the IB4 reactive subpopulation. By late post-natal ages, small diameter unmyelinated DRG neurons are categorised into two types: those with a high affinity for NGF and express TrkA, and those that are not NGF responsive and are characterised by an ability to bind the isolectin IB4. The neuropeptides CGRP and SP are expressed by the TrkA-positive neurons, whilst the IB4 reactive neurons express the neuropeptide somatostatin, the enzyme thiamine monophosphatase (TMPase), the P2X3 receptor and also the GDNF family receptor components: c-ret and GDNF Family Receptor α (GFR) family members. Further research has shown that despite survival responses by GDNF family ligands only occurring postnatally, expression of GDNF family receptors occurs embryonically (Molliver et al., 1997; Baudet et al., 2000). It would, therefore, seem that neurotrophins are essential in embryonic development, although some subpopulations switch dependence to GDNF family members following birth.

By adulthood, sensory neurons can survive both *in vitro* and *in vivo* without the need for neurotrophic factor support (Lindsay, 1988). However, neurotrophic factors continue to have roles following neuronal insult by damage or disease. Roles include axonal regeneration and maintenance of neuronal phenotype. They can also alter the expression of other sensory neuron specific genes in such conditions, producing neuroprotection or enhancing the associated hyperalgesia.

1.6. The biology of the neurotrophic factors

Since the discovery of NGF and the elucidation of the neurotrophic factor hypothesis, several other neurotrophins – “Brain-Derived Neurotrophic Factor” (BDNF), “Neurotrophin-3” (NT-3), “Neurotrophin-4/5” (NT-4/5), “Neurotrophin-6” (NT-6) and “Neurotrophin-7” (NT-7) - have been characterised. Other families of proteins with similar survival promoting effects have also been discovered. These molecules are collectively referred to as neurotrophic factors, although some were initially characterised for their regulatory properties in other systems, e.g. macrophage stimulating protein (MSP) and leukaemia inhibitory factor (LIF).

The main families of neurotrophic factors to date are the neurotrophins (NGF, BDNF, NT-3, NT-4/5, NT-6 and NT-7), the GDNF family (GDNF, persephin, neurturin and artemin), the neuropoietic cytokines (Ciliary neurotrophic factor, CNTF); leukaemia inhibitory factor, LIF; Oncostatin M, OsM; cardiotrophin-1, CT-1; interleukin-6, IL-6; interleukin-11, IL-11; cardiotrophin-like cytokine, CLC; neuropoietin) and two cytokine related factors, also known as plasminogen related growth factors (Hepatocyte Growth Factor, HGF, and Macrophage Stimulating Protein, MSP). These families, the individual members and their receptors are outlined in table 1.

FAMILY	NEUROTROPHIC FACTOR	ABBREVIATION	RECEPTOR (NON-PREFERRED IN BRACKETS)
NGF	Nerve Growth Factor	NGF	TrkA
	Brain-Derived Neurotrophic Factor	BDNF	TrkB
	Neurotrophin-3	NT-3	TrkC (TrkA, TrkB)
	Neurotrophin-4/5	NT-4/5	TrkB
	Neurotrophin-6	NT-6	TrkA
	Neurotrophin-7	NT-7	TrkA
GDNF	Glial Cell Line Derived Neurotrophic Factor	GDNF	GFR α 1 (GFR α 2)
	Neurturin	NTN	GFR α 2 (GFR α 1)

	Artemin		GFR α 3 (GFR α 1)
	Persephin		GFR α 4
Plasminogen – Related Growth Factors	Macrophage Stimulating Protein / Scatter Factor	MSP / SF	STK/Ron
	Hepatocyte growth factor	HGF	Met
Neuropoietic Cytokines	Ciliary Neurotrophic Factor	CNTF	CNTFR α bound to heterodimer of LIFR β and gp130
	Leukemia Inhibitory Factor	LIF	Heterodimer of LIFR β and gp130
	Oncostatin M	OsM	Heterodimer of OsMR and gp130
	Cardiotrophin-1	CT-1	As yet unknown α subunit, bound to heterodimer of LIFR β and gp130
	Cardiotrophin- Like-Cytokine	CLC	CNTFR α bound to heterodimer of LIFR β and gp130
	Interleukin-6	IL-6	IL6R α bound to gp130 homodimer
	Interleukin-11	IL-11	IL11R α bound to gp130 homodimer

	neuropoietin		CNTR α bound to heterodimer of LIFR β and gp130
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Table 1.3. *The main neurotrophic factor families*

As well as effects on neuronal survival, research has demonstrated numerous other roles for NTFs throughout development and in the adult. Effects on neurite outgrowth, regeneration, axonal branching, precursor proliferation, neuronal form and synaptic function have all been described, as well as effects on gene expression and maintenance of neuronal phenotype following neuronal damage. Effects in systems other than the nervous system have also been extensively documented.

The most relevant of these factors and their receptors, their biology, expression patterns and regulation of expression will now be discussed.

1.6.1. The neurotrophins

The neurotrophin family of neurotrophic factors consists of nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and the two fish neurotrophins neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7). They are all produced as precursors which are proteolytically cleaved to produce the mature molecule, made up of a homodimer of approximately 120 amino acid subunits. The family shares approximately 50% sequence similarity (Radziejewski et al., 1992). The crystal structures of the mature neurotrophins have been determined. They reveal a common tertiary fold and cysteine knot (McDonald et al., 1991; Fandl et al., 1994; Robinson et al., 1995; Butte et al., 1998; Robinson et al., 1999).

All neurotrophins have specific and essential roles in the survival, differentiation and gain of phenotype of particular subpopulations of neurons of the CNS and/or the PNS throughout development. Although adult neurons can survive independently of neurotrophic factor support, neurotrophins have been implicated in responses following injury or neuronal damage. They may act sequentially, in synergy or in opposition.

Initial efforts to isolate neurotrophin receptors led to the discovery of the common neurotrophin receptor p75^{NTR}. Initially, this receptor was thought specific for NGF, but further research by Rodriguez-Tebar et al., (Rodriguez-Tebar et al., 1991) revealed it could bind all of the neurotrophin ligands with similar affinity. Despite similar binding strength, the binding rate constants were found to be markedly different, suggesting that p75 may, to some extent, discriminate between ligands (Rodriguez-Tebar et al., 1992).

The Trk family of tyrosine kinase neurotrophin receptors were discovered later. This family consists of TrkA, TrkB and TrkC, with NGF, BDNF and NT-3 binding preferentially to TrkA (Cordon-Cardo et al., 1991; Kaplan et al., 1991, 1991b; Klein et al., 1991a) TrkB (Glass et al., 1991; Squinto et al., 1991; Klein et al., 1991b) and TrkC (Lamballe et al., 1991), respectively. Additionally, NT-3 can also bind, but with lower affinity, to TrkA and TrkB (Squinto et al., 1991; Klein et al., 1991b; Soppet et al., 1991), and NT-4, can also signal via TrkB (Klein et al., 1992; Conover et al., 1995). These binding patterns are illustrated in figure 1.7.

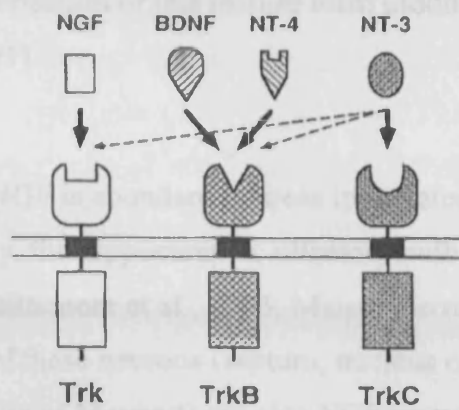


Figure 1.7:

Binding of neurotrophins to their preferred receptors (Bold arrows), and other ligand receptor interactions that can occur (dashed arrows). Taken from Barbacid M, 1994

Upon ligand binding, the Trk receptors form homodimers, allowing phosphorylation of tyrosine residues within the receptor, leading to signal transduction. The Trk family and signalling will be discussed further following descriptions of each neurotrophin.

1.6.1.1. Nerve growth factor (NGF)

The classic neurotrophic factor, NGF, was discovered by chance, when application of a snake venom to chick sensory ganglia was shown to elicit neurite outgrowth (Cohen and Montalcini, 1956). A non-dialyzable, heat labile protein responsible for this neurotrophic activity was purified from the fraction and was named nerve growth factor (Cohen et al., 1960). Angeletti and Bradshaw (Angeletti and Bradshaw, 1971) determined the amino acid sequence of mouse NGF, and subsequently cDNAs of mouse, human, bovine and chick NGF have all been cloned (Scott et al., 1983; Ullrich et al., 1983; Meier et al., 1986; Ebendal et al., 1986). Mouse NGF is initially produced as a 307 amino acid precursor that is cleaved to leave the mature, 118 amino acid protein which contains the cysteine knot motif characteristic of the neurotrophins (Berger and Shooter, 1977; Scott et al., 1983;

Edwards et al., 1988). Dimerisation of this mature form produces biologically active NGF (McDonald et al., 1991).

In the rat nervous system, NGF is abundant in areas innervated by the magnocellular cholinergic neurons, namely, the hippocampus, olfactory bulb and cerebral cortex (Korsching et al., 1985; Whittemore et al., 1986; Maisonpierre et al., 1990). Regions containing the cell bodies of these neurons (septum, nucleus of the diagonal band of Broca and the nucleus basalis of Meynert) are also NGF immunoreactive (Korsching et al., 1985). Accordingly, NGF only supports the survival of basal forebrain cholinergic neurons within the CNS (Chun and Patterson, 1977; Levi-Montalcini, 1987; Hatanka et al., 1988; Hartikka and Hefti, 1988). Other areas of the CNS also express NGF, but at much lower levels (Korsching et al., 1985; Whittemore et al., 1986). Outside the CNS, NGF is expressed by the target tissues of NGF-responsive sensory and sympathetic neurons in proportion to their innervation density (Davies et al., 1987; Heumann et al., 1984; Korsching et al., 1985; Korsching and Thoenen, 1983, 1988; Shelton and Reichardt, 1984; Harper and Davies, 1990).

Recent work has shown that the precursor to NGF, proNGF, selectively induces cell death through activation of p75 by a mechanism that is dependent upon presence of the membrane protein sortilin (Lee et al., 2001; Ibanez, 2002; Nykjaer et al., 2004). This signalling pathway is thought to be important following neuronal injury when expression of both p75 and proNGF are up-regulated (Beattie et al., 2002; Harrington et al., 2004). This pathway has been proposed as the route for elimination of damaged cells.

NGF binds not only to p75, the receptor common for all neurotrophins, but also to the receptor tyrosine kinase TrkA. TrkA was the first member of the Trk family of neurotrophin receptors to be discovered. This 140 kDa protein has a restricted expression, only being detectable centrally in cholinergic neurons of the basal forebrain and the striatum (Vasquez and Ebendal 1991, Holtzman et al., 1992; Merlio et al., 1992; Steininger et al., 1993) and sympathetic neurons and the neurons of the

DRG and cranial ganglia that require NGF for survival (Martin-Zanca et al., 1990; Tessarollo et al., 1993; Schropel et al., 1995). In the DRG of embryonic day 17.5 (E17.5) mice, the majority of neurons express TrkA (Tessarollo et al., 1993), but following birth, a subpopulation of small diameter DRG neurons down-regulate TrkA and become responsive to GDNF family ligands (Molliver et al., 1997). This subpopulation are also immunoreactive for the isolectin-B4 (I-B4), and are therefore often called the “IB4-reactive subpopulation”. Outside the nervous system, TrkA is expressed on T cells and monocytes (Ehrhard et al., 1993, 1993a).

Transgenic mice with null mutations in the TrkA and the NGF genes have facilitated a clearer understanding of the biology of this signalling pathway. Both TrkA^{-/-} and NGF^{-/-} mice die shortly after birth with massive loss of sympathetic neurons and small diameter sensory neurons in DRG and trigeminal ganglia (Smeyne et al., 1994; Crowley et al., 1994). The small diameter sensory neurons that are lost are responsible for transmission of nociceptive information: this is indicated by a failure of both NGF^{-/-} and TrkA^{-/-} mice to respond to noxious stimuli (Smeyne et al., 1994; Crowley et al., 1994). The size of the other main population of neurons that expresses TrkA, the basal forebrain cholinergic neurons of the CNS, is unaffected in both null mutations. This would suggest that NGF/TrkA signalling is not required for the formation and survival of these neurons. However, there is a reduced expression of choline acetyltransferase (ChAT) in the basal forebrain cholinergic neurons in both NGF^{-/-} mice, suggesting that although NGF might not be directly involved with cholinergic neuron survival, it may affect the function of these neurons (Crowley et al., 1994). Because NGF^{-/-} and TrkA^{-/-} mice die shortly after birth, it is not possible to examine the importance of this ligand receptor system post-natally in these mice.

The expression of TrkA by basal forebrain cholinergic neurons has raised the possibility that activation of NGF/TrkA signalling in neurodegenerative disorders associated with loss of such neurons may be therapeutically valuable. The potential for NGF in the treatment of Alzheimer’s disease (AD) was highlighted several years ago by grafts of cells genetically engineered to produce NGF into the brain of

primates. These grafts were shown to inhibit the cholinergic neuron degeneration that is associated with the onset of AD (Tuszynski et al., 1996; Martinez-Serrano et al., 1995). More recently, phase I clinical trials have been carried out. In these trials, fibroblasts, engineered to express NGF, were implanted into the brains of 8 patients with mild AD. No adverse effects due to the presence of NGF were found after 22 months, and its presence led to a decline in the rate of cognitive degeneration (Tuszynski et al., 2005).

The expression of TrkA in the small diameter nociceptive sensory neurons has implicated a role for NGF/TrkA signalling in nociception. In support of this is the finding that NGF^{-/-} and TrkA^{-/-} mice fail to respond to noxious stimuli (Smeyne et al., 1994; Crowley et al., 1994), and that direct administration of NGF leads to pain and hyperalgesia in human and rodents (Dyck et al., 1997; Lewin et al., 1993). Furthermore, in inflammatory conditions such as arthritis, an increase in NGF mRNA and NGF protein have been detected in the inflamed tissues (Aloe et al., 1992; Aloe et al., 1993; Falcini et al., 1996; Halliday et al., 1998; Lowe et al., 1997; Miller et al., 2002). Additionally, application of inhibitors to NGF attenuates pain induced by the inflammatory agents CFA and carrageenan (Woolf et al., 1994; Safieh-Garabedian et al., 1995; McMahon et al., 1995; Dmitrieva et al., 1997; Delafoy et al., 2003). NGF is thought to contribute to the associated hyperalgesia through sensitisation of mast cells (Bischoff and Dahinden, 1992) and through increased production of the neuropeptides CGRP and SP. NGF also potentiates nociceptive signalling pathways by regulating expression of the capsaicin receptor VR1 and the acid sensing ion channel 3 (ASIC3) (Ji et al., 2002; Amaya et al., 2004; Shu and Mendell, 1999; Mamet et al., 2003). In addition to its contribution to inflammatory pain, NGF has a role in promoting neuropathic pain in the chronic constriction injury (CCI) model of this kind of pain (Herzberg et al., 1997; Ro et al., 1999). Taken together, the above findings suggest therapeutic roles for antagonists of NGF in the management of neuropathic and inflammatory pain.

1.6.1.2. Brain-derived neurotrophic factor

BDNF was first isolated from pig brain using its ability to support survival of cultured chick embryonic sensory neurons as an assay (Barde et al., 1982). Survival *in vivo* was not demonstrated until 1988, when BDNF became accepted as a neurotrophic factor similar to NGF (Hofer and Barde, 1988). Porcine, human, mouse and rat BDNF have since been cloned and characterised (Leibrock et al., 1989; Hofer et al., 1990; Jones and Reichardt, 1990; Maisonpierre et al., 1991). Like NGF, BDNF is secreted as a precursor molecule which is proteolytically cleaved to produce the approximately 120 (13-14kDa) amino acid mature active protein (Leibrock et al., 1989) which dimerizes to form a homodimer (Radziejewski et al., 1992). The mature form of BDNF shares approximately 50% homology with NGF (Maisonpierre et al., 1991).

Expression of BDNF is low early in CNS development, but increases with maturation, with notable expression in cortex, hippocampus and cerebellum of adult mice (Maisonpierre et al., 1990). It is also detected, at lower levels, in striatum, hindbrain, midbrain, olfactory bulb and spinal cord (Leibrock et al., 1989; Hofer et al., 1990). In the PNS, BDNF is expressed embryonically by sympathetic ganglia, and sensory ganglia including DRG and trigeminal ganglia (Schechter and Bothwell, 1992). It is also expressed in target tissues of nodose ganglia and vestibular ganglia neurons during development (Robinson et al., 1996). Outwith the nervous system, BDNF mRNA transcripts are detectable in tissues innervated by BDNF responsive neurons, namely those of the heart, lung and skeletal muscle (Maisonpierre et al., 1990, 1991; Buchman and Davies, 1993).

BDNF can support survival of retinal ganglion cells (Johnson et al., 1986; Thanos et al., 1989), basal forebrain cholinergic neurons (Alderson et al., 1990; Knusel et al., 1991) and prevent axotomy-induced degeneration of postnatal rat motor neurons (Sendtner et al., 1992; Koliatsos et al., 1993). It can also promote survival of dopaminergic neurons of the substantia nigra (Hyman et al., 1991; Knusel et al.,

1991; Altar et al., 1992), which would implicate this neurotrophin in the therapeutic treatment of Parkinson's disease. This will be discussed later.

In the PNS, target-derived BDNF has been shown to promote survival of neural crest derived DRG (Lindsay et al., 1995; Davies et al., 1986; Kalcheim et al., 1987) and also populations of sensory neurons insensitive to NGF, i.e. those of the placode-derived nodose ganglia (Davies et al., 1986; Lindsay et al., 1985; Buj-bello et al., 1994).

Experiments on early chick DRG neurons have also shown that BDNF produced by an autocrine route is likely to allow maturation, but not survival, of these neurons prior to target innervation (Davies and Wright, 1995). This autocrine route of BDNF production may also occur in adult DRG neurons for survival purposes (Acheson et al., 1997). Adult mouse DRG neurons can survive in culture independently of exogenous NTFs. However application of antisense oligonucleotides to BDNF, which inhibit expression of BDNF, results in a dramatic loss of neurons (Acheson et al., 1997). This loss could be reversed by application of BDNF but not by any other NTF. Recent work has shown that in the same way as proNGF, proBDNF, the precursor of BDNF, also affects neuronal survival, inducing neuronal apoptosis in cells co-expressing p75 and a co-receptor sortolin (Teng et al., 2005).

Analysis of the product of the *trkb* gene, gp145trkB in NIH 3T3 cells, found that it could be phosphorylated by BDNF (Soppet et al., 1991), indicating that this receptor was the high affinity BDNF receptor. Other groups have confirmed or shown BDNF affinity and signalling for TrkB (Squinto et al., 1991; Soppet et al., 1991; Glass et al., 1991; Klein 1991b). NT-3 can also activate TrkB as well as its preferred receptor TrkC (Klein et al., 1991, 1992; Soppet et al., 1991).

TrkB is a 145kDa protein that shares approximately 69% homology with TrkA (Klein et al., 1989). TrkB can be detected within the mouse embryo at E9.5 (Klein et al., 1990), and unlike the restricted expression profile of TrkA, TrkB shows widespread expression in the CNS, with expression being particularly notable in tissues of the hippocampus, hypothalamus, brainstem, cerebellum and spinal cord

motor neurons (Klein et al., 1989; Merlio et al., 1992). In the PNS, TrkB is also widely expressed, being detectable in almost every peripheral ganglion (Klein et al., 1989; Ninkina et al., 1996; Carroll et al., 1992; Tessarollo et al., 1993; Lamballe et al., 1994). TrkB is also found in Schwann cells (Carroll et al., 1992) and is observed outside the nervous system in lung, muscle and ovaries (Klein et al., 1989).

The importance of BDNF in neuronal development is highlighted by the phenotype of BDNF^{-/-} mice. The majority of BDNF^{-/-} mice die shortly after birth (Ernfors et al., 1994; Jones et al., 1994). Such mice have severe deficits of co-ordination and balance due to the loss of the majority of vestibular sensory neurons (rather than cerebellar dysfunction). They also have reduced numbers of other cranial and DRG sensory neurons. Detailed analysis has shown that there are no defects in motor or sympathetic neurons (Ernfors et al., 1994; Jones et al., 1994). Research using mice with either a conditional mutation in the BDNF gene or who carry a deletion in one copy of the BDNF gene shows that these mice have impaired learning abilities, suggesting a role for BDNF in learning and memory, possibly through effects on long term potentiation (LTP)⁶ (Linarsson et al., 1997; Minichello et al., 1999). Mice with a disruption in the TrkB gene, can survive up to birth. However feeding mechanisms are absent and so most die by Postnatal day 1 (P1) (Klein et al., 1993). The observation that the phenotype of TrkB^{-/-} mice is more drastically affected than BDNF^{-/-} mice is consistent with the finding that NT-4/5 and NT-3 can also signal via this receptor (Conover et al., 1995; Soppet et al., 1991; Klein et al., 1991, 1992).

The effects of BDNF on motor neurons following spinal nerve injury have also been observed in mature adult mice. In this model, application of BDNF following the axotomy of spinal motor neurons attenuated injury-induced alterations in neuronal phenotype (Friedman et al., 1995). BDNF has also been shown to have important effects on neuro-regeneration in adult neurons. When fetal spinal cord was transferred into rat spinal cord following complete spinal cord transection, it was found that addition of exogenous BDNF (or NT-3) promoted axonal growth, allowing some functional restoration of anatomical connections (Coumans et al.,

⁶ LTP is the process in which a synapse can be strengthened (or potentiated) by repetitive stimulation. It is thought to contribute to synaptic plasticity producing an adaptable nervous system.

2001). Furthermore, addition of antibodies against BDNF following peripheral nerve injury significantly attenuated the length of the regenerated nerve by 24% and produced abnormalities in the lamellar layers of the myelin sheath (Zhang et al., 2000).

BDNF also has a nociceptive role, regulating spinal cord excitation. Following inflammatory stimulus and nerve injury, an increase in BDNF contributes to the associated central sensitisation of spinal processing and subsequent hyperalgesia (Chao et al., 1997a, b; Tonra et al., 1998; Michael et al., 1999; Kerr et al., 1999; Ha et al., 2001; Yajima et al., 2002). Evidence has suggested that it may act as a neurotransmitter in the pain pathway of adult animals (Thompson et al., 1999). Antagonists of BDNF might therefore be of therapeutic use in the treatment of hyperalgesia associated with persistent inflammatory states or following nerve damage.

Parkinson's disease (PD) is a neurological disorder associated with the progressive loss of dopaminergic neurons from the substantia nigra pars compacta (SNc). Current treatments involve administration of levodopa (L-Dopa), which aims to replenish the diminishing levels of dopamine, but produces significant, distressing side-effects and ultimately does not address the neuronal degeneration at the route of the disorder. The discovery that BDNF can exert neurotrophic factor effects on dopaminergic neurons (Hyman et al., 1991; Knusel et al., 1991; Altar et al., 1992; Klein et al., 1999), associated with a down-regulation of BDNF mRNA and protein in substantia nigra of sufferers of PD (Mogi et al., 1999; Howells et al., 2000), raised the possibility that BDNF might have a role in the treatment of this disorder. Gene transfer of BDNF into rat nigrostriatal neurons, prior to 6-hydroxydopamine (6-OHDA)⁷ lesion, led to significant reduction in the degeneration of dopaminergic neurons and also corrected behavioural deficits (Sun et al., 2005; Mohapel et al., 2005). Research by Guillin et al., (Guillin et al., 2001, 2003) also showed that TrkB was co-expressed with the D3 dopamine receptor and that BDNF produced by dopaminergic neurons stimulates the production of D3 receptors in the nucleus

⁷ 6-hydroxydopamine (6-OHDA) is a neurotoxin used to specifically kill dopaminergic and noradrenergic neurons

accumbens during development and maintains its expression in the adult. Agonists of the D3 receptor have been shown to inhibit the L-dopa induced dyskinesia, without affecting the beneficial therapeutic effects of this drug (Guillin et al., 2003). BDNF has also been shown to augment effects of levodopa. It is thus clear that BDNF, through regulation of D3 receptor production, is of potential therapeutic use, enhancing the effects of L-Dopa treatment, whilst attenuating troublesome side effects.

1.6.1.3. Neurotrophin-3

NT-3 was the third neurotrophin discovered. The gene encoding it was cloned following comparisons of sequence homologies of BDNF and NGF and the design of oligonucleotides complimentary to regions of such homology (Ernfors et al., 1990; Maisonpierre et al., 1990; Hohn et al., 1990; Rosenthal et al., 1990). Like NGF and BDNF, NT-3 is synthesized as a precursor which is cleaved to generate a mature active protein of 119 amino acids (approximately 13.6kd) with 57.6% and 55.6% sequence identity to NGF and BDNF, respectively (Rosenthal et al., 1990). The mature form exists as a homodimer (Radziejewski et al., 1992). In the developing embryo, NT-3 is expressed very early, peaking at E4.5 and declining steadily thereafter. It is broadly expressed, detectable in peripheral tissues including heart, liver, spleen, kidney and lung. In the CNS, it is expressed at greatest concentrations in the hippocampus and cerebellum (Ernfors et al., 1990; Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990). Expression of NT-3 is initially high in regions of developing CNS, but levels decrease as maturation progresses (Maisonpierre et al., 1990).

NT-3 has neurotrophic factor activity in many of the neuronal populations of the CNS. Embryonic noradrenergic neurons of the locus coeruleus (LC) can be supported in culture by NT-3, while in adult neurones, its presence can prevent 6-OHDA-induced degeneration of such neurons (Arenas and Persson, 1994). NT-3 can support survival of embryonic rat motor neurons (Henderson et al., 1993), but cannot rescue postnatal motor neurons from axotomy-induced degeneration (Sendtner et al.,

1992; Koliatsos et al., 1993). It is also essential for the survival and differentiation of oligodendrocyte precursors (Barres et al., 1993, 1994; Rubio et al., 2004).

In the PNS, NT-3 is important in initial survival of NC-derived trigeminal sensory ganglia just after target innervation and prior to a switch in responsiveness to NGF (Buchman and Davies, 1993; Buj-bello et al., 1994; Wilkinson et al., 1996) (see 1.5.2.). NT-3 can also promote survival of other sensory neuronal containing ganglia including the DRG, parts of the trigeminal mesencephalic nucleus and also the, placode-derived nodose sensory ganglia (Rosenthal et al., 1990; Ernfors et al., 1990; Maisonpierre et al., 1990; Hohn et al., 1990; Wilkinson et al., 1996). Other PNS neurons responsive to NT-3 include the sympathetic ganglia of the paravertebral chain (Zhou and Rush, 1995; Maisonpierre et al., 1990).

NT-3 also influences the development of PNS neuronal precursors, inducing their survival, differentiation and proliferation (Kalcheim et al., 1992; Pinco et al., 1993; DiCicco-Bloom et al., 1993; Verdi and Anderson, 1994; Karavanov et al., 1995; Memberg and Hall, 1995; Elshamy and Ernfors, 1996; Elshamy et al., 1996). NT-3 has also been suggested as having a role very early in PNS neuronal development, contributing to initial ganglia formation (Ockel et al., 1996).

Lamballe et al. (Lamballe et al., 1991) discovered the 145kDa protein receptor, TrkC, by screening a mouse brain cDNA library using the gene encoding TrkA as a probe. TrkC is first detected in the mouse embryo at E9.5 (Lamballe et al., 1991) and shows widespread expression, both throughout development and in the adult, being detectable in similar tissues as its ligand, NT-3 (Lamballe et al., 1994). Like TrkB, TrkC is widely distributed in the brain, with high expression in neocortex, caudate, putamen, brainstem, hypothalamus, hippocampus, cerebellum, and spinal cord motor neurons (Merlio et al., 1992; Tessarollo et al., 1993). Some tissues co-express TrkB and TrkC (Tessarollo et al., 1993). However, although general tissue expression is similar, the two receptors show distinct patterns of expression on specific

subpopulations of neurons, which suggests different survival roles for NT-3 and BDNF (Merlio et al., 1992).

Broad expression of TrkC is also observed in the PNS, detectable to some extent in most ganglia and also within migratory cells of the neural crest (Tessarollo et al., 1993). Expression of TrkC is also observed in the enteric nervous system and in non-neuronal tissues, including the vibrissae and dental papillae, the submandibular gland, the wall of the aorta and the brown adipose tissue surrounding the cervical spinal cord (Tessarollo et al., 1993; Lamballe et al., 1994). The distribution is similar to that of its ligand NT-3.

As stated earlier, NT-3 can also bind to TrkA and TrkB (Soppet et al., 1991; Klein et al., 1991b; Cordon-Cardo et al., 1991; Glass et al., 1991; Ip et al., 1993), albeit with lower affinity than their preferred ligands NGF and BDNF, and can also signal via these receptors. NT-3's effects on survival and neurite outgrowth of sympathetic neurons are mediated through TrkA rather than TrkC (Belliveau et al., 1997).

Mice with a null mutation in the gene encoding NT-3 die within a few weeks of birth and suffer from abnormal limb movement most likely due to a lack of muscle afferent projections to the spinal cord (Farinas et al., 1993; Ernfors et al., 1994). No defects in enteric or motor neurons are apparent, but affected animals show much loss of sensory neuron populations (Liebl et al., 1997). Disruption of the TrkC gene to remove the catalytic portion of the receptor also produces mice with severe proprioceptive sensory neuron loss (Klein et al., 1994). Unlike NT-3^{-/-} mice, some TrkC^{-/-} mice can survive for long periods following birth, and only show 30% loss of sensory neurons rather than the 70% deficit of the NT-3^{-/-} mice (Liebl et al., 1997). This probably illustrates the ability of NT-3 to signal through other Trks. NT-3 supports the survival of both cultured trigeminal and nodose ganglion neurons from TrkC^{-/-} mice, to an extent comparable to NGF and BDNF, respectively (Davies et al., 1995). This suggests that NT-3 can also signal via TrkA and TrkB in the absence of its preferred Trk receptor, TrkC. This has been further confirmed using studies of NIH 3T3 cells expressing various Trks, which illustrate that NT-3 can bind and

signal via TrkA and TrkB, although with lower potency than via TrkC (Cordon-Cardo et al., 1991; Glass et al., 1991; Ip et al., 1993) and by the observation that NT-3 can displace NGF from its preferred receptor (Ernfors et al., 1990).

In the adult, NT-3 has a number of effects following neuronal injury or insult. Thus, the presence of NT-3 has been shown to increase numbers of oligodendrocytes and thus enhance CNS myelin repair following chemical demyelination of CNS neurons (Jean et al., 2003). This action would suggest a potential therapeutic role for NT-3 in the treatment of the debilitating demyelination disorders such as MS. Additionally following injury, NT-3 acts in an antagonistic fashion to the pro-hyperalgesic effects of NGF (Wilson-Gerwing et al., 2005) by competition for TrkA binding sites (Gratto and Verge, 2003) and by decreasing the levels of BDNF (Karchewski, et al., 2002), SP and CGRP (Jongsma Wallin, 2001).

1.6.1.4. Neurotrophin-4/5

A fourth neurotrophic factor, was discovered by polymerase chain reaction (PCR) techniques, using oligonucleotides designed to contain regions conserved in NGF, BDNF and NT-3 (Berkemeier et al., 1991; Hallbook et al., 1991; Ip et al., 1991). This neurotrophin was originally identified in *Xenopus* oocytes (Hallbook et al., 1991) and named neurotrophin-4 (NT-4). Rat and human forms encoding a similar receptor were discovered shortly after but were named neurotrophin-5 (NT-5) (Hallbook et al., 1991; Ip et al., 1992). With approximately 65% identity, it was thought that NT-4 and NT-5 (Berkemeier et al., 1991; Ip et al., 1992), were likely to be identical: the factor is now referred to as NT-4/5. Like other neurotrophin family members, NT-4/5 is secreted as a precursor, although the pro-region of NT-4 is significantly shorter (approximately 60 amino acids) than NGF, BDNF and NT-3 (Berkemeier et al., 1991; Ip et al., 1992). The precursor is cleaved to produce mature active NT-4/5, which shares 50%, 56% and 55% homology to NGF, BDNF and NT-3 respectively (Berkemeier et al., 1991).

Rat NT-4 is widespread, detected in lung, thymus, muscle, ovary, heart and stomach both in the adult and in development (Berkemeier et al., 1991; Ip et al., 1992; Timmusk et al., 1993). Within the brain it is detected in many areas including cortex, pons, cerebellum, hippocampus, olfactory bulb and hypothalamus (Timmusk et al., 1993).

NT-4/5 has been shown to bind to (Klein et al., 1992) and signal (Ip et al., 1992) via TrkB. Weak tyrosine phosphorylation of TrkA was also observed for NT-4/5 in studies using NIH 3T3 cells (Ip et al., 1992).

NT-4/5 exerts neurotrophic factor effects on many subpopulations of neurons. In the CNS, it can promote survival and neurite outgrowth of cultured adult retinal ganglia cells (Cohen et al., 1995). It can also enhance ChAT activity in cultured embryonic cholinergic neurons of the basal forebrain and locus coeruleus (LC), and enhance their survival (Friedman et al., 1993). Effects on motor neurons have also been observed. NT-4/5 can promote survival of cultured corticospinal motor neurons from the neonatal rat (Junger et al., 1997) and rescue injury-induced loss of facial motor neurons *in vivo* (Koliatsos et al., 1994). Application to embryonic motor neurons can also stimulate differentiation to the cholinergic phenotype by up-regulation of (ChAT) (Wong et al., 1995). In the PNS, NT-4/5 can promote survival and neurite outgrowth of embryonic mouse trigeminal neurons *in vitro* at a time coinciding with early stages of target field innervation (Davies et al., 1993; Ibanez et al., 1993). Potent survival effects are also observed for NT-4/5 on cultured embryonic chick DRG neurons (Berkemeier et al., 1991; Ip et al., 1992) and for mouse, but not chick, nodose neurons at a time coinciding with naturally occurring cell death (Davies et al., 1993). Species differences in NT-4 effects are thus apparent, and suggest that NT-4 is not well conserved between mammals and birds (Davies et al., 1993). Additionally NT-4/5 can promote survival and neurite outgrowth of embryonic sympathetic ganglia neurons, but with low potency (Berkemeier et al., 1991; Hallbook et al., 1991).

In the adult, NT-4 is a potent stimulus to axon outgrowth in explanted mouse nodose ganglia (Wiklund et al., 2000). These effects on axonal outgrowth suggest a role for NT-4/5 in regeneration of neurons following injury (Blesch et al., 2004). After thoracic spinal cord injury in rats, application of NT-4/5 significantly improved axonal regrowth of motor axons, coeruleospinal, reticulospinal, and propriospinal neurons (Blesch et al., 2004). Positive effects of NT-4/5 on nerve regeneration were also observed following sciatic nerve transection (Yin et al., 2001; Simon et al., 2003). NT-4/5 regulation of gene expression following injury is also notable in the adult rat. NT-4/5 can prevent axotomy-induced changes in neuronal phenotype following axotomy of spinal motor nerves (Friedman et al., 1995) and also cholinergic hypoglossal motor neurons (Tuszynski et al., 1996)

Mice with a null mutation in the NT-4/5 gene reveal an essential role for NT-4/5 in development of a specific subpopulation of sensory neurons (Conover et al., 1995; Liu et al., 1995). NT-4/5^{-/-} mice are viable and fertile and have a milder neurological phenotype than other neurotrophin knockouts. They have a reduced number of nodose sensory neurons in comparison to wild-type mice (Conover et al., 1995; Liu et al., 1995). NT-4/5^{-/-} mice have abnormal and enlarged neuromuscular junctions with disassembled expression of the acetylcholine receptor (AChR), suggesting a role for NT-4/5 in the maintenance of neuromuscular connections (Belluardo et al., 2001).

1.6.1.5. Neurotrophin-6 and neurotrophin-7

The most recently described neurotrophins, NT-6 and NT-7, are exclusively present in fish. Compared with mammalian neurotrophins, they share greatest similarity with NGF and signal exclusively via TrkA (Lai et al., 1998; Nilsson et al., 1998). In some fish species (pufferfish and salmon) only one NT-6/7 like protein can be found (Dethleffsen et al., 2003). NT-6 and NT-7 have been distinguished as separate factors in other fish species, as they share relatively low sequence similarity (63%) (Nilsson et al., 1998) and their expression patterns are different (Gotz et al., 1994; Lai et al., 1998; Dethleffsen et al., 2003).

NT-6 was isolated from the teleost fish *Xiphophorus* and is expressed as early as stage 13 of embryonic development (Gotz et al., 1994). Although it has not been found in mammals, recombinant purified NT-6 has been shown to act in a similar fashion to NGF, promoting both neurite outgrowth and survival of chick sensory DRG explants, but with a much lower potency (Li et al., 1997). NT-6 differs from other neurotrophin family members in being glycosylated (Li et al., 1997). The biological significance of this glycosylation is yet to be determined.

The fish neurotrophin NT-7 was isolated from both carp (Lai et al., 1998) and zebrafish (Nilsson et al., 1998). Like NT-6, NT-7 is most similar to NGF (with 65% sequence identity) and acts exclusively via TrkA (Lai et al., 1998; Nilsson et al., 1998).

1.6.1.6 Neurotrophin receptors

Neurotrophins signal via two types of receptor: the common receptor p75, and the ligand specific Trk neurotrophin receptors. These receptors have been mentioned briefly whilst discussing the individual neurotrophins. More information about structure, signalling pathways, and splice variants follows in this section.

p75^{NTR}

The p75^{NTR} receptor was the first neurotrophin receptor discovered. p75^{NTR} has been isolated in rat, chicken and human, with all forms showing significant homology (Chao et al., 1986; Johnson et al., 1986; Radeke et al., 1987). Initially, p75^{NTR} was thought to be a specific NGF receptor, but was later shown to bind NGF, BDNF and NT-3 with equal affinity (Rodriguez-Tebar et al., 1991). p75^{NTR} is a distant member of the tumour necrosis factor (TNF) receptor family, containing the characteristic cysteine repeats in the extra-cellular domain. It is approximately 75kDa (Johnson et

al., 1987; Radeke et al., 1987) and contains 4 cysteine residues important for ligand binding (Welcher et al., 1991; Yan and Chao, 1991).

p75^{NTR} is widely expressed throughout the chick and rat brain both during development and in the adult, with highest levels in the cerebellum and septum (Ernfors et al., 1988; Buck et al 1988; Yan and Johnson, 1989). In the PNS of the chick, p75^{NTR} is present in embryonic premigratory neural cells and all the sympathetic, parasympathetic and sensory tissues derived from them (Heuer et al., 1990; Hallbook et al., 1990; Wyatt et al., 1990). Moreover, in chick sensory ganglia, p75^{NTR} expression is increased during development as the first axons reach their targets. It continues to increase throughout target innervation, plateauing when all neurons have reached their target field (Wyatt et al., 1990).

The role of p75^{NTR} is controversial and complex. In addition to regulating Trks signalling and neuronal responsiveness to neurotrophins, p75^{NTR} can also directly act as both an inhibitor and promoter of apoptosis, dependent upon presence and/or absence of neurotrophin ligands. The complex balance between concentration of Trks and p75^{NTR}, their direct and indirect actions and the presence and absence of endogenous or exogenous ligands, determine the cellular outcome of neurotrophin binding at different developmental stages. These factors will be discussed briefly here

p75^{NTR} as a regulator of Trk responsiveness

p75^{NTR} has been shown to alter responsiveness to neurotrophins. Studies using transgenic mice show that NT-3- induced survival of sympathetic neurons via TrkA, is more effective in the absence of p75^{NTR} (Brennan et al., 1999). Such an inhibitory effect on NT-3/TrkA mediated neurite outgrowth was also observed *in vitro* in cultured PC12 cells⁸ (Benedetti et al., 1993; Clary and Reichardt, 1994). Conversely cultured embryonic DRG and SCG neurons from p75^{-/-} mice show a decreased

⁸ PC12 cells are sympathetic neurons.

response to NGF, suggesting p75^{NTR} enhances NGF-induced TrkA signalling (Lee et al., 1994).

p75^{NTR}, a promoter of survival or apoptosis

As well as regulating Trk signalling, p75^{NTR} is able to regulate cellular survival in the absence of Trks. p75^{NTR} appears to both promote survival *and* apoptosis, depending upon the subpopulation of neurons. In the CNS, p75^{NTR} has been shown to promote death of chick retinal cells (Frade et al., 1996; Frade and Barde, 1998), spinal motor neurons (Frade and Barde, 1999), oligodendrocytes (Casaccia-Bonofil et al., 1996) and basal forebrain cholinergic neurons of the mouse (Van der zee et al., 1996; Yeo et al., 1997), whilst enhancing cell survival of rat hippocampal neurons in the presence of glutamate (Bui et al., 2002). In the PNS, p75^{NTR} promotes apoptosis of chick trigeminal neurons (Davey and Davies, 1998) and mouse sympathetic neurons of the SCG (Majdan et al., 2001), whilst enhancing survival of mouse sensory neurons of the DRG in the absence of trophic support (Barrett and Barlett, 1994; Longo et al., 1997).

Several signalling pathways have been implicated in the mediation of the effects of p75^{NTR}. Survival effects are thought to be mediated by the transcription factor NFκB (Carter et al., 1996; Hamanoue et al., 1999; Foehr et al., 2000; Gentry et al., 2000), whilst c-Jun N-terminal kinase (JNK) pathways have been implicated in pro-apoptotic effects (Casaccia-Bonofil et al., 1996; Yoon et al., 1998; Harrington et al., 2002). Induction of the intracellular mediator, ceramide, through hydrolysis of sphingomyelin (Dobrowsky et al. 1994,1995) has also been shown to occur following p75^{NTR} activation.

Binding of the zinc finger protein, neurotrophin receptor interacting factor (NRIF) has been shown to play a role in p75^{NTR}-induced apoptosis (Bamji et al., 1998; Casademunt et al., 1999). Tumor Necrosis Factor Receptor Associated Factor-6

(TRAF-6)⁹ has also been shown to mediate p75^{NTR} signalling via NFκB and JNK signalling (Foehr et al., 2000; Yeiser et al., 2004).

The effects of p75^{NTR} in certain cell types also vary depending on the presence or absence of neurotrophin ligands. In PC12 cells, p75^{NTR} was shown to enhance apoptosis in the absence of NGF (Rabizadeh et al., 1993), but in the presence of NGF, cell death by p75^{NTR} was inhibited (Rabizadeh et al., 1993). The use of antisense oligonucleotides against p75^{NTR} show that the survival response of early DRG neurons (E12-E15) to NGF is lost in the absence of p75^{NTR}. However, by E19 – P2, the absence of p75^{NTR} enhances the survival of DRG neurons in the absence of NGF (Barrett and Bartlett, 1994). These results not only highlight the effects of ligand upon p75^{NTR} - induced apoptosis, but also detail a developmental switch in sensory neurons of the role of p75^{NTR} as an inhibitor of apoptosis to one of promotion.. This role in apoptosis for p75^{NTR} could be important during the developmental periods of naturally occurring cell death that occur within both the PNS and CNS (Bamji et al., 1998; Casademunt et al., 1999).

Mice with a null mutation in the gene encoding the p75^{NTR} receptor are viable and fertile. However, sensory deficits due to defective innervation of TrkA expressing peptidergic sensory neurons lead to an impaired response to heat and noxious stimuli (Lee et al., 1992; Stucky and Koltzenberg, 1997; Bergmann et al., 1997). This could be due to alterations in the response to neurotrophins in the absence of p75^{NTR}, mentioned previously. It would seem that p75^{NTR} plays an essential part of the functional development of, and not just survival of, sensory neurons.

Regulation of p75^{NTR} expression

Several factors, most notably the neurotrophin NGF, have been shown to contribute to the regulation of p75^{NTR} expression. In cultured embryonic and adult sensory neurons, NGF promotes an increase in p75^{NTR} mRNA expression (Wyatt et al., 1993): Lindsay et al., 1990; Verge et al., 1992). Likewise, NGF enhances p75^{NTR}

⁹ Tumour Necrosis Factor (TNF) is a cytokine with a number of proinflammatory actions. TRAF-6 is a protein that can associate with TNF receptor to modify its action.

mRNA expression in neonatal and adult sympathetic neurons both *in vitro* and *in vivo* (Doherty et al., 1988; Miller et al., 1991, 1994; Verge et al., 1992). However, mice with a null mutation in the NGF gene show no alterations in p75^{NTR} expression within trigeminal ganglia (Davies et al., 1995), illustrating that the normal developmental expression pattern of p75^{NTR} is not dependent upon endogenous NGF.

p75^{NTR} expression on Schwann cells increases following nerve transection (King et al., 2000), and the number of myelinated axons and thickness of myelin is significantly reduced in p75^{-/-} mice following sciatic nerve injury, suggesting a role for p75^{NTR} in remyelination following peripheral nerve injury (Song et al., 2006). BDNF and NT-3 increase in p75 on Schwann cells at the lesion site (King et al., 2000), and a role for BDNF in the successful regeneration and remyelination of axons following nerve injury has been suggested previously (Zhang et al., 2000; Song et al., 2006). It is possible that these effects of BDNF are thus mediated through p75^{NTR} signalling on Schwann cells.

The Trk family

The second class of neurotrophin receptors, the Trk (tropomyosin kinase) family, were discovered after p75^{NTR}. This family has of three members: TrkA, TrkB and TrkC, with each member displaying several characteristic features. On the extracellular portion, three leucine repeat motifs are flanked by cysteine rich clusters. Two immunoglobulin (IgG)-like domains, adjacent to the transmembrane region are responsible for neurotrophin binding (Martin-Zanca et al., 1989; Perez et al., 1995) and an intracellular tyrosine kinase domain provides catalytic effects.

The *trk* proto-oncogene was first isolated from a human colon carcinoma biopsy (Martin-Zanca et al., 1989). This 140kDa glycoprotein was referred to as “gp140trk” and subsequently “TrkA”. Other *trk*-related transcripts were isolated by screening a mouse brain cDNA library using a human *trk* probe under relaxed hybridisation

conditions. This led to the discovery of the 145kDa (approximately) TrkB (Klein et al., 1989) and TrkC (Lamballe et al., 1991) receptors.

Splice variants

Several splice variants of the Trk receptors have been characterised. Two isoforms of TrkA exist, TrkAI and TrkAII, which differ by the insertion of a 6 amino acid insert into the extracellular domain (Barker et al., 1993). The presence of the insertion (TrkAII) appears to have no effect on either affinity of ligand binding or on receptor signalling. In the rat and human, only the TrkAII form is expressed in neuronal tissues at appreciable levels. TrkAI expression is found in non-neuronal cells such as mast cells (Barker et al., 1993; Horigome et al., 1993).

Two truncated TrkB transcripts, TrkB.T1 and TrkB.T2, have been observed. They are identical to the 9kb (145kDa) TrkB protein, but lack the cytoplasmic region containing the catalytic tyrosine kinase domain (Klein et al., 1990; Ninkina et al., 1996; Middlemas et al., 1991). This renders these isoforms catalytically inactive. Other isoforms, which contain deletions in the leucine-rich motifs of the extracellular domain, also exist (Ninkina et al., 1997).

The truncated 'inactive' forms of TrkB receptors appear later in development than the complete form (Escandon et al., 1994). The physiological role of these receptors is unsure. Experimental studies have shown that presence of the truncated forms can attenuate the survival response of BDNF, acting via catalytic TrkB. This could be due to competitive effects of two splice variants for BDNF binding, or be due to formation of inactive receptor heterodimers (Eide et al., 1996; Ninkina et al., 1996). Due to the location of the truncated versions on the choroidal plexus and ependymal linings of the cerebral ventricles, it has been proposed that they may mediate the active transfer of the ligand BDNF (or even NT-4/5) around the brain and might aid transfer across the blood brain barrier (Klein et al., 1990). Other suggestions include a role in the recruitment of ligand for the catalytic form of the TrkB receptor, or that these truncated versions are not receptors at all, but may function as cell adhesion

molecules, or ligands for an, as yet, unknown receptor (Klein et al., 1990; Middlemas et al., 1991).

At least four isoforms of the TrkC receptor have been identified. Three contain an insert into the kinase domain, while one is truncated and lacks the intracellular catalytic kinase domain (Tsoulfas et al., 1993; Valenzuela et al., 1993). Insertion of the TrkC isoforms into PC12 cells, reveal that those containing an insert in the kinase domain can still autophosphorylate in response to NT-3. However, it is unable to promote neurite outgrowth (Tsoulfas et al., 1993; Valenzuela et al., 1993; Guiton et al., 1995). It is of note that truncated TrkC is the only isoform found in astrocytes and non-neuronal cells (Valenzuela et al., 1993).

Neurotrophin Receptor signalling:

When Trk receptors bind with ligands they form homodimers. As a result, the autophosphorylation of specific tyrosine residues on the intracellular portion of the receptor can occur (reviewed in Kaplan and Stephens, 1994). The phosphorylated residues act as docking sites for intracellular signalling proteins that carry Src homology-2 (SH-2) domains or phosphotyrosine-binding (PTB) motifs. These adaptor molecules link Trks to signalling pathways, which include the phosphatidylinositol-3 kinase (PI3-K)/Akt kinase, the ras/ERK (extracellular signal-regulated kinase) pathway and the phospholipase C γ (PLC γ) pathways. Activation of these pathways ultimately leads to regulation of gene expression by stimulation of transcription factors.

Mutational studies have shown that the Shc family of proteins are important adaptor molecules for neurotrophin signalling and activation of the ras/ERK pathways (Minichello et al., 1998). The Shc family consists of ShcA, ShcB/SCK and the most recently discovered neuronal Shc (n-Shc) also known as "ShcC" (Nakamura et al., 1996). The neuronal form is active in the CNS, while other members have signalling roles in non-neuronal tissues. n-Shc binds to phosphotyrosine residues on Trks via two domains, either the Src homology domain-2 (SH-2) domain or the

phosphotyrosine binding domain (PTB) (Kavanaugh and Williams, 1994). This allows phosphorylation of tyrosine residues and subsequent binding to the Grb2 (Growth factor receptor bound protein 2) adaptor protein. Activation of the MAPK/ERK signalling pathway then ensues. This pathway involves activation of the G protein “ras”, the serine/threonine kinase “raf”, mitogen and extracellular regulated kinase (MEK) and the extracellular signal-regulated kinase (ERK) (Obermeier et al., 1994; Stephens et al., 1994; Marshall et al., 1995). Activated ERKs can then regulate gene expression via activation of specific transcription factors and produce effects on neurite outgrowth (Vambutas et al., 1995). This is illustrated more clearly in figure 1.8.

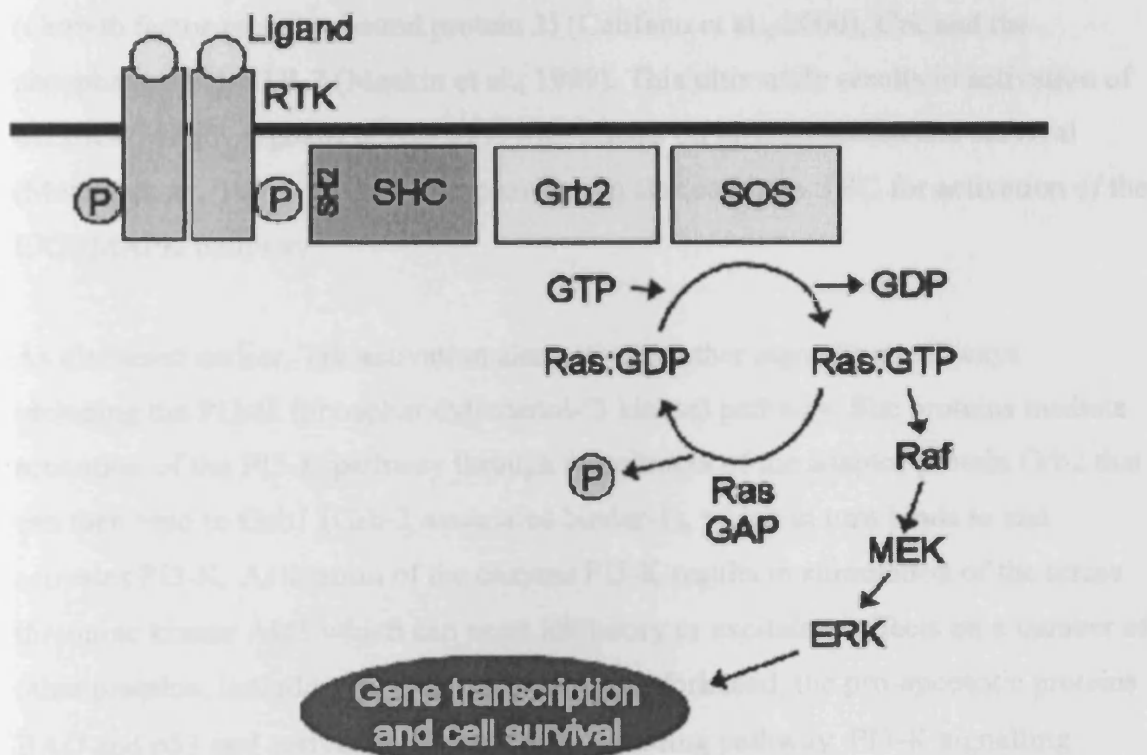


Figure 1.8. Signalling via the ras/ERK pathway. Taken from <http://fbspcu01.leeds.ac.uk/users/bmbatr1/apoptosis13.gif>

Two of the key phosphotyrosine residues required for activation of Ras/ERK signalling cascades were determined using PC12 cells carrying TrkA receptors with

mutations at phosphotyrosine sites Y490 and Y785 (Stephens et al., 1994; Obermeier et al., 1994). Mutation of tyrosine residues individually had no effect, but the combined mutation of both resulted in lack of activation of ERK and attenuation of the subsequent neurite outgrowth. This would indicate that phosphorylation of both Y490 and Y785 is important in activating the Ras/ERK pathway in PC12 cells.

Another adaptor protein, the 90kDa Suc1-associated tyrosine phosphatase-1 (SHP-1) or fibroblast growth factor receptor substrate-2 (FRS), is phosphorylated following neurotrophin binding. It is thought to be of importance in neurotrophin signalling via the ERK/MAPK pathway (Rabin et al., 1993; Ong et al., 1996). SHP-1 competes with SHC for phospho-Y490 on activated TrkA (Meakin et al., 1999). Phosphorylated SHP-1 is then able to bind to other adaptor proteins, including Grb2 (Growth factor receptor bound protein 2) (Califano et al., 2000), Crk and the phosphatase SH-PTP-2 (Meakin et al., 1999). This ultimately results in activation of the ERK/MAPK signalling pathway, with effects on differentiation and survival (Meakin et al., 1999). SHP-1 thus provides an alternative to SHC for activation of the ERK/MAPK pathway.

As discussed earlier, Trk activation also activates other signalling pathways including the PI3-K (phosphatidylinositol-3 kinase) pathway. Shc proteins mediate activation of the PI3-K pathway through recruitment of the adaptor protein Grb2 that can then bind to Gab1 (Grb-2 associated binder-1), which in turn binds to and activates PI3-K. Activation of the enzyme PI3-K results in stimulation of the serine threonine kinase Akt1 which can exert inhibitory or excitatory effects on a number of other proteins, including the transcription factor forkhead, the pro-apoptotic proteins BAD and p53 and activation of the NF κ B signalling pathway. PI3-K signalling produces effects on cell survival and gene transcription with a cascade of other effects (Reviewed in Huang and Reichardt 2001).

Phospholipase C γ 1 (PLC- γ 1) signalling pathways begin with binding of PLC- γ 1 directly to phosphorylated Y785 on activated TrkA (Vetter et al., 1991; Stephens et al., 1994). This leads to the generation of the second messengers inositol tris-

phosphate (IP3) and diacylglycerol (DAG). The increased level of IP3 mobilises Ca^{2+} from storage organelles causing an increase in intracellular Ca^{2+} . This allows activation of Ca^{2+} dependent enzymes including Ca^{2+} regulated forms of protein kinase C (PKC) and Ca^{2+} calmodulin dependent kinases and phosphatases. DAG also stimulates activation of PKC isoforms, including PKC δ , which can induce neurite outgrowth in PC12 cells and can also activate the ERK cascade (Corbit et al., 1999).

A more in-depth look at the functional outcome of each signalling pathways associated with NGF-induced growth and differentiation during development has been studied using mice in which the gene encoding the pro-apoptotic protein Bax has been knocked out (Markus et al., 2002). Neurons from these mice can grow in culture in the absence of NGF, allowing effects of exogenous signalling mediators to be observed. Experimental studies have found that Raf-Erk signalling can mediate axon elongation, whilst PI3K/Akt pathways influence other aspects such as axon branching (Markus et al., 2002).

1.6.2. The GDNF family

The GDNF family of neurotrophic factors are distant members of the transforming growth factor beta (TGF β) superfamily, with all members containing the seven cysteine residues in the same spacing (cysteine knot), characteristic of this family. The GDNF family contains glial cell line-derived neurotrophic factor (GDNF), persephin, neurturin and the most recently discovered artemin. Unlike other TGF β family members, the GDNF family signals via a receptor tyrosine kinase called Ret (Jing et al., 1996; Treanor et al., 1996) rather than the typical serine/threonine kinases utilised by other members of the TGF β family. Receptors for the GDNF ligands (GDNFLs) are multicomponent (Treanor et al., 1996) consisting of the common signalling receptor tyrosine kinase Ret, and a member of the GFR α family: GFR α 1, 2, 3 or 4 which confers ligand specificity (Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996). The GFR α protein is activated by binding of the correct homodimeric GDNF family ligand. It can then form a homodimeric complex that

binds to ret, leading to its dimerisation, phosphorylation and ultimately intracellular signalling (illustrated in figure 1.9).

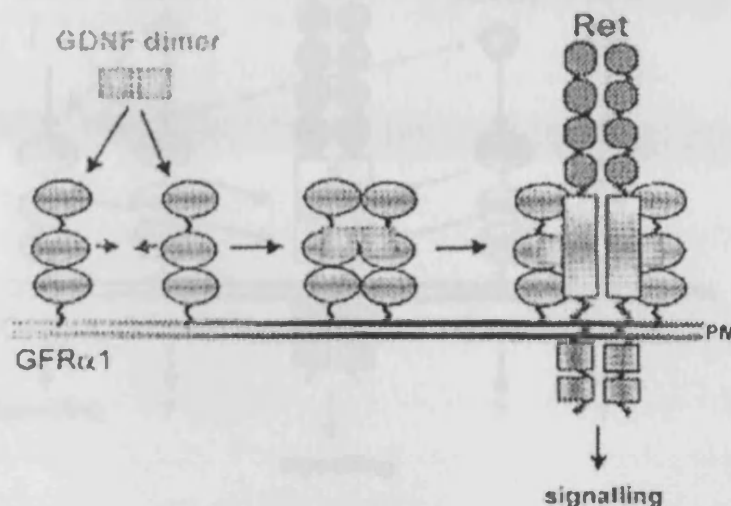


Figure 1.9. *GDNF family signalling.* A homodimer of the GDNF family ligand binds to its preferred GFR α protein, leading to its dimerisation and binding to ret. Ret now dimerises, leading to autophosphorylation of its tyrosine residues and subsequent signalling. Taken from Sariola and Saarma, 2003.

Preferentially, GDNF binds to GFR α 1 (Jing et al., 1996, 1997; Treanor et al., 1996), neurturin to GFR α 2 (Baloh et al., 1997; Buj-Bello et al., 1997; Klein et al., 1997; Sanicola et al., 1997), artemin to GFR α 3 (Baloh et al., 1998a) and persephin to GFR α 4 (Masure et al., 2000; Enokido et al., 1998), although some receptor promiscuity has been observed. Neuronal cultures have revealed that GDNF can also signal through GFR α 2 (Sanicola et al., 1997; Jing et al., 1997), neurturin through GFR α 1 and 4 (Creedon et al., 1997; Jing et al., 1997) and artemin can also bind to GFR α 1 (Baloh et al., 1998b). It is of note that persephin only binds to GFR α 4 (Enokido et al., 1998; Lindahl et al., 2000). These binding affinities are illustrated in

figure 1.10. Despite such receptor cross-talk apparent *in vitro*, analysis of knockout mice has revealed that *in vivo*, each ligand shows a more specific role, binding to its preferential receptor complex.

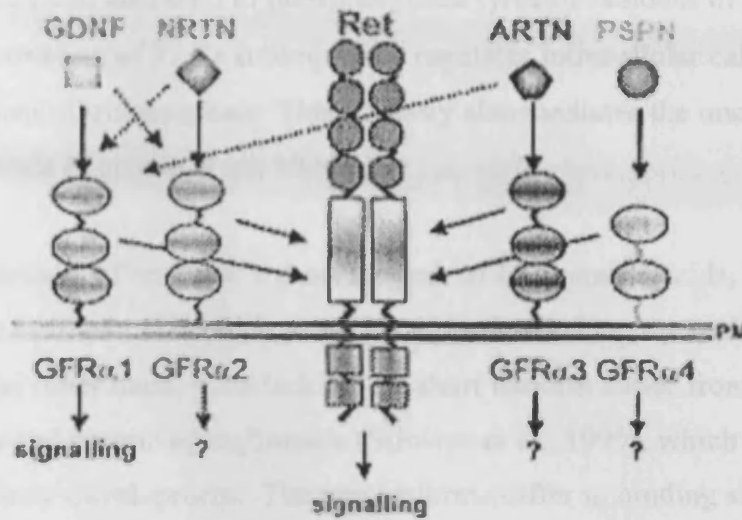


Figure 1.10.

GDNF family ligand binding to preferred GFRα family members (shown in bold arrows). Other ligand/ receptor interactions are also shown (dashed arrows). Taken from Sariola and Saarma, 2003.

Following ret phosphorylation, several intracellular signalling pathways are activated. Most of these pathways are similar to those activated by neurotrophin/Trk binding mentioned previously. Phosphorylation of ret on specific tyrosine residues (Y905, Y1015, Y1062, Y1096) allows the binding of proteins to PTB or SH2 domains on ret (Asai et al., 1996; Arighi et al., 1997; Tsui-Pierchala et al., 2002). These molecules include the shc family, Grb proteins, and the adaptor proteins Crk and Nck (Pandey et al., 1995, 1996; Bocciardi et al., 1997; De Falco et al., 2005). These proteins act as signalling intermediates, and upon binding activate several intracellular signalling pathways such as the MAPK/Akt, PI3K and JNK pathways.

Activation of Shc and Grb2 leads to stimulation of both MAPK/Akt and the PI3K pathways, which are important in neurite outgrowth and survival (Kaplan and Miller, 2000; Encinas et al., 2001; Murakami et al., 1999). Ret signalling via PI3K also stimulates activation of several focal adhesion proteins important in survival signalling, such as focal adhesion kinase (FAK), paxillin and p130Cas (Murikami et al., 1999). PLC γ can also bind to phosphorylated tyrosine residues of ret (Borrello et al., 1996). Activation of PLC γ subsequently regulates intracellular calcium via an increase in inositol trisphosphate. This pathway also mediates the oncogenic activity of GDNF ligands (Borrello et al., 1996).

Two splice variants of ret exist: a short isoform of 1072 amino acids, known as ret9, and a long isoform of 1114 amino acids, ret 51. Mice lacking the ret51 type are normal. On the other hand, mice lacking the short isoform suffer from renal abnormalities and enteric aganglionosis (Srinivas et al., 1999), which suggests a role for ret9 in kidney development. The two isoforms differ in binding sites for shc proteins, with ret51 only containing the SH2 binding site, but the short isoform having both SH2 and PTB (Ohiwa et al., 1997; Lorenzo et al., 1997; Tsui-Pierchala et al., 2002). These isoforms can therefore form different signalling complexes explaining their differing cellular actions. ret51 can associate more strongly than ret9 with the ubiquitin ligase Cbl, allowing faster turnover. Furthermore, it can bind to Crk1 allowing activation of Erk1 and Erk2 (Scott et al., 2005).

GFR α (GDNF family receptor) family members are bound to the outer leaflet of the plasma membrane via a GPI anchor (as shown in figure 1.9.). Such a location suggests the ability to recruit and signal via the cholesterol rich microdomains, known as lipid rafts. Research on GDNF and GFR α 1 signalling has shown that GFR α 1 recruits ret to lipid rafts, where it can then bind to adaptor proteins and other signalling molecules such as src kinases within or outwith the raft (Tansey et al., 2000; Encinas et al., 2001; Paratcha et al., 2001). The formation of lipid rafts is essential for GDNF-induced signalling, differentiation and neuronal survival (Tansey et al., 2000). However, GFR α 4, the receptor component binding persephin, does not

have the ability to recruit ret to lipid rafts (Yang et al., 2004). It is of interest that following GDNF activation, ret preferentially associates with Shc outside of lipid rafts, leading to activation of MAPK/Akt pathways. However, within the rafts, Shc binds with FGF, FRS2, suggesting that GDNF signalling through ret inside and outside of rafts can produce different responses (Paratcha and Ibanez, 2002)

GFL signalling via such GFR α /ret complexes is fairly well characterised. However, expression of GFR α family members is more widespread than that of ret, suggesting that GFLs may be able to signal via another receptor type (Trupp et al., 1997; Golden et al., 1998; Kokaia et al., 1999). It has been shown that in the absence of ret, GDNF family members can signal via the neural cell adhesion molecule (NCAM) in various parts of the nervous system (Paratcha et al., 2003; Enomoto et al., 2004). In the absence of ret, GFR α 1 is able to bind to NCAM, which facilitates binding of the GDNF ligand to NCAM and subsequent activation of the cytoplasmic kinases, Fyn and focal adhesion kinase (FAK) (Paratcha et al., 2003).

1.6.2.1. Glial cell line derived neurotrophic factor

GDNF was initially characterised as a neurotrophic factor for embryonic midbrain dopaminergic neurons, supporting survival and differentiation as well as promoting increased dopamine uptake (Lin et al., 1993). GDNF has since been shown to exert effects on several other CNS neurons including spinal cord motor neurons (Henderson et al., 1994; Oppenheim et al., 1995), facial motor neurons (Yan et al., 1995), central adrenergic neurons (Arenas et al., 1995), cerebellar Purkinje neurons (Mount et al., 1995) and cholinergic neurons of the basal forebrain (Williams et al., 1996; Golden et al., 2003). In the PNS, GDNF supports the survival of cranial parasympathetic neurons and sensory neurons of the nodose ganglia, DRG and trigeminal ganglia (Trupp et al., 1995; Ebendal et al., 1995; Hashino et al., 2001; Fergie et al., 1999; Enomoto et al., 2000; Hashino et al., 2001). GDNF can also promote cell survival and neurite outgrowth from PC12 cells (Chen et al., 2001) via PI3K and MAPK dependent mechanisms, respectively (Chen et al., 2001; Encinas et

al., 2001). GDNF can also support neurons of the enteric nervous system (Hearn et al., 1998; Heuckeroth et al., 1998).

Consistent with these effects, GDNF mRNA is detectable in various areas of the rat brain and developing spinal cord (Schaar et al., 1993; Trupp et al., 1995; Golden et al., 1998). Outside the nervous system, GDNF mRNA expression is detected in developing skin, kidney, bladder, stomach and testis with a lower expression in developing skeletal muscle, lung, ovary and adrenal gland (Trupp et al., 1995; Golden et al., 1998; 1999; Kawakami et al., 2003). Such a high expression in non-neuronal tissues might suggest other important functions for GDNF, as confirmed by studies of the phenotype of mice lacking GDNF or GFR α 1 (Moore et al., 1996; Sanchez et al., 1996; Pichel et al., 1996; Cacalano et al., 1998; Enomoto et al., 1998). In both GDNF^{-/-} and GFR α 1^{-/-} mice, the enteric nervous system is absent and ureters and kidneys do not develop. It is of interest that mice with a null mutation in the gene encoding ret show a similar phenotype. Mice homozygous for the mutation die shortly after birth with renal agenesis or severe dysgenesis. The enteric nervous system is also undeveloped (Schuchardt et al., 1994).

The preferential ligand-binding component for GDNF is GFR α 1 (Jing et al., 1996; Treanor et al., 1996), although GDNF can also bind with lower affinity to GFR α 2 (Sanicola et al., 1997; Jing et al., 1997). GFR α 1 (Jing et al., 1996; Treanor et al., 1996) is widely expressed throughout the body and in the nervous system of embryonic and adult mice (Golden et al., 1998; 1999; Kawakami et al., 2003), although not always co-localised with ret.

Studies of GDNF^{-/-} and GFR α 1^{-/-} mice have permitted further characterisation of the effects of GDNF/GFR α 1 signalling in the nervous system. GDNF^{-/-} mice have reduced numbers of neurons in the DRG, nodose ganglia and sympathetic neurons, but display normal development of hindbrain, noradrenergic, midbrain dopaminergic neurons and motor neurons (Moore et al., 1996; Sanchez et al., 1996). However, more recent studies using *in vivo* transgenic approaches and also *in vitro* experiments using neuronal cultures gave differing results, which are discussed here.

GDNF regulation of motor neuron number has been extensively studied. A reduction in the majority of spinal and cranial motor neurons has been observed in GDNF^{-/-} and GFRα1^{-/-} mice (Oppenheim et al., 2000; Garces et al., 2000; Cacalano et al., 1998). This role for GDNF in motor neuron development was also reflected in GDNF over-expressing mice, which display enhanced motor neuron survival (Oppenheim et al., 2000). Furthermore, *in vitro* studies using embryonic cultures of mammalian and avian motor neurons found that GDNF could support survival (Henderson et al., 1994; Oppenheim et al., 1995; Soler et al., 1999) by a PI3K dependent pathway (Soler et al., 1999).

Survival of DRG sensory neurons was found to be unaffected in GDNF^{-/-}, GFRα1^{-/-} and GDNF over-expressing mice (Oppenheim et al., 2000). A study of GFRα1 deficient mice showed no neuronal losses within peripheral ganglia, including SCG and nodose (Enomoto et al., 1998). On the other hand, *in vitro* experiments have shown a role for GDNF in the survival of a specific subpopulation of developing sensory neurons after birth. GDNF was found to only support the survival of 10% of cultured rat embryonic DRG neurons (Kotzbauer et al., 1996; Matheson et al., 1997). However, a much greater proportion were rescued in cultured DRG neurons from newborn rats (Matheson et al., 1997; Bennett et al., 1998), which illustrates the switch in dependence from neurotrophins to GDNF ligands which occurs postnatally for a subpopulation of nociceptive neurons (Molliver et al. 1997; Baudet et al., 2000). There was an additive effect of NTN and GDNF on the number of surviving neurons, but no additive effect for cultures containing either of these neurotrophic factors and artemin. This suggests that GFRα1 is expressed by a subpopulation of neurons distinct from those expressing the neurturin receptor component GFRα2, and that GFRα3 is expressed by both GFRα1 and GFRα2 expressing neurons (Baudet et al., 2000).

1.6.2.2 Neurturin

The second member of the GDNF family, neurturin (NTN), was originally identified as a survival factor for cultured sympathetic neurons (Kotzbauer et al., 1996). It has since been shown to act as a neurotrophic factor for other populations of neurons including sensory neurons of nodose and DRG (Kotzbauer et al., 1996; Forgie et al., 1999), cranial parasympathetic neurons (Forgie et al., 1999; Hashino et al., 2001), spinal motor neurons (Klein et al., 1997), embryonic and adult dopaminergic neurons (Horger et al., 1998; Tseng et al., 1998) embryonic basal forebrain cholinergic neurons (Golden et al., 2003) and enteric neurons and glia (Heuckeroth et al., 1998). Like GDNF, active NTN is produced by cleavage of the 195 amino acid precursor molecule, preproNTN. The amino acid sequence of the, 100 amino acid, mature NTN, shares 42% similarity with GDNF (Kotzbauer et al., 1996).

NTN transcripts are detectable in the cerebral cortex, striatum, brain stem and pineal gland of the developing nervous system (Widenfalk et al., 1997). It is widely expressed in other areas of the body. Throughout development, high levels of NTN mRNA are detectable in pituitary, bladder, intestine and testis, and moderate levels in adrenal gland, kidney, ovary, thyroid and spleen. This would suggest that NTN might be involved in the maintenance of these peripheral organs, as well as a target derived neurotrophic factor for innervating neurons (Golden et al., 1999; Xian et al., 1999; Kawakami et al., 2003). In the adult, expression is much less widespread, being detected predominantly in the gut, testis and oviduct (Golden et al., 1999).

GFR α 2 is the preferred ligand-binding portion of the NTN receptor. Mammalian GFR α 2 was isolated by several groups (Baloh et al., 1997; Buj-Bello et al., 1997; Klein et al., 1997; Sanicola et al., 1997; Widenfalk et al., 1997). Expression is more widespread than NTN throughout the nervous system. GFR α 2 is detectable in both the developing and the adult nervous system in areas of cortex, cerebellum, thalamus, hypothalamus and brain stem. In peripheral sensory neurons, GFR α 2 is expressed

predominantly in 80% of IB4 reactive small diameter neurons (Bennett et al., 1998), but is barely detectable in TrkA-expressing nociceptors. At various stages of development, GFR α 2 is also present in other tissues, including bladder, heart, digestive tract, respiratory system, skin, bone and endocrine glands (Golden et al., 1999; Kawakami et al., 2003).

Although neurturin preferentially signals via GFR α 2, it can also bind to GFR α 1 and GFR α 4 (Creedon et al., 1997; Jing et al., 1997). The recent identification of a cell-line, NG108-15, that endogenously expresses ret and GFR α 1, but not GFR α 2 or GFR α 4 has proved useful in the elucidation of the individual effects of NTN on GFR α 1 (Lee et al., 2006). It has been demonstrated that application of GDNF, but not NTN could promote survival of NG108-15 cells through a MAPK signalling pathway. However NTN, but not GDNF was shown to promote neurite outgrowth (Lee et al., 2006). It appears that NTN and GDNF have differential effects via the GFR α 1-ret complex and the biological responses that occur are determined by ligand concentration and receptor availability.

Mice with null mutations in the neurturin or GFR α 2 genes are viable and fertile, but show defects in parasympathetic cholinergic neurons, with poor innervation in the lacrimal and salivary glands (Heuckeroth et al., 1999; Rossi et al., 1999). Noxious heat transduction is also lost in this null mutant due to loss of IB-4 reactive nociceptive neurons (Stucky et al., 2002).

In culture, parasympathetic neurons are initially dependent upon GDNF for survival, but switch to NTN at a later stage of embryonic development (Forgie et al., 1999; Enomoto et al., 2000; Hashino et al., 2001). In developing DRG neurons, NTN is able to promote survival and enhance neurite outgrowth (Kotzbaur et al., 1996; Yan et al., 2003). Additionally, NTN can stimulate axon outgrowth from DRG of young adult mice (Paveliev et al., 2004).

Outside the nervous system, a role for NTN in the immune system has been suggested by the discovery that T cells, B cells and monocytes can all produce NTN

(Vargas-Leal et al., 2005). Quantitative PCR also reveals that these cells express significant levels of GFR α 2 transcripts (Vargas-Leal et al., 2005).

1.6.2.3. Persephin

Persephin (PSP), the third member of the GDNF family discovered, is cleaved from the 156 amino acid precursor, preproPSP (Milbrandt et al., 1998). The mature cleaved form has only 96 amino acids and shares 40% identity with GDNF and NTN. It is ubiquitously expressed throughout the CNS, but at very low levels (Milbrandt et al., 1998; Jaszai et al., 1998).

Culture studies have shown that PSP is able to promote survival and differentiation of embryonic basal forebrain cholinergic neurons, with an efficacy comparable with NGF (Golden et al., 2003). This suggests a role for PSP in the development of such neurons and thus in normal cognitive function. No survival effects on peripheral neurons have been observed.

GFR α 4 comprises the ligand-binding portion of the PSP receptor. Mammalian GFR α 4 (Masure et al., 2000; Lindahl et al., 2000) was isolated following earlier characterisation of the avian form (Enokido et al., 1998; Thompson et al., 1998). Mammalian GFR α 4 has a markedly different sequence from the rest of the GFR α family and also lacks the first cysteine rich domain observed in GFR α 1-3. It also shares only 37% sequence similarity with the avian form (Masure et al., 2000). Unlike other GFR α family members, it does not recruit into lipid rafts upon ligand binding (Yang et al., 2004). The mammalian form is detected in both the developing and mature nervous system, and in testis and thyroid gland. It is also expressed at very low levels in developing and adult kidney, muscle spleen and liver (Lindahl et al., 2000; Masure et al., 2000). It is alternatively spliced in a tissue dependent fashion with a GPI linked isoform being exclusively expressed in juvenile thyroid C cells and parathyroid gland, but another transmembrane, non-GPI anchored form is present in new born and adult thyroid, parathyroid pituitary and adrenal gland

(Lindahl et al., 2000). In the peripheral nervous system, GFR α 4 mRNA expression has been noted within the sympathetic chain ganglia and SCG and also in sensory neurons of the trigeminal ganglia and DRG (Lindahl et al., 2000). In adult DRG, transcripts do not encode the GPI anchored form (Paveliev et al., 2004).

Much evidence indicates the potential for PSP in the treatment of Parkinson's disease through protection of dopaminergic neurons. PSP is also able to support survival of cultured midbrain dopaminergic neurons to an extent comparable with GDNF (Akerud et al., 2002). *In vivo*, PSP is localised to nigrostriatal dopamine neurons and its receptor, GFR α 4 is detected in midbrain dopaminergic neurons (Akerud et al., 2002). Moreover, two weeks following 6-OHDA-induced injury, an increase in PSP mRNA is observed in the ipsilateral striatum. (Zhou et al., 2000). The use of rodent models of PD has further indicated therapeutic potential for PSP. In one study, neural stem cells were genetically engineered to over-express PSP and were grafted into the striatum of a rodent model of PD (Akerud et al., 2002). Within the first month, PSP dispersed successfully throughout the striatum, and in mice injured by 6-OHDA injections, the loss in dopamine neurons and behavioural impairment was prevented (Akerud et al., 2002). Furthermore in mice subject to OHDA lesioning, enhanced dopamine dependent behaviour was observed. The ability of PSP to act only via one receptor, GFR α 4, combined with its slightly more restricted expression pattern in comparison to other GDNF family members, might make it a more attractive candidate for the treatment of this neurodegenerative disorder than other members of the GDNF family.

Studies on PSP^{-/-} mice have indicated a role for PSP in neuroprotection following ischaemic insult. Phenotypically, PSP^{-/-} mice seem normal. They are viable, healthy and display no behavioural or developmental impairments. However, following focal ischaemia, the mice show increased cerebral infarction when compared with wild-type litter-mates (Tomac et al., 2002). Such effects could be attributed to the loss of regulatory effects of PSP on glutamate-induced Ca²⁺ influx. Without regulation, a dramatic influx in Ca²⁺ could lead to neuronal damage.

Mutations in the GFR α 4 gene have recently been implicated in the rare inherited cancer syndrome, multiple endocrine neoplasia 2 (MEN2) also known as Sipple's syndrome). It leads to medullary carcinoma of the thyroid gland and pheochromocytoma (neuroendocrine tumour of the adrenal gland resulting in excessive secretion of catecholamines). This disorder has been attributed in part to mutations in the ret receptor gene. However the phenotypic variability in MEN2 sufferers who carry the same ret mutation, combined with the finding that a small minority of patients do not have mutations in the ret gene, suggest that other factors are involved. The GFR α 4 gene has been implicated by the overlapping expression of ret and GFR α 4 (but not other GFR α family members), in normal and malignant thyroid medullary cells (Lindahl et al., 2001). Two particular mutations were found in patients suffering from this disorder. The first was a single base substitution upstream of the coding region, altering the reading frame of the receptor. The second, was a 7 base pair insert that would alter the formation of ret signalling complexes through a shift in membrane binding (Vanhome et al., 2005). It is thought that these mutations might contribute to MEN2 in the absence of a ret mutation, or that they might alter the ret mutation phenotype (Vanhome et al., 2005).

1.6.2.4. Artemin

The 113 amino acid mature protein, artemin (ART) was the last of the GDNF family to be cloned (Baloh et al., 1998b; Masure et al., 1999). The rodent form was cloned by exploring DNA databases using NTN as a query (Baloh et al., 1998b). Masure et al., (Masure et al., 1999) isolated and characterised the human form of artemin and subsequently named it "enovin". For the purpose of this thesis however, the rodent form will be discussed. Artemin has 45% sequence identity with NTN and PSP, and slightly less similarity (36%) with GDNF. It was first isolated for its ability to support the survival of sympathetic and sensory neurons in culture, but has also been shown to promote survival of dopaminergic neurons signalling via the GFR α 1-Ret receptor (Baloh et al., 1998b). This highlights the cross talk between receptor types. Artemin mRNA is present at low levels in fetal and adult brain. In the CNS, artemin transcripts are detectable in basal ganglia and thalamus. Its expression in non-

neuronal tissues is observed in pituitary gland, placenta and trachea of the adult and in the kidney and lung of the fetus (Baloh et al., 1998).

Only artemin can bind to GFR α 3, with no other GFLs having this affinity. The amino acid sequence of GFR α 3 is 32% identical with GFR α 1 and 37% identical with GFR α 2 (Naveilhan et al., 1998; Baloh et al., 1998a; Widenfalk et al., 1998; Worby et al., 1998). Unlike GFR α 1 and GFR α 2, whose expression is found in overlapping populations of the adult rodent CNS, expression of GFR α 3 is primarily in the PNS. It is predominantly expressed by small diameter nociceptive sensory neurons, the majority co-expressing TrkA, CGRP and VR1. A significant proportion are IB4 reactive (Baloh et al., 1998a; Naveilhan et al., 1998; Widenfalk et al., 1998; Worby et al., 1998; Orozco et al., 2001). GFR α 3 immunoreactivity is barely detectable in the adult, although during development prominent expression can be detected in the DRG and trigeminal sensory ganglia where it is found on a mixed subpopulation of neurons that also express GFR α 1 or GFR α 2 (Baloh et al., 1998a; Naveilhan et al., 1998; Widenfalk et al., 1998; Worby et al., 1998; Baudet et al., 2000). It is also expressed on sympathetic neurons (Baloh et al., 1998a; Widenfalk et al., 1998; Worby et al., 1998), and on non-neuronal immature Schwann cells (Widenfalk et al., 1998).

Prominent expression during development and production of artemin by Schwann cells suggest that artemin influences the development of PNS neurons (Baloh et al., 1998b). Artemin expression is detectable in blood vessels embryonically, at a time when sympathetic neurons are using blood vessels as the pathway to reach their final target tissues (Enomoto et al., 2001). Study of *ret*^{-/-} mice, *artemin*^{-/-} mice and GFR α 3^{-/-} mice reveal defects in sympathetic neuron migration, axonal outgrowth and neuronal survival (Nishino et al., 1999; Enomoto et al., 2001; Honma et al., 2002; Andres et al., 2001). Furthermore, *in vitro*, early embryonic SCG cultures show enhanced neurite outgrowth in the presence of artemin (Yan et al., 2003). Artemin also promotes proliferation of sympathetic neuroblasts (Andres et al., 2001).

Artemin is also important in the development of sensory neurons, but at slightly later stages. It can support the survival of a proportion of cultured sensory DRG neurons at early postnatal stages and also enhance neurite outgrowth (Yan et al., 2003). Neurons rescued correspond to the IB4 reactive population of neurons (approximately 35% of total DRG) that switch dependence from NGF to GDNF family ligands after birth (Baloh et al., 1998b; Widenfalk et al., 1998). It is of particular interest that by late postnatal ages, artemin signalling is able to inhibit survival effects of other GDNF family members. Experiments by Baudet et al., (Baudet et al., 2000) using cultures of P15 DRG neurons demonstrated that artemin, in combination with GDNF and/or neurturin, supported the survival of significantly fewer neurons than with either factor alone. It is unclear why this effect is observed at these ages, but it has been postulated that possible formation of heterodimeric complexes might prevent receptor binding, or, that artemin might possibly signal via another receptor in order to promote inhibitory effects (Baudet et al., 2000).

In the adult, it has been suggested that artemin might have a role following neuronal injury. Following sciatic nerve axotomy, a dramatic increase in GFR α 3 expression in DRG neurons occurs such that all small diameter DRG neurons were found to be GFR α 3 immuno-reactive (Bennett et al., 2000). An increase in artemin production from Schwann cells was also apparent (Baloh et al., 1998b). These findings suggest a possible role for artemin in injury associated neuropathic pain. This is supported by the observation that administration of artemin following SNL leads to a reversal of nerve-injury related pain behaviour and the associated alterations in biochemical phenotype (Gardell et al., 2003). Artemin antagonists are therefore of potential therapeutic benefit in the treatment of neuropathic pain. A role for artemin following neuronal trauma and in nerve regeneration is also implicated following the observation that it is able to stimulate axon outgrowth in cultured DRG neurons taken from the young adult mouse (Paveliev et al., 2004). It is thus of potential therapeutic benefit following neuronal trauma.

1.6.3. The plasminogen-related growth factors

Plasminogen-related growth factors (or cytokine-related factors) are a new family of growth factors related to the blood proteinase plasminogen. This family contains just two factors: hepatocyte growth factor/scatter factor/plasminogen-related growth factor 1 (HGF/SF/PRGF1) and macrophage stimulating protein/plasminogen-related growth factor 2 (MSP/PRGF2). These factors will be referred to as “HGF” and “MSP”.

Both of these factors have evolved from the same ancestral gene as plasminogen and apolipoprotein that consisted of an N-terminal corresponding to the plasminogen activation peptide (PAP), at least 3 copies of the kringle domain¹⁰ and a serine protease domain (Donate et al., 1994). It is of interest that activation of plasminogen and apolipoprotein requires cleavage of the N terminal domain. This does not occur for HGF and MSP, suggesting that this portion is important for their function (Matsumoto et al., 1991; Okigaki et al., 1992; Donate et al., 1994). Like other members of the family, HGF and MSP are secreted as inactive single chain precursors (pro-MSP and pro-HGF). These precursors are cleaved by trypsin-like serine proteases to produce the biologically active molecules (Naldini et al., 1992). Proteolysis produces a disulphide linked $\alpha\beta$ chain heterodimer, the α chain containing the kringle subunit (62 kDa for HGF and 53kDa for MSP) and the β chain forming the serine proteinase-like subunit (32kDa for HGF and 62kDa for MSP) (Skeel et al., 1991). This is shown for MSP in figure 1.11. (taken from Leonard and Danilkovitch, 2000). Unlike plasminogen and apolipoprotein, MSP and HGF have no enzymatic activity due to amino acid substitutions in catalytic triad of the β subunit (Yoshimura et al., 1993).

¹⁰ Kringle domain – a conserved sequence in a triple disulphide loop structure

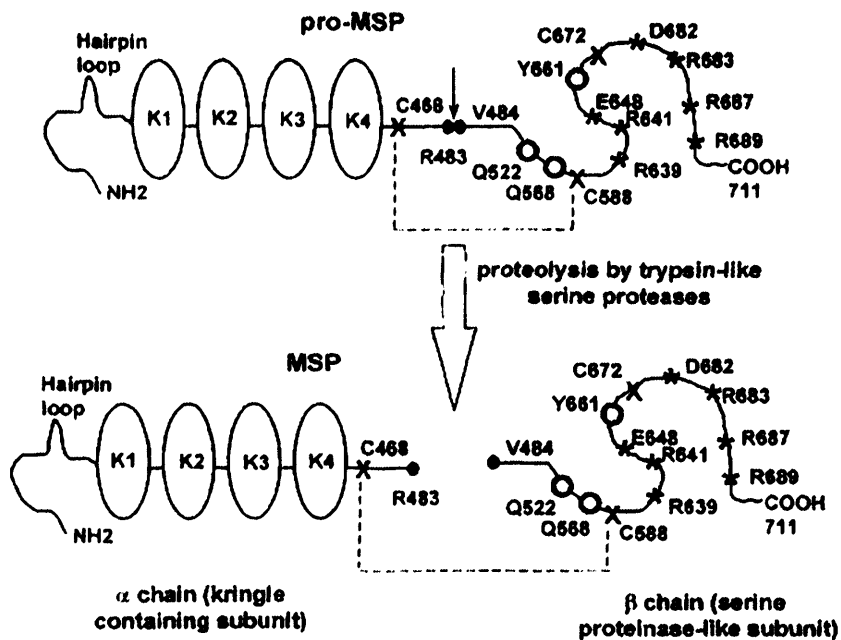


Figure 1.11 The cleavage of MSP from its precursor pro-MSP by trypsin-like serine proteases (Taken from Leonard and Danilkovitch, 2000)

1.6.3.1. Hepatocyte growth factor

Hepatocyte growth factor (HGF), also known as scatter factor (SF), is a pleiotropic factor which was discovered independently by several groups as a molecule that was able to trigger motility, proliferation and morphogenesis in a variety of epithelial and other cells (Ebens et al., 1996; Montesano et al., 1991; Shima et al., 1991; Weidner et al., 1991; Gheradi et al., 1989; Nakamura et al., 1989; Miyazawa et al., 1989; Rubin et al., 1989; Stoker et al., 1987). HGF has since been found to have a wide variety of roles. Thus during embryogenesis, HGF is required for development of many organs and tissues including liver, kidney, lung, gut, skeletal muscle and placenta (Matsumoto et al., 1996; reviewed in Birchmeier and Gherardi, 1998). In the adult, it supports the regeneration of organs such as liver, lung and kidney (Ueki et al., 1999; Matsumoto et al., 1996).

HGF is found in both the developing and mature CNS and PNS where it displays neurotrophic factor-like activity (Thompson et al., 2004; Hamanoue et al., 1996;

Maina et al., 1997; Maina and Klein, 1999; Davey et al., 2000; Ebens et al., 1996; Wong et al., 1997; Yamamoto et al., 1997). It is localised both in neurons and in non-neuronal cells including Schwann cells, for which it is a mitogen (Krasnoselsky et al., 1994), and microglia (Hamanoue et al., 1996). Thus, HGF may affect neurons both directly, through receptors on neurons, and indirectly, through actions on non-neuronal cells. It would seem, for example, that survival and maturation of embryonic rat dopaminergic mesencephalic neurons is promoted by HGF secreted from microglia (Hamanoue et al., 1996). During development, HGF itself can directly support the survival of a subpopulation of motoneurons of a similar size to that supported by either BDNF or CNTF (Ebens et al., 1996; Wong et al., 1997; Yamamoto et al., 1997).

In the PNS, HGF has no effect on sensory neurons on its own, but enhances neurotrophic factor effects of NGF and CNTF on differentiation, axonal outgrowth and survival *in vitro* (Maina et al., 1997; Maina and Klein, 1999; Davey et al., 2000). Mice which carry a signalling defect in the HGF receptor (*met*) gene have intercostal nerves that are significantly shorter and contain less branching than their wild type litter mates, further suggesting that HGF signalling is important in the development of sensory neuronal populations (Maina et al., 1997). HGF is also important in development of other peripheral neurons. It can promote survival and neurite outgrowth of mature sympathetic neurons *in vitro* (Thompson et al., 2004) and also enhance *in vitro* effects of NGF-induced neurite outgrowth (Maina et al., 1998). The use of pharmacological inhibitors has shown that effects on survival and growth of sympathetic neurons are actioned through PI-3 kinase and MAP kinase dependent mechanisms (Thompson et al., 2004).

The HGF receptor is encoded by the *c-met* proto-oncogene, a transmembrane protein with tyrosine kinase activity. This oncogene was identified by immunoblot analysis (Bottaro et al., 1991) and confirmed as the receptor for HGF/SF by further experiments, which included ligand induced tyrosine phosphorylation of the β subunit and co-precipitation (Naldini et al., 1991).

The importance of HGF signalling has been highlighted by the generation of mice with a null mutation in the gene encoding HGF. HGF^{-/-} mice die as embryos, suggesting an essential role for HGF signalling in development (Schmidt et al., 1995; Uehara et al., 1995). Livers from embryos are much reduced in size. Embryos also lack muscles of the forelimbs and diaphragm, confirming an important role for HGF in organogenesis and tissue development. Furthermore, placental function is severely impaired, so that it is unable to invade maternal uterine tissue in order to expand the placenta, implicating HGF in placental invasive growth. The early lethality of this defect in these mice prevents the investigation into roles for HGF in the adult.

1.6.3.2. Macrophage stimulating protein

Macrophage stimulating protein (MSP) was originally characterised as a chemotactic factor for macrophages (Leonard and Skeel, 1978). It has since been isolated from human blood plasma. Cloning (Skeel et al., 1991; Yoshimura et al., 1993) has allowed further characterisation and the identification of additional functions. MSP has been shown to modulate the growth of various cell types, including the enhancing of proliferation of keratinocytes (Wang et al., 1996); the growth of bone marrow megakaryocytes (Banu et al., 1996) and resorption of bone by osteoclasts (Kurihara et al., 1996; Kurihara et al., 1998).

MSP has 45% sequence identity with HGF. As a result, it is often termed “HGF-like protein”. MSP exerts its effects via the tyrosine kinase receptor *recepteur d’origine nantis* (Ron), also known in the mouse as stem cell derived tyrosine kinase (STK). The cDNA encoding human Ron was first isolated from a transformed foreskin keratinocyte cell line (Ronsin et al., 1993). The murine form, STK was later isolated from hematopoietic stem cells (Iwama et al., 1994). For the purpose of this study, Ron/STK will be referred to only as “Ron”. Ron is structurally related to the HGF receptor, c-Met proto-oncogene, displaying 63% sequence identity in the intracellular region (Ronsin et al., 1993). In the adult, Ron transcripts have been shown to be detectable in almost all tissues with the exception of spleen and heart (Guadino et al., 1995). During development Ron can be detected in liver, lung, kidney bone, adrenal

glands, digestive tract, testis and the brain (Quantin et al., 1995; Guadino et al., 1995). This widespread expression would suggest an important role for Ron signalling in the development of many tissues. In the immune system, Ron expression is tightly restricted to certain types of macrophages. Peritoneal, skin, liver and bone macrophages have all been found to express the receptor (Iwama et al., 1995; Kurihara et al., 1996; Nanney et al., 1998). However macrophages from the lung, spleen or bone marrow show no expression (Iwama et al., 1995). Additionally, blood monocytes, neutrophils and lymphocytes all do not express this receptor (Iwama et al., 1995). During late development, in the mouse, the Ron receptor is detectable at particularly high levels in the trigeminal ganglia and hypoglossal nucleus (Gaudino et al., 1995; Quantin et al., 1995).

In the nervous system MSP has been shown to act as a neurotrophic factor for many populations of neurons. MSP exerts neurotrophic factor-like effects on motor neurons promoting both survival and neurite outgrowth in cultured embryonic chick hypoglossal motor neurons (Schmidt et al., 2002). In the PNS, MSP can support the *in-vitro* survival of sensory neurons from the mouse (Forgie et al., 2002). This survival effect has also been observed for early embryonic sympathetic neurons *in vitro*. However this effect is lost by birth, coinciding with a decrease in Ron mRNA in sympathetic targets (Forgie et al., 2003). In adult mouse hypoglossal neurons, MSP has also been shown to prevent motor neuron atrophy following axotomy (Stella et al., 2001). No studies on other populations of adult neurons have been done.

Mice with a null mutation in the MSP gene have been found to develop normally and are viable. They suffer from liver abnormalities, shown by lipid containing vacuoles present in the cytoplasm of hepatocytes. However hepatic function is unaffected and mice can still survive into adulthood (Bezzera et al., 1998). Ron/STK^{-/-} mice show different effects. Mice carrying a homologous mutation die embryonically, which has confirmed an essential role for Ron signalling in the development of numerous tissues and organs. In order to facilitate further study, mice in which the tyrosine kinase portion of the receptor had been removed, were generated. These knockout

mice were viable and appeared healthy. However, they displayed enhanced tissue damage in response to acute and cell-mediated inflammation (Waltz et al., 2000; reviewed in Wang et al., 2002). In addition, peritoneal macrophages produced enhanced levels of nitric oxide (NO) in response to the inflammatory mediator, interferon (IFN)-gamma, but levels of pro-inflammatory cytokines were unaffected (Correll et al., 1997). A significant increase in inducible –nitric oxide synthase (iNOS)¹¹ within the ovary was also observed, producing higher levels of NO, which led to a decrease in ovulation rate (Hess et al., 2003).

These observations indicate a normally inhibitory effect of MSP/Ron signalling on NO production, allowing regular ovulation rates and also an attenuation of the harmful effects of the cellular immune response that can lead to tissue damage. This effect has also been observed *in vitro*. In this study, physiological concentrations of MSP, applied to mouse peritoneal macrophages, were found to inhibit lipopolysaccharide-(LPS)-induced production of inducible NOS (iNOS) and subsequent NO production (Wang et al., 1994; Chen et al., 1998). Expression of other pro-inflammatory molecules has also been found to be negatively regulated by MSP/Ron signalling. Cyclooxygenase-2 (COX-2) and prostaglandin E₂ (PGE₂) are both produced from macrophages to enhance pathophysiological effects of inflammation. MSP has been found to inhibit the LPS-induced production of both COX-2 and PGE₂ from macrophages (Zhou et al., 2002).

More recent studies have revealed that expression of Ron is also regulated by inflammation. Levels of Ron have been found to be decreased by the inflammatory mediators' lipopolysaccharide (LPS) and IFN γ (Wang et al., 2000). Further investigation has revealed that these inflammatory mediators stimulated NO production, which subsequently attenuated Ron expression via suppression of the Ron gene promoter activities (Wang et al., 2000). Taken together, these results

¹¹ iNOS – inducible Nitric Oxide Synthase is one of a family of several NOS's. As mentioned previously, this family are responsible for the synthesis of Nitric Oxide (NO). iNOS is found predominantly in the immune system, where it is used by macrophages to defend the body from pathogens.

would therefore suggest that cytoplasmic signalling via the Ron receptor is important for inflammatory responses, but is not essential for survival.

Genetically modified mice, which over-express Ron, have also highlighted additional effects of this receptor. Over-expression of Ron in lung epithelia, which normally show barely detectable levels, resulted in the formation of multiple pulmonary tumours (Chen et al., 2000; Chen et al., 2000). An increased expression of Ron has also been shown in colon (Chen et al., 2000), breast (Maggiara et al., 1998) and ovarian (Maggiara et al., 2003) tumours. Epithelial cells from such tissues normally show barely detectable levels of this receptor. It is thought the abnormal up-regulation of this receptor and the increased signalling can enhance invasive activity of cells and protect them from apoptosis. A role for Ron in the progression of carcinomas to the invasive metastatic phenotype is thus suggested and provides a possible therapeutic target for the treatment of such carcinomas.

1.6.4. The neurotrophic cytokines

The neurotrophic cytokine family (also known as gp130 cytokines or the interleukin-6 family of cytokines) contains ciliary neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF), oncostatin M (OsM), cardiotrophin-1 (CT-1), interleukin-6 (IL-6), interleukin-11 (IL-11), cardiotrophin-like cytokine (CLC) and neurotrophin. All members of the family share low sequence homology. However, a similar tertiary structure has suggested a possible common ancestral gene (Bazan et al., 1991; Bruce et al., 1992). With the exception of LIF, which is introduced in a small section of chapter four, this family is not studied in this thesis. Only brief information will therefore be provided about each factor, with an outline of the receptor complex. LIF is discussed in more depth in the introduction to Chapter Four.

The neurotrophic cytokines are often referred to as the gp130 family, because all utilise the common receptor signalling subunit, gp130. gp130 then forms hetero- or homo-dimers with additional α and/or β receptor subunits dependent upon the ligand involved. The two interleukins, IL-11 and IL-6 form homodimers of gp130 following

binding of their own supplementary subunits, IL6R α and IL11R α (Barton et al., 2000; Murakami et al., 1993). CNTF, LIF, CT-1, CLC and neuropoietin form heterodimers comprising of gp130 and the β receptor subunit (LIFR) (Gearing et al., 1991). CNTF, CLC and neuropoietin must also firstly bind to the α subunit, CNTFR α , which allows the formation of the high affinity complex with LIFR β and gp130 (Davis et al., 1991, 1993; Gearing et al., 1991; Ip et al., 1993; Stahl and Yancopoulos, 1994; Elson et al., 2000). CT-1 also requires an alpha subunit, but this protein is yet to be identified (Pennica et al., 1995a; Pennica et al., 1996a). The OSM ligand binds to the heterodimeric complex of the OSM receptor subunit, OSMR and gp130, without the need for LIFR β (Ichihara et al., 1997; Lindberg et al., 1998; Heinrich et al., 2003)

Neither gp130 nor LIFR contain tyrosine kinase activity, so in order for the receptor complex to become active, membrane bound janus kinases (JAKs) must phosphorylate tyrosine residues in the cytoplasmic region of gp130. Signal transducers and activators of transcription (STAT) family members can then bind to these phosphorylated residues via their SH2 domain. Phosphorylated STAT dimers are then translocated to the nucleus where they initiate transcription of target genes (reviewed in Heinrich et al., 1998). gp130 cytokine family members show many neurotrophic factor-like effects, promoting survival of both embryonic sensory neurons (Horton et al., 1998; Their et al., 1999) and motor neurons (Arce et al., 1999).

1.6.4.1. Ciliary neurotrophic factor

CNTF was originally discovered as a trophic factor that promotes the survival of chicken embryo ciliary ganglion neurons (Adler et al., 1979). It has since been shown to promote survival of a range of neurons including certain chick and rodent sympathetic and sensory neurons (Barbin et al., 1984; Horton et al., 1996) and chick embryonic motor neurons (Arakawa et al., 1990). CNTF can also regulate differentiation of sympathetic neurons. The presence of CNTF can induce ChAT expression in primary cultures of rat sympathetic neurons, together with a reduction

in tyrosine hydroxylase (TH)¹² levels, promoting differentiation to a cholinergic phenotype (Saadat et al., 1989). CNTF mRNA is present at high levels within the sciatic nerve, spinal cord, optic nerve and olfactory bulb, with lower expression in other brain areas (Stockli et al., 1991; Dobrea et al., 1992).

1.6.4.2. Leukaemia inhibitory factor

Leukaemia inhibitory factor (LIF) (also known as cholinergic differentiation factor, CDF), is widely expressed throughout the mammalian nervous system and has pleiotropic activity in several adult and embryonic systems. In the hematopoietic system, LIF induces the proliferation of hematopoietic stem cells (Fletcher et al., 1990; Leary et al., 1990) as well as the differentiation of leukaemic cells (Tomida et al., 1984) and megakaryocyte progenitor cells (Metcalf et al., 1990). LIF affects bone resorption (Abe et al., 1986) and also inhibits adipogenesis by negative regulation of lipoprotein lipase (Mori et al., 1989). Other inhibitory effects on cell differentiation are observed for kidney epithelial cells (Tomida et al., 1990) and also for embryonic stem cells, where the inhibitory effects of LIF has been shown to maintain their developmental potential (Smith et al., 1988; Williams et al., 1988). In the nervous system, LIF displays neurotrophic factor-like activity with effects on both nerve differentiation and survival on several subpopulations of neurons (Yamamori et al., 1989; Murphy et al., 1991; Murphy et al., 1993). Other effects and regulation of LIF will be discussed in more depth in chapter four.

1.6.4.3. Oncostatin M

Oncostatin M (OsM) was originally isolated as an inhibitor of tumour progression (Zarling et al., 1986). However, it has since been shown to have neurotrophic factor effects in the nervous system. OsM can promote the survival and differentiation of oligodendrocytes (Vos et al., 1996), while in the PNS it can support the survival of certain subpopulations of sensory neurons (Horton et al., 1996). This survival role is

¹² TH is an enzyme used in the body in the manufacture of adrenaline. It catalyses the conversion of L-tyrosine, to the dopamine precursor, dihydroxyphenylalanine (DOPA).

further shown in a study of $OsM^{-/-}$ mice, which display a loss in a subpopulation of VR1 and P2X3 expressing DRG neurons in comparison to wild-type litter-mates (Morikawa et al., 2004). OsM has also been shown to affect neuronal differentiation of PNS neurons, enhancing ChAT activity in sympathetic neurons, and promoting differentiation towards a cholinergic phenotype (Rao et al., 1992).

1.6.4.4. Cardiotrophin-1

Cardiotrophin-1 (CT-1) was originally cloned from mouse embryoid body cDNA library (Pennica et al., 1995, 1996). The human form has also been identified and shares 80% identity (Pennica et al., 1996b). CT-1 is found predominantly in the heart, although is also expressed in many other areas including fetal kidney and lung and in adult skeletal muscle, prostate and ovary (Pennica et al., 1995b, 1996b). CT-1 was originally identified as a factor that induces cardiac myocyte hypertrophy (Pennica et al., 1995). It can also promote survival and differentiation of several populations of neurons including rat dopaminergic neurons, chick ciliary neurons (Pennica et al., 1995b), motor neurons (Arce et al., 1998) and sensory neurons of the nodose and trigeminal ganglion (Horton et al., 1998).

1.6.4.5. Interleukin-6

Interleukin-6 (IL-6) (also known as B cell stimulating factor-2 (BSF-2)) was discovered in 1986 as factor that regulates immunoglobulin production from B lymphocytes (Hirano et al., 1986). IL-6 can also support survival of several subpopulations of neurons including sensory neurons (Horton et al., 1996), catecholaminergic neurons from both fetal and postnatal rats and also postnatal cholinergic neurons (Kushiman et al., 1992a, 1992b, Hama et al., 1989). Additionally it can enhance ChAT activity in cholinergic neurons.

1.6.4.6. Interleukin-11

Interleukin-11 (IL-11) was cloned in 1990 (Paul et al., 1990) and initially considered a hematopoietic cytokine. It can also affect non-hematopoietic systems stimulating

osteoclasts, megakaryocyte maturation, platelet maturation and inhibition of adipogenesis (Teramura et al., 1992; Yang et al., 1993; Keller et al., 1993; Girasole et al., 1994). IL-11 transcripts have been found in a wide range of tissues, but highest levels are observed in the testis and brain, particularly the hippocampus (Du et al., 1996). The high expression in hippocampus concurs with its ability to stimulate proliferation of a hippocampal neuronal progenitor cell line *in vitro* (Du et al., 1996). Also in the nervous system, IL-11 enhances the *in vitro* survival of rat postnatal DRG but only in the presence of its soluble receptor component ILR α (Their et al., 1998).

1.6.4.7. Cardiotrophin-like cytokine

Cardiotrophin-like cytokine (CLC) (also named novel neurotrophin-1/B cell-stimulating factor-3 (NN1/BSF-3)) was originally identified as a protein with the capacity to bind to gp130 and for its ability to stimulate B-cells (Shi et al., 1999; Senaldi et al., 1999). Highest expression of CLC has been found in the spleen and lymph node (Shi et al., 1999; Senaldi et al., 1999), but it is also detectable in a variety of other tissues including thymus, lung, uterus, ovary and testis (Senaldi et al., 1999). It has been found to have highest homology to CNTF and CT-1, and to show similar activities to other family members, including an ability to promote survival of chick embryo motor and sympathetic neurons (Senaldi et al., 1999). CLC has also been shown to stimulate differentiation of astrocytes (Uemura et al., 2002).

1.6.4.8. Neuropoietin

The remaining member of the family, neuropoietin, has only been recently discovered, following a structural profile-based computational screen (Derouet et al., 2004). It has been shown to share similarities with CNTF, CT-1 and CLC. It is not expressed in adult tissues, but high specific expression is found in embryonic neuroepithelia (Derouet et al., 2004). Neuropoietin has been found to support survival of embryonic motor neurons and to stimulate the proliferation of neural precursors *in vitro* when associated with epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) (Derouet et al., 2004). This effect, when

combined with the specific embryonic expression, would implicate an important role for neuropoietin in the development of the nervous system.

1.7 Aims and Objectives

In this study the neurotrophic factor regulation of genes within mouse sensory neurons is to be studied at various ages. The thesis thus falls neatly into three chapters which explore expression at a) embryonic ages (chapter 2), b) postnatal ages (chapter 3) and in c) the adult (chapter 4).

Several genes have been chosen that are required for a number of key functional properties of subsets of sensory neurons, and in this study I hope to gain more knowledge about their regulation and patterns of expression at various stages of development.

Previous work on expression of the selected genes has largely focused on the adult and postnatal DRG, therefore I want to initially determine if regulation of expression by neurotrophic factors differs in the embryonic mouse and also to explore expression within other sensory neuronal ganglia.

In the embryonic mouse those genes chosen are important in determining the functional characteristics of sensory neurons. Although work on the regulation of expression of these genes has been explored in postnatal and adult mice, I want to identify if the *initial induction of expression* of such genes at early embryonic stages is regulated by neurotrophic factors. Knockout mice in which genes encoding neurotrophic factor receptors or the neurotrophic factors themselves have been chosen as a suitable and reliable method for this work, and due to the low levels of RNA available for study at this young age the highly sensitive methods of competitive RT-PCR and also real-time QPCR were chosen as the most suitable techniques for quantification of gene expression.

Postnatally, as mentioned, research has focussed on expression of genes within the DRG. In this chapter, as well as reconfirming previous published findings, I wanted to explore gene expression and neurotrophic factor regulation in other sensory neuronal ganglia. To date very little has been known about the regulation of

expression of nociceptive neurons markers in the, neurogenic placode-derived, nodose ganglion, either under normal conditions or following nerve lesion/trauma so in this chapter I wanted to explore how expression and regulation of genes differed in this ganglia from regulation in neural crest derived trigeminal and dorsal root ganglia. Once again I chose to use knockout mice, however neurotrophin and neurotrophin receptor knockout mice do not survive to postnatal ages, so the use of double knockout mice in which the proapoptotic gene Bax had also been removed was employed. *In vitro* cultures were also set up for comparison and the sensitive and efficient method of real-time QPCR was, once again, chosen for quantification.

In the final chapter I wanted to explore gene expression in adult sensory neurons and in addition to the mRNAs examined in previous chapters, I wanted to also examine the transcriptional regulation of a number of other genes that may play important roles both in determining normal nociceptive thresholds, and in the generation of inflammatory and neuropathic pain conditions.

Since all the genes selected have important roles in determining nociceptive thresholds, it seems important to establish the neurotrophic growth factor, or combination of growth factors, that set these steady state expression levels. This is of particular importance, since changes in the expression of these genes, that occur during inflammation and following nerve trauma, and which may be causally related to pathological pain conditions, are likely to be driven, at least in part, by a change in the availability of neurotrophic factors that normally regulate their steady state levels in the “normal” adult. Since adult rodent DRG neurons can survive in culture independently of neurotrophic factors (Lindsay, 1988), they allow a direct comparison of the effects of specific neurotrophic factors on regulating gene expression as a true ‘no neurotrophic factors control’ can be set up without the need for caspase inhibitors to prevent apoptosis.

In many respects, culturing neurons can be regarded as a model of axotomy and/or peripheral nerve injury. Indeed, adult DRG cultures have been extensively used in the fields of inflammatory and neuropathic pain research to examine the regulation of

a number of genes that have previously been implicated in the aetiology of neuropathic pain following *in-vivo* nerve lesion/axotomy/crush models. As such my results may provide an insight into alterations in gene expression occurring in situations of nerve injury and the neurotrophic factor regulation that can enhance or attenuate such effects. This work would therefore be of potential therapeutic benefit, providing further background to research in the fields of nerve injury and neuropathic pain.

Chapter 2

Nerve growth factor regulation of the early induction of sensory neuron gene expression in the developing mouse embryo

2.1. Introduction

It has previously been demonstrated that NGF/TrkA signalling is necessary to maintain the expression of several sensory neuron specific genes (e.g. CGRP, SP, Nav1.8, Nav1.9), within mouse DRG neurons, normally in the neonatal period and also following neuronal damage, axotomy or inflammation in the adult (Patel et al., 2000; Dib-Hajj et al., 1998; D'Arcangelo G et al., 1993). However, whether NGF/TrkA signalling is required for the initial induction of expression of these genes is unclear. In addition, few studies have addressed the role of NGF/TrkA signalling in the regulation of gene expression in other sensory populations, such as those within the nodose ganglia or trigeminal ganglia. In this chapter the role of NGF and TrkA signalling in the initial induction of expression of the neuropeptides Calcitonin Gene Related Peptide (CGRP) and Substance P (SP), and the sensory neuron specific, tetrodotoxin resistant (TTX-R) sodium channels Nav1.8 and Nav1.9, in trigeminal ganglia and DRG, will be explored.

2.1.1. Calcitonin gene related peptide (CGRP)

Calcitonin gene related peptide (CGRP) is a 37 amino-acid neuropeptide that is expressed throughout the peripheral and central nervous system (Amara et al., 1992; Rosenfeld et al., 1993; reviewed in Ishida-Yamamoto and Tohyama, 1989). It is predominantly located in small sensory unmyelinated, C and myelinated A δ fibres in the periphery, often colocalised in C fibres with the tachykinin peptides, SP and neurokinin A (Lundberg et al., 1985; Lee et al., 1985; Skofitsch et al., 1985). CGRP is usually located in nerves closely associated with blood vessels, suggestive of a cardiovascular role, which will be discussed later (Sexton et al., 1991; Brain et al., 1996; Wimalawansa et al., 1997). It belongs to the calcitonin family of peptides that

consists of calcitonin (CT), adrenomedullin (AM), amylin (AMY) and the two isoforms of CGRP, α -CGRP and β -CGRP. Although primary sequence homology between peptides is weak, they do show significant similarities at the secondary structure level. They all have a six amino acid ring structure at the N termini and are amidated at the C terminal. A region of potential amphipathic α helix also lies adjacent to the N terminal (Reviewed in Breiner et al., 1988; Poyner et al., 2004).

α -CGRP is the product of a calcitonin/ α -CGRP transcript (CALC1) that is alternatively spliced to allow tissue specific production of either CT or α -CGRP (Amara et al., 1982; Rosenfeld et al., 1983). Calcitonin is a potent inhibitor of bone resorption and is produced in abundance in parafollicular C-cells (Amara et al., 1982; Rosenfeld et al., 1983). α -CGRP peptide and transcripts are widely distributed throughout both the CNS and PNS. (Amara et al., 1992; Rosenfeld et al., 1993; reviewed in Ishida-Yamamoto and Tohyama, 1989).

β -CGRP was isolated later, and found to differ from the α isoform by just one amino acid in the rat (Amara et al., 1995), and three amino acids in the mouse (Thomas et al, 2001). This high degree of sequence homology is observed at both the nucleotide and amino-acid levels, but despite the similarity, β -CGRP is the product of a separate gene that lacks the exon encoding CT and is thought to have arisen by partial gene duplication.

Despite almost perfect sequence homology, the two CGRP isoforms show different expression profiles. The isoforms do coexist in many tissues, however α -CGRP is found predominantly in the CNS and PNS (Amara et al., 1995), whilst β -CGRP predominates in the enteric nervous system (Mulderry et al., 1988; Schutz et al., 2004) and pituitary gland (Jonas et al., 1985; Petermann et al., 1987).

Developmental switches in expression also occur. In adult rats, α -CGRP is the abundant isoform in both DRG neurons and motor neurons of the spinal cord (Amara et al. 1985, Gibson et al., 1988), however, during embryonic development, transcripts of the β - isoform are initially predominant, with a switch during perinatal

stages (Terrado et al., 1999). Using *in-situ* hybridization, Terrado et al. found that, β -CGRP mRNA, but not α -CGRP mRNA was expressed in cells of both trigeminal ganglia and DRG at E14. α -CGRP mRNA was not co-expressed with β -CGRP mRNA, until E16.

The recent sequencing of the mouse β - isoform (Thomas et al, 2001) and the production of mice that lack a functional α -CGRP gene has led to further investigation into the distinct expression of both α - and β - isoforms in the mouse. Homozygous α -CGRP knockout mice are viable and fertile, with no obvious abnormalities (Lu et al., 1999). In DRG of the adult α -CGRP^{-/-} mouse, little or no CGRP transcript (due to β -CGRP) was observed in comparison to wild-type (Zhang et al., 2001; Schutz et al., 2004), suggesting a predominance of the α - isoform in accordance with the data from rat.

Receptors

CGRP receptors are divided into two categories CGRP₁ and CGRP₂. Differentiation between the subtypes was permitted by the discovery of the receptor antagonist CGRP₁₂₋₃₇ and the receptor agonists [Cys(ACM)2,7]h α CGRP and [Cys(Et)2.7]h α CGRP (Dennis et al., 1989; Dennis et al., 1990; Dumont et al., 1997). CGRP₁ receptors show more sensitivity to CGRP₁₂₋₃₇ than CGRP₂ receptors (Dennis et al., 1989; Dennis et al. 1990), whilst CGRP agonists are more potent at CGRP₂ receptors. Although this classification still exists, it is not universally accepted with many still questioning the selectivity of the CGRP agonist, [Cys(ACM)2,7]h α CGRP (Poyner and Marshall, 2001).

A breakthrough in the structure determination of these receptors was made following the discovery of the rat calcitonin like receptor (CL) (Njuki et al., 1993), and two years later human CL (Fluhmann et al., 1995). These receptors could not bind CGRP and were deemed orphan receptors. However the cloning of the human CGRP₁ receptor (Aiyar et al., 1996), which showed high sequence homology with CL,

combined with work by McLatchie et al (McLatchie et al.,1998) led to the realisation that CL must be combined with a receptor activity-modifying protein (RAMP) in order to produce a functional CGRP receptor.

The RAMPS are a family of single transmembrane domain proteins that consist of RAMP1, RAMP2 and RAMP3 (McLatchie et al., 1998). CL is a seven-transmembrane spanning G protein coupled protein which, when combined with RAMP1, produces a CGRP receptor that is antagonised by CGRP₈₋₃₇ (CGRP1). Combined with RAMP2 it produces an amylin receptor and with RAMP3 another AM receptor, that may also have the ability to bind β CGRP (Reviewed in Poyner et al., 2002).

Physiological Role

As few studies have discriminated between isoforms, the subsequent background information provided here will refer to both isoforms, α - and β -, as CGRP, unless otherwise mentioned. CGRP is distributed widely throughout the CNS and PNS, regulating the biological function of tissues including those of the gastrointestinal (GI), respiratory, endocrine and CNS. CGRP has a number of physiological roles within the body most notably with regards to its cardiovascular effects, hence the wide expression in nerves closely associated with blood vessels. Both isoforms of CGRP are potent arterial and venous vasodilators through activation of the CGRP₁ receptor (Brain et al. 1986). At the cellular level effects via two pathways have been suggested. The first is endothelium dependent and the second, endothelium independent.

In the endothelium independent pathway has CGRP produces an increase in cAMP via adenylyl cyclase. This stimulates the production of protein kinase A (PKA), which can open K⁺ channels, in turn activating Ca²⁺ sequestration and producing the relaxation of smooth muscle (Crossman et al., 1990; Hirata et al., 1988; Nelson et al., 1990). The majority of tissues appear to use this endothelium dependent pathway, including rat perfused mesentery (Han et al., 1990), cat cerebral artery (Edvinsson et

al., 1985) and porcine coronary artery (Yoshimoto et al., 1998). Exceptions however do exist which require the presence of endothelium and activation of the smooth muscle relaxant, nitric oxide (NO). In this model, CGRP once again activates PKA through increases in adenylyl cyclase and cAMP. PKA activates endothelial nitric oxide synthase (eNOS), stimulating the production of NO, which produces subsequent muscle relaxation through guanylate cyclase and accumulation of cGMP (Brain et al., 1985; Gray and Marshall, 1992a, 1992b).

The intravenous administration of CGRP and use of antagonists have shown that CGRP is associated with hypotension, as a result of such vasorelaxant effects. It has also been found to promote positive effects on the force of muscular contractions and the rate of heart contraction (Ando et al., 1990; Gardiner et al., 1991; Bell and McDermoot, 1994). In contrast intracerebroventricular administration increases blood pressure in rats due to actions on sympathetic nerves and the release of norepinephrine (Fisher et al., 1983). These general effects of CGRP will be discussed here with regards to effects in other tissues.

CGRP and blood pressure

Studies of the role of CGRP in maintenance of basal blood pressure have produced conflicting results. Intravenous injection of the CGRP antagonist into adult rodents or dog has shown no effect on resting blood pressure (Gardiner et al., 1989; Shen et al., 2001), however studies in transgenic mice have produced varying results. Lu et al., (Lu et al., 1999) found no change in basal blood pressure in α CGRP^{-/-}, however mice in which the gene encoding both α CGRP and calcitonin is knocked out, show an increase in both systolic and mean arterial pressure (Gangula et al., 2000). This was originally thought to be due to effects of additionally removing calcitonin, however other studies by Oh-hashii et al., (Oh-hashii et al., 2001) in which solely α CGRP is knocked out, show an increase in mean arterial pressure and heart rate.

CGRP and migraine

A role for CGRP in the onset of migraine and also cluster headache was suggested as in both conditions an increased release of CGRP into cranial circulation is observed (Goadsby et al., 1990, 1994). Furthermore, addition of exogenous CGRP induces delayed migraine like headache in sufferers (Lassen et al., 2002). It is proposed that CGRP promotes vasodilation of cerebral blood vessels, which allows stimulation of perivascular sensory nociceptive nerve fibres producing the subsequent pain (Goadsby et al., 2000). The use of CGRP antagonists in the treatment of migraine has much therapeutic potential and has been shown to be clinically effective (Brain et al., 2004; Goadsby et al., 2005).

Protective effects of CGRP in heart conditions

The heart is innervated by CGRP containing fibres (Gulbenkian et al., 1993; Sun et al., 1993), and the local production of CGRP has been shown to be protective in many heart conditions. Indeed an increase in CGRP immunoreactivity is observed following myocardial infarction, suggesting release as a protective mechanism (Preibisz et al., 1993; Roudenok et al., 2001). In patients with congestive heart failure, infusion of CGRP can produce an increase in cardiac output and a decrease in blood pressure through vasodilation (Gennari et al., 1990; Shekhar et al., 1991). Additionally effects of CGRP are useful in patients suffering from angina, a condition in which coronary arteries are narrowed by several factors including stress or fatty deposits, resulting in severe chest pain in situations when the heart rate is increased. The use of CGRP was illustrated by clinical trial, in which CGRP was administered to exercising angina sufferers. CGRP produced dilation of coronary arteries, which delayed the onset of myocardial ischemia induced by the exercise (Uren et al., 1993).

Pulmonary hypertension

Constriction of lung arterioles leads to pulmonary hypertension and is caused by a misbalance in endogenous vasodilators and constrictors. Indeed a decrease in blood CGRP was observed in a rat model of pulmonary hypertension (Keith et al., 2000). Exogenous CGRP has also been shown to prevent development of pulmonary hypertension (Tjen et al., 1992, 1998) and to relax precontracted pulmonary arteries *in vitro* (Martling et al., 1990).

CGRP and blushing

Blushing, such as that observed in menopausal women, is believed to be due to a release of CGRP and its subsequent vasodilatory effects in skin arterioles. Indeed elevated levels of CGRP were noted in serum and urine during attacks of hot flushes and sweating in menopausal women (Wyon et al., 1998, 2000).

CGRP, inflammation and hyperalgesia

The distribution of CGRP on small diameter nociceptive neurons of the PNS (Molliver et al., 1997; Snider and McMahon, 1998; Lewin et al., 2004; Lewin and Moshourab, 2004; Green et al., 2004) suggests a role for CGRP in pain. An increase in CGRP is observed in primary sensory neurons following application of inflammatory stimuli such as complete Freund's adjuvant (CFA), suggesting a role for CGRP in inflammation (Donaldson et al., 1992; Donnerer et al., 1992; Woolf et al., 1994). Experiments have revealed roles in both the immune response to inflammation and also within the associated hyperalgesia.

Vasodilator effects of CGRP allow enhanced activities of inflammatory cells, through increased blood flow, and thus circulating cells such as neutrophils, to the area (Buckley et al., 1991). It can also directly and indirectly affect production of chemotactic compounds from macrophages. The presence of CGRP attenuates



release of interleukin-1 (IL-1), but produces an increase in production of IL-10 (Torii et al., 1997). It can also indirectly enhance LPS induced release of IL-6 from macrophages through induction of NO (Tang et al., 1999). As well as macrophages CGRP has been shown to be chemotactic for human T lymphocytes (Foster et al., 1999) and can also attenuate lymphocyte activity (McGillis et al., 2002). A role for CGRP in the cellular response to inflammation is thus likely.

Studies of mice with null mutations in the α -CGRP gene have illustrated a role for CGRP in the inflammation-associated hyperalgesia. $\text{CGRP}^{-/-}$ mice, in which the gene encoding both α -CGRP and calcitonin has been disrupted, are less sensitive to thermal hyperalgesia, failing to develop secondary hyperalgesia following knee joint inflammation (Zhang et al., 2001). Mice, in which α -CGRP, specifically, is knocked out, show a decrease in response to inflammatory stimuli including capsaicin, formalin and carrageenan (Salmon et al., 2001).

Additionally CGRP has also been shown to potentiate oedema formation when co-injected with histamine, a potent mediator of vascular permeability that is released following application of inflammatory stimuli (Brain et al., 1992). It also enhances the release of SP, thus promoting the inflammatory response and associated hyperalgesia (Oku et al., 1987; Brain et al., 1989).

Nerve damage and neuropathic pain

In contrast to inflammatory stimuli, following axotomy or transection of sciatic nerve a decrease in expression of both isoforms of CGRP peptide and transcripts is observed in adult lumbar DRG (Noguchi et al., 1990; Mulder et al., 1997; Sterne et al., 1998; Shi et al., 2001; Shadiack et al., 2001). Interestingly axotomy of the cervical vagus to identify alterations within nodose ganglia, showed no difference in CGRP immunoreactivity (Helke et al., 1995). This illustrates how the sensory neurons from different populations are phenotypically different and often regulated by different factors.

Neurotrophic Regulation

The regulation of CGRP mRNA and peptide expression in sensory neurons of the DRG, by NGF has previously been studied in adult and neonatal rodents.

As mentioned, sciatic nerve axotomy or transection in adult rats results in the loss of target field trophic support and a massive decrease in CGRP mRNA and peptide. This decrease in CGRP expression can be prevented by application of exogenous NGF, both *in-vivo* and *in-vitro* when cultured neurons can be used as a model of axotomy (Lindsay et al., 1989; Verge et al., 1995; Jiang and Smith, 1995; Price et al, 2005). Sequestering endogenous NGF with anti-NGF antibodies or TrkA IgG also reduces CGRP expression, inducing axotomy like changes (Shadiack et al, 2001; Christensen and Hulsebosch, 1997; McMahon et al, 1995). The addition of exogenous NGF or NGF over expression has also been shown to stimulate the expression of CGRP in intact and capsaicin lesioned DRG neurons of the adult rodent (Ma et al, 1995; Schuligoi and Amann, 1998; Schicho et al, 1999; Tandrup et al., 1999; Price et al., 2005) and cause a prolonged hyperalgesia (Lewin and Mendell, 1993). All of the above studies only measured the expression of the α CGRP isoform.

NGF regulation of CGRP has also been shown postnatally by the use of anti-NGF antiserum and TrkA/Bax and NGF/Bax double knockout mice (Tonra and Mendell, 1998; Patel et al, 2000). TrkA and NGF single knockout mice have a dramatic phenotype, with massive sympathetic and sensory neuronal loss resulting in death shortly after birth (Smeyne et al., 1994; Crowley et al., 1994). By additionally knocking out the gene that encodes the pro-apoptotic protein Bax, Patel et al., found that cells normally lost in the single knockout survive. These surviving neurons are also phenotypically identical to those that would be lost (Patel et al, 2000).

Postnatally, the expression of CGRP and SP was dramatically reduced in DRG of TrkA/Bax and NGF/Bax null mutant mice (Patel et al.2000). However the study of alpha and beta isoforms individually was not addressed in this publication.

Effects of the GDNF neurotrophic factor family member, artemin on CGRP expression have been observed in a model of neuropathic pain. Following sciatic nerve ligation, the associated down-regulation in CGRP mRNA observed in rat DRGs was partially reversed by the presence of artemin (Gardell et al., 2003). Additionally, in uninjured neurons, intrathecal administration of GDNF produced an increase in CGRP immunoreactivity in the GDNF receptor expressing proportion of DRG neurons (Ramer et al., 2003).

Fibroblast growth factor 2 (FGF2) can also regulate CGRP expression in sensory and motor neurons. Analysis of mice carrying null mutations in FGF2 gene and also in its receptor, FGFR3, showed a decrease in the loss of CGRP normally observed following injury (Jungnickel et al., 2005). Within motor neurons, an increase in CGRP is observed following lesion. This can be abolished upon application of bFGF (Piehl et al., 1995, 1998).

In this chapter TrkA and NGF knockout mice were used to study the regulation of α - and β - CGRP mRNA expression by NGF in both DRG and trigeminal ganglion neurons during embryonic development rather than postnatally or in the adult

The arrival of new Real-time PCR technology in the lab allowed me to extend this study further. Real-time PCR is a highly sensitive and fast form of quantitative PCR (QPCR) and the computer package Beacon Designer permitted the production of more efficient and specific PCR primers that could distinguish between the two closely related CGRP isoforms, allowing me to look at the expression of alpha and beta CGRP separately, alongside studying the expression of other interesting sensory neuron specific genes. Any competitive data produced for CGRP could also be reconfirmed.

2.1.2. Substance P (SP)

Substance P (SP) is an 11 amino acid neuropeptide of the tachykinin family. It was first synthesised by Susan Leeman and colleagues back in 1971 (Chang et al., 1971),

following deduction of the amino acid sequence as Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met.NH₂ (Tregear et al., 1971). Expression studies revealed that SP is confined to the CNS and PNS, where it is localised in cell bodies and nerve terminals (Hokfelt et al., 1975; Cuello et al., 1978). Predominant expression is within sensory nuclei particularly within small diameter non-myelinated sensory neurons and thinly myelinated A-delta nociceptive neurons that express TrkA and are NGF responsive (Hokfelt et al., 1975; Cuello et al., 1978; Skofitsch and Jacobowitz, 1985; Lee et al., 1985). Within the CNS it is also detected at high levels in the thalamus, hypothalamus and extrapyramidal system and at lower levels in the spinal root ganglia, caudoputamen and globus pallidus, but is not detectable in the majority of the cortex (Cuello et al., 1978). The expression pattern corresponds with a role as a widespread neurotransmitter and this was confirmed by work of Otsuka and Konishi who illustrated SP release into the spinal cord after application of electrical stimuli (Otsuka and Konishi, 1976).

SP belongs to a family of small, biologically active, peptides collectively called tachykinins. SP is the most widely characterised member of this peptide family, however other members include Neurokinin A (NKA) and Neurokinin B (NKB). SP, NKA and NKB are all produced by alternative splicing of a precursor gene. SP and NKA are derived from the same precursor gene, preprotachykinin (PPT)-A, whilst NKB is generated from a separate gene PPT-B. Both PPT-A and PPT-B originate from the same common ancestral gene by duplication (Carter et al., 1990).

Alternative splicing of the PPT-A gene (Figure 2.1.) generates four transcripts α , β , δ and γ PPT-A mRNAs (Carter et al., 1990). The sequence for SP is present in all four, however the sequence for NKA is only present in β and γ splice forms (Carter et al., 1990; Holzer and Holzer-Petsche, 1997).

The cloning of the first neuropeptide receptor, that of Substance K, by Masu et al., (Masu et al., 1987) was a breakthrough, allowing the subsequent cloning of many more neuropeptide receptors, including those of SP and the other tachykinins, namely the neurokinin (NK) receptors. The family of NK receptors consist of NK₁, NK₂ and NK₃, which bind preferentially to SP, NKA and NKB (Regoli et al., 1987;

Maggi et al., 1987). Ligand promiscuity does occur however dependent upon ligand concentration and receptor availability. The preferred SP receptor, NK₁ is located throughout the CNS with highest levels in olfactory bulb, hippocampus, striatum and superior colliculous. It is also detected to a lesser extent in areas including the substantia nigra, cerebral cortex and within the dorsal horn of the spinal cord (Shults et al., 1984; Dam et al., 1986)

Because of the small size of SP, amplification via PCR is impossible. In this study, part of the precursor PPT-A was amplified instead. Primers were designed that detected only β and γ PPT-A mRNA in the portion that encodes the active SP peptide. They did however also span the region encoding NKA. The product produced is therefore representative of the precursor for both these neuropeptides and not a true representation of SP mRNA expression alone

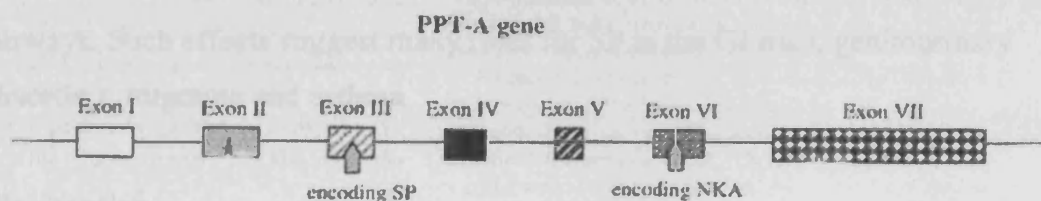


Figure 2.1 Diagram to represent the organization of the PPT-A gene with its seven exons. Exon III encodes SP and exon VI encodes NKA. There are 4 splice variants produced from this gene. α PPT-A has all exons except VI, β contains all exons, the δ transcript is missing exons IV and VI and the γ form is missing exon IV. Taken from Qian et al., 2001.

Substance P knockout

Mice with null mutations in the tachykinin 1, preprotachykinin gene and also in the NK₁ receptor gene are all viable and fertile, but have defects in nociceptive transmission and cellular responses to inflammatory stimuli (Cao et al., 1998; De-Felipe et al., 1998; Zimmer et al., 1998; Ahluwalia et al., 1999; Mansikka et al., 1999; Bester et al., 2001; Kidd et al., 2003). This suggests a role for SP in transmission of nociceptive information particularly following inflammatory stimuli.

This will be discussed whilst examining the physiological roles of this neuropeptide and its effects in peripheral tissues.

Physiological roles

SP and neurogenic inflammation

SP is a sensory neurotransmitter that is produced particularly following inflammatory stimulation (Donaldson et al., 1992; Donnerer et al., 1992; Woolf et al., 1994), and effects produced by release of SP from nerve endings of capsaicin-sensitive sensory neurons is referred to as 'neurogenic inflammation'. The released SP causes vasodilation of arterioles and plasma protein extravasation (Ferrante and VadeBoncouer, 1993). It also has many tissue specific effects including smooth muscle contraction/relaxation in iris and bladder and bronchoconstriction of the airways. Such effects suggest many roles for SP in the GI tract, genitourinary disorders, migraine and asthma.

Nociception

SP is synthesised and located in a subpopulation of small diameter DRG neurons, implicating a nociceptive role. It is released from sensory neurons in response to inflammatory stimuli (Duggan et al., 1987; Oku et al., 1987; Schaible et al., 1990), alongside an increase in transcripts of its preferential receptor NK₁, within the superficial dorsal horn (Krause et al., 1995). It is thus implicated in nociceptive transmission and the hyperalgesia associated with application of inflammatory stimuli (Hokfelt et al., 1975). An increase in SP production is also observed in diseases of chronic inflammation (Oku et al., 1987; Krause et al., 1995).

Much evidence implicates a role for SP in the hyperalgesia associated with inflammation. Intrathecal administration of SP results in hyperalgesia (Moochhala et al., 1984). Application of NK₁ antagonists results in a loss in the increased

excitability in response to noxious thermal stimulation and also in a loss of 'wind-up', (a concept in which following repetitive stimulation, the response will increase markedly, so that the response to the last stimuli is much greater than that to the first stimuli, despite being the same strength) (Thompson et al., 1995; Radhakrishnan et al., 1998). Furthermore ablation of SP neurons results in a loss of capsaicin responsiveness and the thermal hyperalgesia and mechanical allodynia associated with neuropathic or inflammatory pain (Nichols et al., 1999; Khasabov et al., 2002).

Further information on the functional role for SP in peripheral inflammation has been gained following the generation of mice with a null mutations in the NK₁ receptor and the PPT gene. In both NK₁^{-/-} and PPT^{-/-} mice, hyperalgesia develops normally (De Felipe et al., 1998; Cao et al., 1998; Zimmer et al., 1998; King et al., 2003), however NK₁^{-/-} mice display a loss of hyperalgesia in response to more chronic phases of inflammatory disease (Kidd et al., 2003). Furthermore such mice show a loss of response to noxious chemical signalling (Laird et al., 1998; Mansikka et al., 1999), and also display a loss of intensity coding of nociceptive stimuli and a decrease in 'wind-up', alongside a decrease in stress-induced analgesia (De-Felipe et al., 1998; Bester et al., 2001).

Migraine

SP is present in cerebral arteries of several species (Edvinsson et al., 1983). Upon release it can produce arterial relaxation and plasma extravasation, suggestive that SP signalling contributes to the onset of migraine (Moskowitz et al., 1992; Moussaoui et al., 1993). Such effects are proposed to be mediated by NK₁ receptors, as SP-induced plasma extravasation in cerebral arteries of the rat and guinea pig can be prevented by application of antagonists to NK₁, but not NK₂ (Mousanni et al., 1993; O'Shaughnessy et al., 1994).

Asthma and chronic bronchitis

A role for SP in the inflammation of the airways associated with asthma and chronic bronchitis has been suggested. Neurogenic inflammation of the airways leads to bronchoconstriction and in some cases bronchorelaxation (Figini et al., 1996), as well as secretion from seromucous glands (Geppetti et al., 1993) and release of mediators from the epithelium such as prostaglandins and NO. It is proposed that SP and neurogenic inflammation produce the airway hyperresponsiveness following allergen challenge (Bertrand et al., 1996). Application of NK₁ antagonists abolishes hyperresponsiveness and microvascular permeability in animal models (Mashito et al., 1999; Schuiling et al., 1999). Analysis of autopsy, biopsy and bronchoscopy tissue samples from asthmatics support such a role for SP in development of bronchial hyperresponsiveness. A decrease in SP immunoreactivity in tracheal (Lilly et al., 1995) and lung (Howarth et al., 1991) tissue of asthmatic illustrates an enhanced release of SP, which is reflected by the observation of an increase in SP immunoreactivity in sputum collected from asthmatics following antigen challenge (Nieber et al., 1992).

Gastrointestinal (GI) tract

The gut is innervated by extrinsic and intrinsic enteric neurons, which regulate motility and fluid secretion. These neurons are likely to contain SP (Costa et al., 1986). SP has been shown to contract all parts of the GI tract in all mammals including man, despite regional variations in SP expression between species (reviewed in Bartho and Holzer, 1994; Bartho et al., 1995). It can also stimulate plasma extravasation in post capillary venules of stomach, small and large intestines and pancreas, via NK₁, in mouse (Figini et al. 1997) and in stomach, small intestine and pancreas of the rat (Nicolau et al., 1993). Such effects, illustrate the role of SP and regulation of neurogenic inflammation in the GI tract.

Alterations in SP signalling have been postulated to contribute to diseases of the GI tract. An increase in NK₁ receptor sites is observed in inflammatory bowel disease

(Holzer, 1998), Crohns disease and ulcerative colitis (Mantyh et al., 1988). Studies of NK1^{-/-} mice also suggest a role for SP signalling via NK₁ in proinflammation and severity of pancreatitis (Saluja et al., 1999; Grady et al., 2000).

Genitourinary tract

SP immunoreactive fibres are present in the bladder (Maggi et al., 1988). SP increases motility and plasma extravasation in the genitourinary tract (Hua et al., 1987; Maggi et al., 1987; Maggi et al., 1988) and is thought to contribute to cystitis. In interstitial cystitis the number of NK₁ receptors around small blood vessels is increased alongside NK₁ receptor transcripts within the bladder endothelium (Marchand et al., 1998; Buffington et al., 1998). Furthermore, NK₁ receptor antagonists inhibit the onset of the early phase of plasma extravasation in xylene-induced cystitis (Maggi et al., 1988b).

Immune System

SP can regulate activity of many cells of the immune system affecting the cellular response to inflammation. SP can stimulate interleukin-8 (IL-8) synthesis and release (Serra et al., 1994); is chemotactic for neutrophils and eosinophils *in vitro* (Cazlan et al., 1993; Roch-Arveiller et al., 1986); is required for IL-1beta induced neutrophil accumulation in response to inflammation (Ahluwalia et al., 1999) and can contribute to antibody dependent cell mediated toxicity (Wozniak et al., 1993). Additionally it has indirect effects on cells of the immune system, producing a priming effect on responses mediated by other molecules such as platelet activating factor (PAF) and recombinant human interleukin 5 (rhIL-5) (El-Shazly et al., 1996; Wiedermann et al., 1991).

SP, nerve injury and neuropathic pain

Transection of the sciatic nerve results in a down-regulation of SP expression within DRG (Nielsch et al., 1987; Zhang et al., 1995; Sterne et al., 1998). Such a down-regulation is not observed within the nodose following axotomy of cervical vagus (Helke et al., 1995), suggesting that SP expression in this neuronal population is regulated differently from that in sensory neurons of the DRG. Additionally the inflammation induced increase in SP observed in DRG of a mouse model of arthritis, is also attenuated following nerve section (Ahmed et al., 1995). Such effects are thought to be due to a loss of target derived factors such as NGF which regulate expression of SP.

Neurotrophic factor regulation

SP is co-expressed with CGRP in the same subpopulation of sensory neurons, and both neuropeptides appear to be regulated by NGF. Exogenous NGF stimulates the expression of SP and CGRP in intact DRG of the rat (Schuligoi and Amann, 1998; Tandrup et al., 1999). and both neuropeptides are massively down-regulated in $TrkA^{-/-}/Bax^{-/-}$ and $NGF^{-/-}/Bax^{-/-}$ mice (Patel et al., 2000). The axotomy-induced decrease in SP in wild-type mice can also be prevented by NGF administration (Lindsay et al., 1989; Zhang et al., 1995).

2.1.3. Voltage gated sodium channels (VGSC), Nav1.8 and Nav1.9

Sodium channels allow the generation and propagation of the compound action potential in electrically excitable tissues such as nerve, muscle and the heart. Depolarisation of tissues is caused by Na^{+} ion diffusion through voltage gated sodium channels (VGSCs).

Sodium channel structure

Mammalian VGSCs are multimers, composed of a central pore forming α subunit (approx 260kDa) and auxiliary β subunits. The alpha subunit forms the ion pore and is responsible for voltage sensing of the channel. Multiple isoforms of the alpha subunit exist in different regions of the brain and peripheral nervous system. These are outlined in figure 2.2. Isoforms differ in their kinetic properties and expression pattern. Sensitivity to the neurotoxin, tetrodotoxin (TTX), a toxin from the puffer fish, is used to pharmacologically distinguish between α subunits. Only three of the subunits show TTX resistance (TTX-R), Nav1.5, Nav1.8 and Nav1.9, the remaining channels are TTX sensitive (TTXS) (Black et al., 1996; Akopian et al., 1996; Sangameswaren et al., 1996; Dib-Hajj et al., 1998; Tate et al., 1998; Plummer and Meisler, 1999). Interestingly the genes encoding these three TTX-R subunits all exist on the same chromosome (Chromosome 3p21-24 in mice), suggesting an evolutionary link between these channels (Plummer and Heisler, 1999 and Goldin et al., 2000). Future references to sodium channels will be referred to as the gene (e.g. SCN10A) or the new nomenclature (e.g. Nav1.8).

β subunits act as accessory subunits, modulating the channel kinetics and membrane localisation (Catterall., 1992; Isom et al., 1994; Kazen-Gillespie et al., 1999; Isom et al., 2001). Three such subunits have been identified, these are β 1 (SCN1B, 36kDA), β 2 (SCN2B, 33kDa) (Isom et al., 1994; Catterall., 1992) and the most recently discovered β 3 (Morgan et al., 2000).

Table 1. Mammalian Sodium Channel α Subunits

Type	Former Name	Genbank Number ^a	Gene Symbol	Chromosomal Location	Splice Variants	Primary Tissues
Na _v 1.1	rat I HBSCI GPBI SCN1A	X03638 (r) X86362 (h) AF003372 (gp)	SCN1A	Mouse 2 Human 2q24	Na _v 1.1a	CNS PNS
Na _v 1.2	rat II HBSCII HBA	X03639 (r) X81149 (r) X86361 (h) M94055 (h)	SCN2A	Mouse 2 Human 2q23-24	Na _v 1.2a	CNS
Na _v 1.3	rat III	Y00766 (r)	SCN3A	Mouse 2 Human 2q24	Na _v 1.3a Na _v 1.3b	CNS
Na _v 1.4	SkM1, μ 1	M26643 (r) M81758 (h)	SCN4A	Mouse 11 Human 17q23-25		sk. muscle
Na _v 1.5	SkM2 H1	M27902 (r) M77235 (h)	SCN5A	Mouse 9 Human 3p21		Uninnervated sk. muscle, heart
Na _v 1.6	NaCh6 PN4 Scn8a Cer111	L39018 (r) AF049239 (r) AF049240 (r) U26707 (m) AF049617 (m) AF050736 (h) AF226968 (h) AF003373 (gp)	SCN8A	Mouse 15 Human 12q13	Na _v 1.6a	CNS, PNS
Na _v 1.7	NaS hNE-Na PN1	U35238 (rb) X82835 (h) U79568 (r) AF000368 (r)	SCN9A	Mouse 2 Human 2q24		PNS Schwann cells
Na _v 1.8	SNS PN3 NaNG	X92184 (r) U53633 (r) Y09106 (m) U60590 (d)	SCN10A	Mouse 9 Human 3p22-24		DRG
Na _v 1.9	NaN SNS2 PN5 NaT SCN12A	AF059030 (r) AJ237852 (r) AF118044 (m) AB031389 (m) AF126739 (h) AF188679 (h) AF109737 (h) AF150882 (h)	SCN11A	Mouse 9 Human 3p21-24	Na _v 1.9a	PNS
Na _v	Na _v 2.1 Na-G SCL11 Na _v 2.3	M91556 (h) M96578 (r) Y09164 (r) L36179 (m)	SCN7A SCN6A ^b	Mouse 2 Human 2q21-23		heart, uterus, sk. muscle astrocytes, DRG

^a The letter in parentheses after each accession number indicates the species of origin for the sequence, as follows: h, human; r, rat; rb, rabbit; m, mouse; gp, guinea pig; d, dog.

^b This gene was originally assigned symbols SCN6A and SCN7A, which were mapped in human and mouse, respectively. The two most likely represent the same gene, and the SCN6A symbol will probably be deleted.

Figure 2.2. Nomenclature and location of mammalian sodium channel α subunits.

Taken from Goldin et al., 2000

The use of in situ hybridization and RT-PCR has revealed that nine of the ten alpha subtypes and all three of the beta subtypes are present in sensory neurons (Black et al., 1996; Renganathan et al., 2002; reviewed in Lai et al., 2004). Expression profiles of different subunits vary considerably. Of the TTXR channels, Nav1.8 is highly expressed in small diameter neurons, with some expression in medium and large neurons, whilst Nav1.9 is only expressed only in small diameter neurons (Black et al., 1996). The remaining TTX-R channel, Nav1.5, is predominantly a cardiac channel, however some expression has been observed in embryonic DRG (Renganathan et al., 2002). TTX-S channels in sensory neurons include Nav1.6 and Nav1.7 whose expression is detectable to some extent in all sensory neurons (Tzoumaka et al., 2000). Nav1.2 can be found in all sensory neurons, but at barely detectable levels. Other sodium channels have a more restricted expression profile (Black et al., 1996). Nav1.1 is preferentially expressed in large diameter neurons and Nav1.3 is developmentally regulated, showing high expression embryonically, but very low expression in the adult rat (Waxman et al., 1994).

These voltage gated sodium channels collectively, are responsible for the current flow required within sensory neurons to propagate the compound action potential. Electrophysiological results indicate that there are two general sodium currents in DRG, one sensitive to TTX (TTX-S), and one unaffected by TTX (TTX-R) (Kostyuk et al., 1981; Caffrey et al., 1992; Roy and Narahashi, 1992). The TTX-S current is predominant in large sensory neurons, however small neurons additionally contain the TTX-R component, which compliments the restricted expression profiles of the TTX-R channels, Nav1.8 and Nav1.9 in small diameter neurons (Roy and Narahashi 1992; Rush et al., 1998). Additionally, more in-depth, work has recently shown that within small diameter sensory neurons TTX has no effect on sodium currents in IB4 positive neurons, but can inhibit AP generation in approximately 50% IB4 negative neurons (Wu et al., 2004). This provides more information of the distinct expression of TTX-S and TTX-R channels within subclasses of small diameter sensory neurons, peptidergic neurons containing a compliment of both TTX-S and TTX-R, but IB4 reactive small diameter neurons containing just TTX-R channels. The presence of the

TTX-R current only in a sub-population of small sensory neurons suggests a function for TTX-R VGSCs in nociception. This will be discussed further later.

Information on the discrete functions of each VGSC, within sensory neurons, has been hindered by the lack of specific channel blockers. Some insight has been gained using novel approaches and pharmacological techniques. Most of the VGSCs giving rise to the TTX-S current have a low activation threshold (in the region of -50mV and -40mV) with fast activation and inactivation. The application of TTX to distal axons, blocks nerve impulse conduction, evidence that TTX-S channels are required to mediate action potential generation in both myelinated and unmyelinated axons (Brock et al., 1998; Gold et al., 2003).

Several subtypes of the TTX-R current exist within the DRG, these are termed TTX-R1,2 and 3. TTX-R1 has a high threshold for activation (approximately -36mV) and steady state inactivation. It activates and inactivates relatively slowly, but recovers from inactivation rapidly such that when other channel subtypes are inactivated by depolarisation, low levels of activity can be sustained by this current (Ogata et al., 1993; Elliott et al., 1993 Rush et al., 1998). There is evidence to suggest that Nav1.8 is the sodium channel underlying this current. Injection of Nav1.8 into Nav1.8^{-/-} mice produces a TTX-R current with characteristics identical to this TTX-R1 (Akopian et al., 1999). These null mutant mice also no longer display the all-or-nothing action potentials characteristic of such neurons, suggesting a role for TTX-R1 and Nav1.8 in action potential generation electrogenesis (Renganathan et al., 2001).

TTX-R2 has unique biophysical properties with slow activation kinetics and a persistent nature with very low threshold for inactivation (between -70mV and -90mV) (Cummins et al., 1999). The properties of this current suggest that it is unlikely to contribute to the action potential upswing. However, its slow inactivation and its regenerative properties suggest a role in determination of membrane resting potential (Cummins et al., 1999; Herzog et al., 2001; Baker et al., 2003). The finding that this current still persists in Nav1.8^{-/-}, and indeed is further enhanced following GDNF infusion in the null mouse indicates that another TTX-R channel underlies

this current (Cummins et al., 1999; Cummins et al., 2000). This is most likely Nav1.9, due to its similar activation kinetics (Baker et al., 2003; Priest et al., 2005; Reviewed in Wood et al., 2004).

The TTX-R3 current has very similar kinetics to that of the TTX-S current, with low threshold of activation, combined with rapid rates of activation and inactivation (Rush et al., 1998). The physiological and pharmacological profile of this current suggests that the predominantly cardiac channel, Nav1.5 carries it (Renganathan et al., 2002). Nav1.5 is developmentally regulated, suggesting this current is only seen within DRG embryonically.

In this thesis it was decided to initially focus on the expression of the two sensory neuronal predominant TTX-R VGSCs, Nav1.8 (also known as SNS/PN3) and Nav1.9 (also known as NaN).

Expression

Nav1.8 (SNS/PN3) was cloned from rat DRG, and shown to display 65% identity to the rat cardiac TTX-R channel, Nav1.5 (Sangameswaran et al., 1996; Akopian et al., 1996). It is encoded from the gene *Scn10a* and is sensory neuron specific. It is found both in small diameter non-myelinated sensory neurons (C-fibres) and in 10% of small diameter myelinated axons corresponding to A-delta nociceptors (Sangameswaran et al., 1996; Amaya et al., 2000). Of such small diameter unmyelinated neurons, Nav1.8 is expressed equally in the peptidergic TrkA positive population and in the IB4 reactive populations (Benn et al., 2001).

The second TTX-R alpha subunit, Nav1.9 (NaN/SNS2) is encoded from the *Scn11a* gene (Dib-Hajj et al., 1998). It exhibits only 42-53% similarity to Nav1.8. Nav1.9 is predominantly located in small diameter sensory neurons, but is also expressed at low levels within the CNS (Black et al., 1996; Dib-Hajj et al., 1998; Tate et al., 1998; reviewed in Lai et al, 2004). Within sensory neurons, Nav1.9 is expressed specifically within unmyelinated small C fibres that are peptidergic and

immunoreactive for TrkA. It is not detectable in neurons with myelinated axons (Amaya et al., 2000; Benn et al., 2001; Fang et al., 2002). Such a specific expression within the unmyelinated nociceptive neurons is suggestive of a role for Nav1.9 in pain. This will be discussed later.

Physiological function

Both channels are resistant to TTX and are important in the TTX-R currents within sensory neurons. As discussed, Nav1.9 is implicated to underlie the persistent TTX-R2 current that is thought to play a role in determination of membrane resting potential. Nav1.8 is responsible for the TTX-R1 current that has been implicated in action potential generation within nociceptive neurons of the DRG (Reneganathan et al., 2001). Working in conjunction with TTX-S channels, Nav1.8 produces the characteristic shape of the nociceptive action potential, which displays a rapid depolarisation with a prominent shoulder during the falling phase. The upstroke of the nociceptor action potential is produced by an influx of positive charge through both TTX-R channels (Approximately 58%) and TTX-S channels (approximately 40%), with a small contribution (2%) from Calcium channels. TTX-S channels then rapidly inactivate, but the TTX-R1 current remains to sustain low levels of activity (Ogata et al., 1993; Elliott et al., 1993 Rush et al., 1998). This, in combination with Ca^{2+} influx, produces the characteristic nociceptor AP shoulder.

Nav1.8, Nav1.9 and pain

Both Nav1.8 and Nav1.9 are strongly implicated in the molecular mechanisms of nociception, as discussed above. In the following section I will discuss in turn the roles of these channels in neuropathic pain as a result of neuronal injury and in inflammatory pain.

Following nerve damage and axotomy of peripheral DRG neurons, an increase in excitability and enhanced repetitive firing has been observed, alongside a reduction in action potential threshold (Zhang et al., 1997). Such effects are thought to underlie

the neuropathic pain associated with nerve injury and have been attributed partly to alterations in sodium channel expression and conductance. An increased density in TTX-S channels following axotomy is observed, producing an increased sodium conductance within DRG thought to contribute to the increased excitability in the axotomised neurons (Zhang et al., 1997; Black et al., 1999). This has been attributed to an up-regulation of the normally silent VGSC Nav1.3, and is discussed further in chapter 4 (Waxman et al., 1994; Cummins and Waxman, 1997; Sleeper et al., 2000).

Alongside alterations in TTX-S channels, alterations in the TTX-R channels Nav1.8 and Nav1.9 are also observed. In contrast, expression of these channels is decreased within small diameter neurons of the DRG following peripheral axotomy or sciatic nerve section (Okuse et al., 1997; Cummins et al., 1997; Tate et al., 1998; Dib-hajj et al., 1998; Novakovic et al., 1998; Sleeper et al., 2000; Decosterd et al., 2002). Such a down-regulation of TTX-R channels is thought to contribute indirectly to the hyperexcitability of sensory neurons, following axotomy, through the interaction of TTX-R currents with TTX-S sodium conductances (Zhang et al., 1997; Cummins et al., 1997; Sleeper et al., 2000). The persistent TTX-R current carried by Nav1.9, is thought to contribute a depolarising influence to the resting membrane potential of sensory axons (Cummins et al., 1999). The down-regulation of Nav1.9 following injury would therefore produce a hyperpolarizing shift in resting membrane potential. This shift is proposed to relieve the inactivation of TTX-S channels, resulting in an increase in TTX-S currents and subsequent neuronal excitability (Sleeper et al., 2000). Loss of the slow inactivating TTX-R current carried by Nav1.8 has also been illustrated, by computer simulation, to result in a lowered AP threshold and spontaneous/repetitive firing in the absence of stimulation (Elliot, 1997; Schild and Kunze, 1997). Down-regulation of both Nav1.8 and Nav1.9 channels is therefore implicated in the neuronal hyperexcitability that contributes to neuropathic pain following nerve damage.

A direct link between Nav1.8 and neuropathic pain, was demonstrated by Lai et al., (Lai et al., 2002), who used antisense oligonucleotides to 'knock down' the expression of Nav1.8. In such mice, neuropathic pain induced by spinal nerve injury

was no longer observed, but effects of acute pain and responses to non-noxious stimuli remained (Lai et al., 2002). A down-regulation of Nav1.9 mRNA is also observed in three separate models of neuropathic pain (Abe et al., 2002).

Of therapeutic interest were results following experiments to study the electrophysiological properties of Nav1.8 by cloning this channel into xenopus oocytes (Chevrier et al., 2004). The local anaesthetic, lidocaine was found to have a strong affinity for Nav1.8, binding to the slow inactivated state of the VGSC, where it is thought to block nociceptor firing and prevent the proposed positive effects of Nav1.8 on neuropathic pain. Such LA sensitivity was not observed for other channels, namely Nav1.7 (Chevrier et al., 2004), which correlates with results of Roy and Narahashi (Roy and Narahashi, 1992) who found that TTX-R currents were more sensitive to lidocaine than TTX-s channels in native DRG neurons. These results suggest the TTX-R channels, particularly Nav1.8, are suitable targets for the development of drugs to treat neuropathic pain. Despite such findings, recent work by Nassar et al., (Nassar et al., 2005) shows conflicting results. In this study cre-lox transgenic mice, in which Nav1.8 and Nav1.7 are both specifically knocked out in nociceptive neurons, show normal generation of neuropathic pain, despite a lack of inflammatory pain symptoms.

A role for TTX-R channels in inflammatory pain has also been investigated. The generation of mice with null mutations in sodium channel genes has been useful in further characterising such a role. Nav1.8^{-/-} mice appear normal, and are viable and fertile, however, behaviourally such mice display a pronounced analgesia to noxious mechanical stimuli, have small deficits in noxious thermoception and show a delayed development of inflammatory hyperalgesia (Akopian et al., 1999; Ogata et al., 2001). Such mice also display an increase in current densities of TTX-S channels, implicating an up-regulation of TTX-S currents within the DRG to compensate for the loss of TTX-R flow (Akopian et al., 1999; Ogata et al., 2001). Such an up-regulation in TTX-S currents was also observed following the down regulation of

TTX-R channels in models of nerve damage and neuropathic pain, as mentioned previously.

Inflammatory pain caused, for example, by CFA injection into adult rat skin, produces a dramatic increase in Nav1.8 protein expression in DRG ;within 48 hours (Gould et al., 1998; Tanaka et al., 1998; Gould et al., 2004; Coggeshall et al., 2004). This is observed in both small and large neurons, and both myelinated and unmyelinated axons (Gould et al., 2004; Coggeshall et al., 2004). The inflammation-induced, enhanced level in small neurons persists. However as allodynia subsides, the expression of Nav1.8 within large neurons decreases back to baseline (Gould et al., 2004). It has been proposed that the increase in Nav1.8 in *larger* neurons is likely to be responsible for the hyperalgesia observed in such inflammatory states. The persistent up-regulation in small diameter neurons is proposed to produce prolonged increased sensitivity that could provide a protective role, to ensure vigilance during healing (Gould et al., 2004). Further evidence of a role in inflammatory pain was provided by the use of antisense oligonucleotides to attenuate Nav1.8 expression. Injection of such oligonucleotides into rats, prevented PGE2 induced hyperalgesia and increased the threshold to mechanical nociceptive stimuli (Khasa et al., 1998). Interestingly no increase was observed in Nav1.9 in inflammatory states, suggesting that unlike Nav1.8, Nav1.9 plays no role in the peripheral sensitisation early in inflammation (Coggeshall et al., 2004).

The dynamic alteration in Sodium channel levels in response to injury or inflammation is suggestive of a role in the associated neuropathic/inflammatory pain. A TTX-R current only found in small nociceptive neurons also would indicate a role in nociceptive transmission. The precise mechanisms by which such channels could mediate effects are yet to be elucidated. However the limited expression of the sodium channels, Nav1.8 and Nav1.9 to sensory neurons, provides strong therapeutic potential, allowing the manipulation of specific subpopulations of sensory neurons.

Regulation of Na channel expression by neurotrophic factors

The increase in NGF released *in vivo* as part of the inflammatory response is thought to mediate such increases in Nav1.8 in small DRG neurons. Indeed, administration of NGF has been shown to increase Nav1.8 protein expression in DRG and produce a decrease in paw withdrawal latencies characteristic of hyperalgesia. Furthermore pre-blocking NGF with anti-NGF antibodies prevented the NGF-induced effects on paw withdrawal latencies and significantly reduced expression of Nav1.8 protein (Gould et al., 2000).

In addition to altering expression of Nav1.8 in response to inflammation, NGF also regulates the TTX-R current and TTX-R sodium channel expression following axotomy of adult DRG neurons. As mentioned, axotomy of DRG neurons produces a decrease in TTX-R currents (Cummins and Waxman, 1997; Sleeper et al., 2000) in response to a decrease in expression of the sodium channels Nav1.8 (Dib-Hajj et al., 1996; Okuse et al., 1997) and Nav1.9 (Dib-Hajj et al., 1998a; Tate et al., 1998). Following axotomy of DRG neurons, retrograde transport of NGF from peripheral targets is prevented. This reduction of NGF at the site of injury is thought to be responsible for several of the axotomy related phenotypic changes in DRG neurons, including the reduction in TTX-R current and channels. Much evidence supports this. NGF has been shown to restore the down-regulation of TTX-R currents following axotomy of DRG neurons *in vitro* (Aguayo and White, 1992; Black et al., 1997; Fjell et al., 1999) and *in vivo* (Dib-Hajj et al., 1998), attributable to positive effects of NGF on TTX-R channel expression (Black et al., 1997; Dib-Hajj et al., 1998).

GDNF has also been shown to promote similar effects following axotomy both *in vivo* and *in vitro* (Fjell et al., 1999; Cummins et al., 2000). GDNF treatment of cultured rat DRG produced an up-regulation in levels of both Nav1.8 and Nav1.9 mRNAs, alongside increasing both the slowly inactivating and the persistent TTX-R currents (Cummins et al., 2000). *In vivo*, infusion of GDNF to axotomised DRG neurons also resulted in an increase in Nav1.8 and Nav1.9 protein levels and an up-regulation in TTX-R currents (Cummins et al., 2000). Another GDNF family

member, artemin, has also been shown to reduce the SNL-induced down-regulation of Nav1.8 protein in a rat model of experimental neuropathy (Gardell et al., 2003). NGF, GDNF and artemin therefore all prevent axotomy/nerve damage induced changes in sodium channel expression, which are thought to contribute to the onset of neuropathic pain. A clearer understanding of the role and pathways involved with such neurotrophic factor effects would therefore be of interest for the design of novel drugs in the treatment of neuropathic pain.

Neurotrophic factor-induced regulation of Nav1.9 has also been observed in other populations of neurons. BDNF has been shown to evoke TTX-R currents in hippocampal neurons mediated by Nav1.9 (Blum et al., 2002), and it is proposed that such currents play a role in long term potentiation (LTP) (Kovalchuck et al., 2002). Interestingly these Nav1.9 generated currents differ from those seen in sensory neurons being smaller; readily blocked by the neurotoxin, saxitoxin; and only observed in TrkB positive neurons (Blum et al., 2002). Furthermore HEK293 cells transfected using cDNA clones encoding Nav1.9 with or without TrkB, showed characteristics of both types of current (Blum et al., 2002). The ability of Nav1.9 to mediate a different current according to cell type could be attributable to post-transcriptional regulatory steps, for example trans-splicing (Akopian et al., 1999); post-translational modulation of alpha subunits e.g. phosphorylation by PKA (Gold et al., 1999); or through modulation by accessory β subunits (Isom et al., 2001, reviewed in Wood et al., 2004).

Little has been done on the effects of neurotrophic factors on TTX-R channels in development. In cultured embryonic DRG neurons it was found that the expression of both Nav1.8 and Nav1.9 mRNA was decreased following NGF withdrawal, suggesting NGF is required for their expression (Zur et al., 1995; Klein et al., 2003). However, the interpretation of findings is complicated by the fact that the neurons are dependent on NGF for survival and that NGF withdrawal of NGF leads to the death of these neurons.

Other functions

Despite the generally healthy and viable phenotype of the Nav1.8 null mutant, a surprising role for incorrect expression of Nav1.8 has been found in sufferers of multiple sclerosis (MS). Nav1.8 is normally expressed within the PNS, but *not* in the brain, however in a mouse model of MS, Nav1.8 mRNA and protein were expressed in cerebellar Purkinje cells (Black et al., 2001). This was also found in post-mortem tissue from patients with MS, but not in control tissue from subjects with no neurological disorders. These results suggest that expression of Nav1.8 is altered within neurons in the brain of MS sufferers. This abnormal expression may contribute to characteristic traits of the disorder, such as ataxia (Black et al., 2001)

2.1.4. NGF and TrkA knockout mice

In this chapter gene regulation by NGF was explored using mice with null mutations in the NGF gene. TrkA^{-/-} mice were also used to investigate NGF signalling via this receptor tyrosine kinase. Homologous recombination methods in embryonic stem cells allowed the generation of mice with a deletion in the coding sequence of the NGF gene (Crowley et al., 1994) and the TrkA gene (Smeyne et al., 1994). Histological analysis of mice homozygous for NGF disruption revealed a severe cell loss in both sensory and sympathetic ganglia, alongside an inability to respond to noxious mechanical stimuli (Crowley et al., 1994). Similarly, TrkA knockout mice had severe sensory and sympathetic neuropathies, most dying within one month of birth (Smeyne et al., 1994). Because of this drastic phenotype, I studied gene expression in these mice at embryonic stages before the onset of excessive neuronal cell death and after normal expression of TrkA and NGF occurs. A small time window between E12 and E16 was possible for these experiments.

2.2. Materials and methods

2.2.1. Transgenic mice

The TrkA and NGF knockout mice used were those originally generated by homologous recombination by Smeyne et al., 1994 and Crowley et al, 1994. Embryos of the required stage were obtained from overnight matings of TrkA and NGF heterozygote mice. The date of identification of vaginal plug was set to be embryonic day 1. Pregnant females were killed by carbon dioxide asphyxiation followed by cervical dislocation at 13, 14, and 15 days of gestation. Embryos were carefully removed from the amniotic sac and the precise stage of development was determined by the criteria of Theiler (Theiler K, 1972).

2.2.2. Dissections

Dissections of trigeminal, nodose and DRG from staged embryos were performed under a stereo-microscope, using a fibre-optic light source that allows illumination of specimens without overheating. Dissections were carried out in filter sterilised Liebowitz-15 (L-15) medium (pH 7.3) supplemented with penicillin (60mg/l) and streptomycin (100mg/l).

Dissections were carried out using forceps and scissors (E15) followed by tungsten needles to remove adherent connective tissue. For younger ages (E13, E14), tungsten needles were used for the whole dissection. Tungsten needles were made from 0.5mm diameter tungsten wire electrolytically sharpened in 1M KOH whilst being held in chuck-grip platinum wire holders (Davies AM, 1988). All tools were flamed with alcohol to sterilise prior to dissection.

Dorsal Root Ganglia (DRG) Dissection

The head and tail of the embryo were severed so just the trunk region remained. Skin and internal organs were then removed to leave just the spinal column. Cuts were made along the length of the spinal column by inserting one blade of the scissor (or one needle) inside the column and cutting along the ventral aspect. This was done on either side to allow the removal of the ventral section of the spinal column. The DRG were now visible in gaps between where ribs were to form. Ganglia were gently isolated, and any adherent nerves and connective tissue cut off using needles. A Pasteur pipette was used to transfer collected ganglia to 1.5ml micro-centrifuge tubes. Ganglia were stored at -80°C until genotyping was complete.

Trigeminal Dissection

The dissection of these ganglia varied with the age of the mouse. From embryonic day 15, the top of the skull was removed using fine scissors and the brain gently removed using forceps. The two trigeminal ganglia were now visible on either side of the midbone at the base of the skull. The ganglia were removed using forceps and tungsten needles were used to clean them of any adherent connective tissue. At younger ages (E14 and below) the dissection was carried out by initially using tungsten needles (E13) or scissors (E14) to make two coronal incisions between the maxillary and mandibular processes of the first brachial arch, one just above the eye and the second below the eye. The opaque structures of the two trigeminal ganglia were now visible just behind either eye in this tissue slice. They were freed from this tissue slice by slowly removing the surrounding tissue with tungsten needles. See figure 2.3.

Both types of ganglia were dissected from each individual mouse, collected in 1.5ml micro-centrifuge tubes and stored at -80°C until genotyping was complete. Following genotyping, ganglia from knockout and wild-type mice had RNA extracted from them prior to RT-QPCR analysis of gene expression.

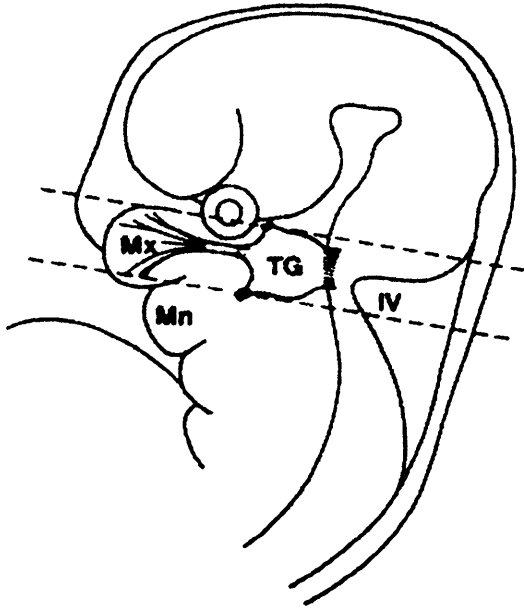
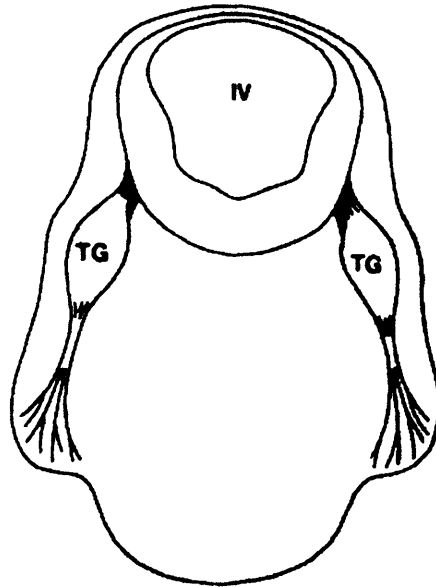
A**B**

Figure 2.3 : Dissection of the trigeminal ganglia

A: The lateral aspect of E11 mouse head. Dotted lines represent the location of the transverse incisions that are made to obtain a tissue slice containing the trigeminal ganglia (TG), B: The rostral aspect of the tissue slice containing the TG.

(Mx - Maxillary process; Mn - Mandibular process; IV - fourth ventricle.

Taken from Davies, 1995

2.2.3. Genotyping

Genotyping was used to determine whether dissected mice were knockout, wild-type or heterozygote for the gene of interest. To avoid contamination, all DNA extractions and Polymerase Chain Reactions (PCRs) were carried out in a DNA free environment, following standard procedures to minimise exposure to DNases and contaminating DNA species.

DNA Extraction

DNA was extracted from a small amount of the remaining tissue of each dissected embryo using a Nucleospin Tissue DNA extraction kit (Macherey-Nagal, Germany), following the manufacturers protocol. In brief, tissue was broken down by overnight incubation at 56°C in a micro-centrifuge tube containing lysis buffer and 25 µg/ml proteinase K. The following day, tubes were centrifuged at 10,000RPM for 5 minutes, to pellet the undigested tissue and 200µl of the supernatant was transferred to a fresh 1.5 ml micro-centrifuge tube along with an equal volume of dH₂O. 200µl of this diluted solution was added to an equal volume of buffer B3 and the samples were incubated at 70°C for 10mins. Samples were centrifuged again, at 10,000rpm for 10mins, and the supernatant transferred to a spin tube with a DNA binding filter. Bound DNA was washed to remove contaminating proteins and salts, using an ethanol based solution and centrifugation. Finally, the DNA was eluted from the membrane by the addition of 120µl of low ionic-strength elution buffer (supplied by the manufacturer) that had been pre-heated to 70°C. Extracted DNA was stored at –20C whilst awaiting genotyping by PCR.

PCR

PCR reactions were designed to distinguish whether individual embryos from heterozygote X heterozygote crosses contained just knockout alleles, just wild type alleles or were heterozygous for both. PCR reactions were performed in duplicate from the DNA sample of each mouse. Details of reaction conditions and primer

sequences are outlined below. A master mix was made up for each reaction, 19 μ l of which was added to 1 μ l DNA. Mastermix recipes are outlined below.

	NGF	TRK A
10 \times hot taq buffer (GeneSys Ltd)	2 μ l	2 μ l
5mM dNTPs (Promega)	1 μ l	1 μ l
WT primers (MWG)	0.34 μ l	0.2 μ l
KO Primers (MWG)	0.67 μ l	0.2 μ l
Common Primers (MWG)	0.5 μ l	0.23 μ l
Hot Start Taq (GeneSys Ltd.)	0.13 μ l	0.2 μ l
25mM MgCl ₂ (GeneSys Ltd.)	4 μ l	1 μ l
Mr solution (GeneSys Ltd.)	6.61 μ l	-
dH ₂ O (GIBCO)	6.61 μ l	13.167 μ l

Table 2.1 Reaction mix for genotyping reactions

The combination of 3 primers could potentially produce 2 products, one for the wild type allele and one for the knockout allele. DNA from Heterozygote animals contained both products.

Primer sequences are outlined below

TrkA:

P095-4 (wild type): 5'-CGG ACC TCA GTG TTG GAG AGC TGG-3'

P096-0 (mutant): 5'-CAC CCT GCA CTG TCG AGT TTG C-3'

P097-0 (common): 5'-GCT CCC GAT TCG CAG CGC ATC G-3'

NGF:

NGF1 (wild-type) 5'-ACA GAT AGC AAT GTC CCA G-3'

NGF 2 (mutant) 5'-TCT GGA TTC ATC GAC TGT G-3'

NGF C (common) 5'-GGT GCT GAA CAG CAC ACG-3'

Two drops of mineral oil were layered on top of each PCR reaction to prevent evaporation of reagents during thermocycling. The Taq polymerase used for genotyping had been genetically altered to be inactive unless heated at 95°C for 15 minutes. Such “hot start” polymerases reduce the amount of mispriming in the first PCR cycle and, therefore, give cleaner, more efficient PCR reactions. Initially samples were heated to 95°C for 15 minutes to activate taq. A variable number of PCR cycles were then performed to amplify the product, the exact number of cycles depending on the concentration of the DNA and the amplification efficiency of the primer sets used. Each cycle followed the same general pattern. First, tubes were heated to 95°C to denature secondary structure in the DNA, and separate double-stranded DNA into single-stranded DNA. Next, the reaction was cooled to the specific annealing temperature of the primer set, to allow primers to anneal. The annealing temperature was determined according to primer sequence and size. Finally, the reaction was heated to 72°C to allow the taq DNA polymerase to extend primers and synthesize a complementary copy of the single stranded DNA template. Following cycling, samples were heated to 72°C for 10mins for a final extension step to ensure all products of the PCR reaction were full length.

Reaction conditions for both knockout strains are outlined below.

	NGF	TRKA
Denaturation	95°C 15mins	95°C 15mins
Denaturation	95°C 40s	95°C 40s
Annealing	55°C 1min 10s	65°C 1min 10s
Extension	72°C 1min 50s	72°C 1min 40s
No. Cycles	45	38
Final Extension	72°C	72°C
Hold	4°C	4°C

Table 2.2 Reaction conditions for genotyping reactions

Visualisation of Products

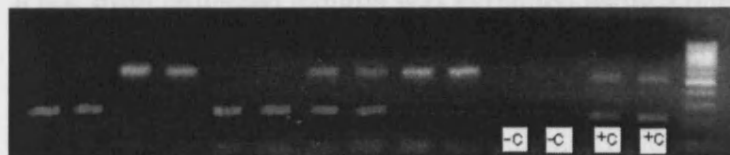
Once the PCR reaction was completed, the PCR products were visualised by adding 4ul of 6X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll in dH₂O) and running 10ul on a 2% agarose gel, made with TAE buffer and containing 1µg/ml ethidium bromide (Sigma).

The wild type lower band and knockout upper band could be visualised in the gel using a UV gel documentation system (Biogene). Figures 2.4. and figure. 2.5. show representative gels of genotyping products from NGF and TrkA mice, respectively. Expected PCR product sizes are outlined below

	NGF	TRKA
Wt band size	190	400
KO band size	610	800

Table 2.3 Band sizes for genotyping reactions

NGF



1 2 3 4 5 6 7 8 9 10

Figure 2.4: Genotyping gel showing the three possible genotypes of embryos from $NGF^{+/-}$ crosses. Lanes 1, 2, 5 and 6 show the PCR product from the wild-type DNA. Lanes 3, 4, 9 and 10 show the PCR product from the knockout DNA. Lanes 7 and 8 show the products from the heterozygote DNA. The size of the products is shown with respect to a 1kb ladder. (N.B. -C shows the negative controls in which DNA extractions were done with no tissue. +C is a positive control using DNA shown to be heterozygote previously).

TrkA

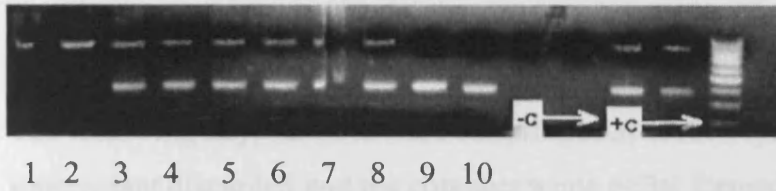


Figure 2.5: Genotyping gel showing the three possible genotypes of embryos from *TrkA*^{+/-} crosses. Lanes 1 and 2 show the PCR product from the knockout DNA. Lanes 3, 4, 5, 6, 7 and 8 show the PCR product from the heterozygote DNA. Lanes 9 and 10 show the products from the wild-type DNA. The size of the products is shown with respect to a 1kb ladder.

As the expression of the genes investigated in this chapter is low in young embryos, knockout and wild-type ganglia were pooled separately to give a more concentrated RNA sample following RNA extraction. 6 ganglia per replicate were pooled for E13, 4 for E14 and 3 or 4 ganglia for E15. 3 or 4 replicates were collected for each type of ganglia at each age (n = 3-4).

2.2.4. RNA extraction

RNA from collected ganglia was extracted using a phenol-based method described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). 500µl of Solution D lysis buffer (4M guanidine thiocyanate, 25mM tri-sodium citrate pH 7.0, 0.5% N-lauroylsarcosine, 0.1M β mercaptoethanol), was added to each tube containing ganglia, and the ganglia were homogenized by passing up and down through a 25 gauge needle. The following was then added sequentially to the lysed cell solution: 1µl 10mg/ml *E-coli* transfer-RNA (Sigma); 50µl sodium acetate, pH 4.4; 500µl water-saturated acidic phenol (Sigma); 150µl of 25:1 chloroform: isoamyl alcohol (Sigma). The solution was mixed vigorously and tubes left on ice for 1-5 minutes before centrifugation at 13,000rpm for 20 minutes. Following centrifugation the RNA solution had separated into two layers, the upper aqueous phase containing total RNA. This top layer, approx. 450µl, was transferred to a clean 1.5ml micro-centrifuge tube and twice the volume of 100% ethanol (AnalR grade) was added. The

samples were vortexed and put at -20°C overnight to allow precipitation of total RNA.

The following day, samples were centrifuged at 13,000 rpm for 20 minutes, the supernatant discarded and the compact white pellet formed washed in 70% ethanol. The pellet was allowed to air-dry before the addition of a DNase solution. (N.B In order to prevent false-positive results following RT-PCR, the samples were extensively DNased to remove any contaminating genomic DNA). 50 μl of the following DNase solution was added to each pellet: 10mM Tris pH 7.5 (Sigma), 6mM MgCl_2 (Promega), 20mM Vanadyl-ribonucleoside complex (VRC, an RNase inhibitor) (Sigma), and 5 μl of 7,500 units/ml RNase-free DNase I (Pharmacia). The samples were then put in a 37°C water bath for 2-3 hours to allow degradation of any contaminating genomic DNA. Following the DNase step an RNA extraction kit was used to purify the RNA further (RNaid kit from BIO 101). 180 μl binding solution (3M NaClO_3) and 20-30 μl , dependent of the amount of starting tissue, RNA binding (RNaid) matrix was added to samples. Tubes were incubated at room temperature for 10 - 25 minutes, with occasional shaking to allow RNA to bind to the matrix. The matrix containing bound RNA was then sedimented by brief centrifugation at 13,000rpm and the supernatant was discarded. The matrix was washed with 700 μl wash solution (RNaid kit, Amersham), centrifuged, the supernatant discarded and the wash step repeated. The RNA was then eluted by addition of 30-100 μl DEPC-treated dH_2O and heating the samples to 65°C for 2-3 minutes. Samples were centrifuged at 13,000rpm for 1 minute, to sediment the matrix, and the supernatant containing the RNA was pipetted off carefully into new RNase-free micro-centrifuge tubes and stored at -80°C until required.

2.2.5. Competitive RT- PCR

RT-PCR is a sensitive method of detecting rare mRNAs in small amounts of tissue. The method of competitive RT- PCR described below allows the expression levels of specific mRNAs to be accurately quantified. The technique involves spiking the RT reaction with a known concentration of a synthetic competitor cRNA, which is

identical to a portion of the mRNA that is being quantified, but with the addition of 3-4bp between the PCR primer binding sites. The mRNA and competitor cRNA are both converted to cDNA with equal efficiency in the same reverse transcription (RT) reaction. During the subsequent PCR reaction, competitor cDNA and cDNA from the endogenous target mRNA are amplified with the same primers. Because the competitor cDNA and cDNA from the endogenous target are virtually identical they are amplified with equal efficiency. For this reason, the ratio between the RT-PCR products of the cRNA and mRNA at the end of the PCR reaction, observed by separating them on a polyacrylamide gel, staining with SYBR Gold and viewing under UV, accurately reflects the ratio of cRNA competitor and endogenous mRNA before the RT reaction. If the amount of the cRNA added to each RT reaction at the beginning of the RT-PCR assay is known and an imaging system is used to quantify the ratio between the SYBR Gold stained RT-PCR products, then the initial amount of mRNA can be easily calculated. This use of competitor cRNAs rather than competitor cDNAs makes a more accurate and reliable assay, since it takes account of variation in the efficiency of reverse transcription between individual reactions.

2.2.5.1. Production of competitors

The cRNA competitors used to assay GAPDH and CGRP mRNAs were transcribed *in-vitro* from a competitor cDNA that had been constructed from a cDNA clone of a portion of CGRP and GAPDH mRNAs.

General Method

RT-PCR to clone the competitor

The cDNA clones were made by RT-PCR amplification of E14 mouse trigeminal ganglia total RNA, with GAPDH or CGRP specific primers.

8µl of E14 mouse trigeminal RNA was reverse transcribed in a 40µl mixture below:

REAGENT	VOLUME
5× RT Superscript buffer (Gibco)	8µl
5mM dNTPs (MBI Fermentas)	4µl
100µM Random Hexanucleotides	4µl
0.1M DTT	4µl
dH ₂ O	12µl

Table 2.4 Reverse Transcription reaction for producing cDNA clones.

The reaction mix was heated for 2 minutes at 90°C followed by addition of 2µl Superscript reverse transcriptase. Samples were incubated for 90 minutes at 37°C and then the temperature increased to 95°C for 6 minutes to stop the reaction and degrade the RNA template.

PCR was used to amplify cDNAs of interest. Primer sequences were based on published sequence information and were selected carefully to ensure amplification of a region between 400 – 800bp. Primer sequences are shown in table 2.5.

GENE	PRIMERS	FRAGMENT SIZE
GAPDH	Forward: 5'-CTT CAT TGA CCT CAA CTA CAT G-3' Reverse: 5'-GGC ATG GAC TGT GGT CAT-3'	401bp
CGRP	Forward: 5'-AAG AGT CAC CGC TTC GCA AGC A-3' Reverse: 5'-AGC TCC TGT CAA AGG GAG AAG-3'	628bp

Table 2.5 Primer sets and fragment sizes for cDNA fragments to produce competitor species

50µl reactions were set up comprising of:

REAGENT	VOLUME
Reverse Transcription Product	5µl
10× Hot Start Taq buffer (Gibco)	4.5µl
5mM dNTPs (MBI Fermentas)	2µl
50pmoles primers (MWG-Biotech)	5µl
Hot Start Taq polymerase (Gibco)	0.5µl
dH ₂ O	33µl

Table 2.6 PCR reaction reagents for amplification of DNA fragments

3 drops of mineral oil was layered on the top of each reaction and the tubes transferred to the heating block of a PCR machine.

The following protocol was run:

STEP	TEMPERATURE	TIME
Activation of Taq	95°C	10 minutes
Annealing at 55°C (for both GAPDH and CGRP)	55°C	60 seconds
Synthesis	72°C	90 seconds
Extension	72°C	10 minutes

Table 2.7 PCR protocol for amplification of cDNA clones

Products were run on a 1% agarose gel and the band of interest cut out using a razor blade. Purification of the gel fragment was done using the Gene Clean®II kit and DNA recovered in 20µl dH₂O.

The cDNA fragment generated was cloned into pGEM T-vector (Promega) following the manufacturers protocol. This ligation mixture was then used to transform competent *E.coli* XLI cells (Stratagene).

Transformation of Competent Cells

1/10 volume of the ligation mixture was added to competent cells and left on ice for 1 hour. Cells were then heat-shocked by placing in a 42°C waterbath for 90 seconds, before returning to ice for 5 minutes. 800µl LB broth was added to cells and tubes incubated at 37°C for 1 hour, before plating onto LB agar (Gibco) dishes. The LB agar contained 100mg/ml ampicillin; hence only colonies containing the transformed vector with ampicillin resistance will grow. Dishes were placed at 37°C overnight to facilitate growth.

Analysis of Transformants

Single colonies were picked from plates and used to inoculate 3ml LB broth containing 100mg/ml ampicillin. Tubes were placed in a shaking incubator overnight at 37°C. Plasmid DNA was now extracted from the resulting bacterial suspension using a QIAprep Spin Miniprep kit (Qiagen) and eluted in 50µl dH₂O. To check for presence of the insert, 5µl of plasmid DNA was digested with appropriate restriction enzyme for 1 hour in a 20µl reaction. The digest was run on a 1% agarose gel to ensure products of the correct size had been generated.

Modification of Cloned DNA to Produce Competitor DNA

40µl of remaining plasmid DNA was cut at a certain unique restriction site by incubation overnight with specific restriction endonuclease, HindIII for GAPDH and BAMHI for CGRP. The reaction contained 5µl 10× buffer (chosen according to the restriction enzyme being used), 2µl dH₂O and 3µl restriction enzyme. The restriction

enzyme was chosen for each reaction to allow a single cut in cDNA that leaves a 4-5 bp 5' overhang.

Following digestion, plasmids were purified using the Gene Clean® II kit. The overhang was now filled in and blunt ended by the use of Klenow polymerase (see figure 2.6). A 20µl reaction was set-up containing 15µl plasmid DNA 2µl 10× klenow buffer and 3µl 5mM dNTPs. After mixing, 1µl of Klenow enzyme was added, and the reaction incubated at room temperature for 5 minutes. The reaction was stopped by the addition of NaI from the GeneClean® II kit and the DNA was purified again.

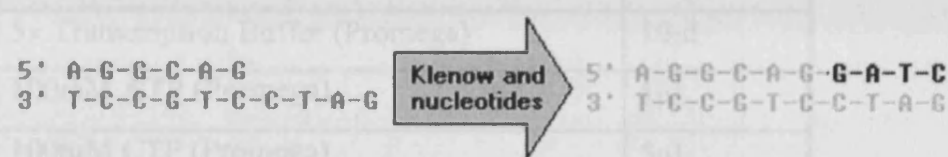


Figure 2.6: The use of klenow polymerase to fill in missing base pairs of a DNA overhang.

Taken from Bowen, 1999

The blunt ends were then re-ligated using the reagents of p-GEM T-vector kit. 9µl of the newly filled-in DNA were incubated overnight at 4°C with 10µl 2× ligase buffer and 1µl of 400u/µl T4 DNA ligase. Following ligation, the Gene Clean®II kit was used to remove DNA ligase and T4 ligase buffer and the DNA was recovered in 20µl dH₂O. This resulted in a competitor cDNA that was 3-4bp longer than the original native cDNA.

Linearisation and Purification

To produce cRNA from the competitor cDNA, the plasmid was first linearised at the 3' end of the inserted DNA by overnight digestion with an appropriate restriction endonuclease (Sal I for gapdh and Nco I for CGRP). Enzymes were chosen for their ability to produce blunt ends, or 5' overhangs and to cut only once, as far away from the insert site as possible. Gel electrophoresis was used to visualise the digest to ensure complete linearisation. The DNA band corresponding to the linearised cDNA

was then cut out of the gel with a razor blade and purified using the Gene Clean®II kit following the manufacturers protocol.

In-Vitro Transcription

cRNA was transcribed *in-vitro* from the linearised competitor cDNA using T7 RNA polymerase (Promega). 2×50µl reaction mixtures were set up containing the following, added sequentially:

REAGENT	VOLUME
5× Transcription Buffer (Promega)	10µl
100mM ATP (Promega)	5µl
100mM CTP (Promega)	5µl
100mM GTP (Promega)	5µl
100mM UTP (Promega)	5µl
100mM DTT (Promega)	5µl
RNAguard (Pharmacia)	2µl
dH ₂ O	6µl
Plasmid DNA (1-5µg)	7µl
30-50u T7/SP6 RNA polymerase (Promega)	2µl

Table 2.8 Reagents for In-Vitro Transcription of competitor cDNAs

After thorough mixing, the reaction was incubated for 1 hour at 37°C. After this time 2µl more of T7/SP6 polymerase was added to the reaction, which was then incubated for a further hour. The remaining plasmid DNA was now degraded by the addition of 2µl VRC and 5µl of 7,500u/ml DNase I, and incubation for 2 hours at 37°C.

The RNA transcripts were then purified, as previously, using the RNaid kit (Bio 101) (See 2.2.3) and eluted in 100µl DEPC water. The integrity and approximate size of the purified cRNA was determined by gel electrophoresis on a 1% agarose DEPC treated gel in a DEPC treated gel tank (DEPC (di-ethyl-pyrocabonate) treatment

effectively removes contaminating RNases). Next, a spectrophotometer was used to determine the concentration. The remaining RNA was then diluted with water and precipitated at a concentration of 1ng/ μ l by the addition of an appropriate volume of water, 0.1 volume of 3M sodium acetate (pH5.5), 3 volumes of ethanol and 20ng E.coli tRNA for every 1ng of transcript. The ethanol-precipitated transcript was stored at -20°C until use.

2.2.5.2 The competitive RT-PCR assay

Reverse Transcription (RT)

Initially total RNA was reverse transcribed to cDNA. Competitor cRNAs were included in the reverse transcription reaction along with the competitor for the housekeeping gene GAPDH.

The protocol used for reverse transcription is outlined below. To 1 μ l of RNA sample the following was added:

REAGENT	VOLUME FOR 1 REACTION
5 \times buffer (GIBCO, included in superscript kit)	8 μ l
100 μ M Random Hexanucleotides (RH) (Pharmacia, Amersham)	4 μ l
5mM dNTPs (Fermentas)	4 μ l
0.1M DTT (GIBCO, included in superscript kit)	4 μ l
Competitor RNA	1 μ l
GAPDH competitor RNA	1 μ l
dH ₂ O	16 μ l

Table 2.9 Reverse transcription reaction reagents for competitive PCR

The mixture was heated to 90 $^{\circ}\text{C}$ for 2 minutes to allow any secondary structures within the RNA to be broken down. After rapid cooling on ice, 1 μ l of Superscript

reverse transcriptase (GIBCO) was added to all samples, excluding those samples that were to serve as no RT negative controls (these samples contained everything but enzyme, and were used to check for the presence of contaminating genomic DNA). Immediately after the addition of enzyme, samples were incubated at 37°C for 1 hour, and finally the reaction was halted by heating the sample to 95°C for 6 minutes, resulting in denaturation of the reverse transcriptase and degradation of the RNA template.

Polymerase Chain Reaction (PCR)

PCR reactions were carried out for CGRP and GAPDH separately. 5µl cDNA was used in a 40µl reaction as below.

A mastermix was made up using the following protocol:

REAGENT	VOLUME
10× buffer (Helena biosciences)	4µl
Primers 1:1 ratio of forward and reverse 50pmoles (MWG)	4µl
5mM dNTPs	1µl
Taq polymerase, 5U/µl (Helena Biosciences)	0.125µl
dH ₂ O	30.875µl

Table 2.10 *PCR reagents for competitive PCR*

After mixing, mineral oil was layered on the top of each sample and tubes transferred to the heating block of a PCR machine. Samples were initially denatured by heating to 95°C for 1 minute, followed by annealing (53°C GAPDH, 59°C CGRP) for 60 seconds and then synthesis at 68°C for 60seconds. Annealing and synthesis steps were then repeated (24 cycles for GAPDH and 32 cycles for CGRP) before a final extension step at 68°C for 10mins.

Primers used were designed to span the region of the competitor insert so that two separate products would be produced, the product from the cRNA being 4bp bigger than that of the native product. The primers are shown below:

Calcitonin Gene Related Peptide (CGRP):

Forward: 5'-TCT GCT GAG CAG ATC AGG AG-3'

Reverse: 5'-GGG CTG TTA TCT GTT CAG GC-3'

GAPDH:

Forward: 5'-TCC AGT ATG ACT CCA CTC AC-3'

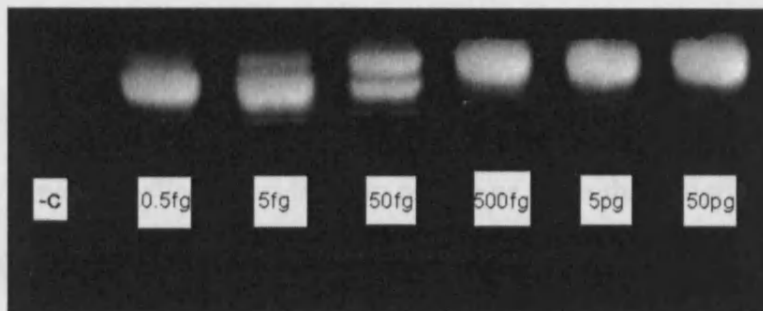
Reverse: 5'-TCC TGG AAG ATG GTG ATG G-3'

Electrophoresis of Products

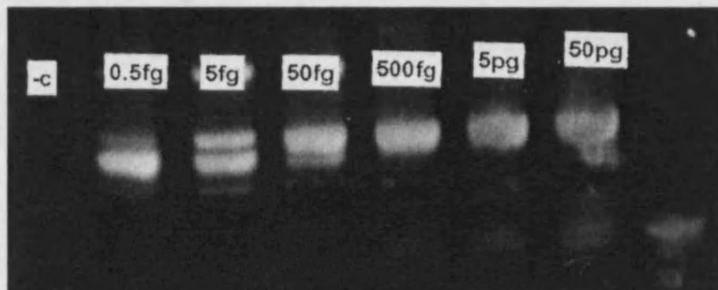
PCR products were run on an 8% (29:1) polyacrylamide gel to allow the very small differences in size of product and competitor cDNA to be visualised. Following electrophoresis, gels were stained for 15 minutes in the dark with SYBR Gold solution at a 10,000 × dilution (molecular probes). SYBR Gold is a non-isotopic UV-sensitive dye that intercalates between double stranded DNA, allowing PCR products to be visualised under UV light. PCR product bands were visualised using a UV video imaging system (Biogene) and the intensity of the adjacent bands were compared via densitometry using phoretix 1D quantifier software. As the concentration of competitor added to the reaction was known, the amount of mRNA in samples could be calculated by the ratio of the intensity of the competitor RT-PCR product band compared to the mRNA RT-PCR product band. This value was then normalised against GAPDH to account for any differences in starting levels of total RNA between different RT-PCR reactions.

Initially, a competitor titration reaction was carried out to determine the optimum amount (fg) of each competitor species to use in the PCR reaction (CGRP and GAPDH). Ideally, an approximately equal amount of competitor and target product

should be amplified, as would be shown by 2 bands of similar intensity on the gel. Such titration results are shown in figure.2.7. More than a five-fold difference in amplified product to amplified competitor cannot be detected accurately by the imaging software.



A



B

Figure 2.7. Titration reactions to determine levels of CGRP competitor species to use in RT-PCR reactions.

0.5fg to 50pg of CGRP competitor was used to determine the most suitable amount to use with E14 trigeminal RNA samples (A) and E14 DRG samples (B). The RT-PCR product of the competitor is the higher molecular weight band at the top and the RT-PCR product of the CGRP mRNA is the lower band. 50fg was the most suitable competitor concentration to use for trigeminal RNA and 5fg for DRG RNA, as at these competitor concentrations both competitor and mRNA RT-PCR products have a similar intensity.

2.2.6. Real time quantitative PCR – Stratagene MX3000P

A new Q-PCR machine, Stratagene's MX3000P, was acquired in the lab allowing validation of previous competitive RT-PCR results and also enabled quantitative

study of expression levels of additional mRNAs using a method that has a broader dynamic range and is quicker to optimise reactions for new mRNAs. Additionally, reactions are set-up and run in a 96-well format and PCR products do not have to be resolved by gel electrophoresis, making it significantly faster than competitive RT-PCR.

Unlike competitive RT-PCR used previously, real-time PCR monitors the progress of the PCR reaction continuously through the use of dual-labelled fluorescent probes, single labelled hybridisation probes or, as in the majority of the reactions performed in this project, a dye (SYBR green) that fluoresces upon binding to double-stranded DNA. SYBR green generation of fluorescence is significantly cheaper and more sensitive than probe systems, but is not specific to any one PCR product (see figure 2.8). SYBR green is a double stranded (ds) DNA-binding dye. It is thought to bind in the minor groove of double-stranded DNA and upon binding its fluorescence increases over a hundred-fold.

SYBR Green

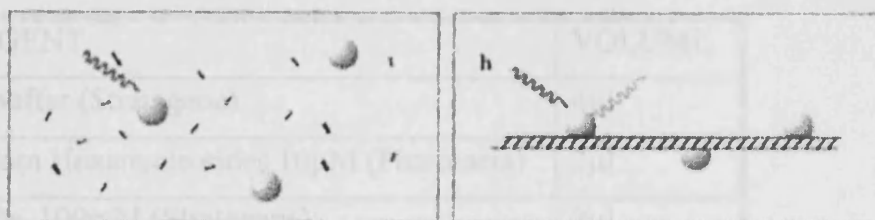


Figure 2.8.: SYBR green is a non-specific fluorescent dye that, upon binding to any double-stranded DNA, fluoresces.

Taken from Rasmussen, 2001

After each PCR cycle the fluorescence, and hence product accumulation, is recorded and represented graphically on a PC attached to the PCR machine. A sigmoidal curve illustrates the reaction. It shows the initial build up of the product followed by the exponential amplification, and then the plateau when either one or all reagents are used up or become limiting, or single stranded template re-annealing becomes the dominant kinetic process (figure 2.9A). By monitoring the number of cycles required

to produce a threshold amount of product, the relative quantity of mRNA of interest within a sample can be calculated. The process is outlined in more detail below.

2.2.6.1 Reverse transcription

For reactions on the Stratagene MX3000P, cDNA was produced using a Stratagene reverse transcription enzyme, Stratascript, rather than the GIBCO Superscript used previously. In initial pilot studies, Stratascript was found to produce consistently higher yields of cDNA than Superscript and was found to be significantly more cost effective. In addition, the amount of cDNA produced shows a stronger linear relationship with reference to the amount of input total RNA than with Superscript. Importantly, active Superscript that carries over into the QPCR reaction was found to inhibit QPCR much more strongly than Stratascript (data not shown).

5 μ l RNA was transcribed to cDNA using Stratascript reverse transcriptase (Stratagene) in the following 40 μ l reaction.

REAGENT	VOLUME
10 \times buffer (Stratagene)	4 μ l
Random Hexanucleotides 10 μ M (Pharmacia)	2 μ l
dNTPs, 100mM (Stratagene)	2 μ l
Stratascript Enzyme (Stratagene)	0.4 μ l
dH ₂ O (GIBCO)	31.6 μ l

Table 2.11 Reaction mix for Stratagene Reverse Transcription

Samples were incubated at 37°C for 1hr 15 minutes. Unlike previous reverse transcription reactions for competitive RT-PCR, a final 95°C step was not included because the QPCR enzyme used was a “hot start” enzyme and required 10 mins at 95°C as the first step of the PCR reaction.

2.2.6.2 Real-time quantitative PCR

Real time QPCR was carried out using the Stratagenes' MX3000P. A mastermix was set up and 22.5 μ l added to 2.5 μ l of cDNA.

Per sample:

REAGENT	VOLUME
10 \times buffer (stratagene)	2.5 μ l
MgCl ₂ 20mM (stratagene)	1.5 μ l, 2 μ l or 2.5 μ l to give a final concentration of 3mM, 4mM or 5mM respectively.
15 μ M forward and reverse primer mix (MWG)	0.5 μ l
Rox reference dye at a dilution of 1/500 in dH ₂ O (Stratagene)	0.4 μ l
SYBR Green at a dilution of 1/4000(Molecular Probes)	0.25 μ l
dNTPs 20mM (Stratagene)	1 μ l
Taq (Stratagene)	0.33 μ l
dH ₂ O	15.8 μ l, 15.3 μ l or 14.8 μ l (volume dependent on MgCl ₂ concentration)

Table 2.12 Reaction mix for Real-Time PCR

Samples were initially heated to 95°C for 10 minutes to denature DNA secondary structure, degrade Stratascript and RNA and activate the “hot start” taq. The cycling stage now began with samples being heated to 95°C, cooled to the primer annealing temperature (determined by the primer sequence being used), and then heated back up to 72°C to allow the DNA polymerase to extend the primers and synthesize a copy of the template DNA. This cycle was repeated 40 times to ensure ample fluorescent product build up. A fluorescence measurement was taken from all wells at the end of each annealing step

As SYBR green can bind to all double-stranded DNA, the specific product, non-specific products and primer dimers are all detected equally well. This problem can be ameliorated by using a “hot start” taq polymerase and careful primer design. For this project ‘Beacon Designer’ software (Biosoft International) was used for primer design. Following cycling, due to the non-specificity of SYBR green, it was important to include a melting curve to ensure that only the correct product had been amplified. The samples were gradually heated from 65°C to 95°C at 1° C per minute with continuous monitoring of fluorescence. A rapid drop in fluorescence is recorded at the temperature that the PCR products separate or melt. If the rate of change of fluorescence is plotted against temperature a sharp peak is obtained at the melting temperature of each PCR product. A good PCR reaction will have only one clean peak at the expected melting temperature of the correct PCR products (as determined by computer software) and no additional peaks at lower temperature corresponding to primer artefacts (see figure 2.9B). Reactions that did not show a melting curve with these characteristics were not used for data analysis.

The concentration of SYBR green added to the reaction was optimised in a series of pilot experiments as preliminary experiments showed that at lower SYBR green concentrations insufficient fluorescence is emitted, but at higher concentrations, the PCR reaction was inhibited. A 1/4000 dilution seemed to produce optimum results (data not shown).

In some cases, when primer artefacts confounded obtaining good quantitative data, specific Molecular Beacon probes were used in place of SYBR Green. Such Molecular Beacons are designed so that they only bind to the specific amplified product of the gene of interest. In this case no melting curve was required. A probe for beta CGRP was often used. The sequence for this probe is given below, designed on Beacon Designer and produced by Biosearch Technologies, Inc.

5'Quasar 670 d(CGC GAT AAA TAT GAT GGT GTC TCC CAC TGG ATC GCG)
BHQ-2 3'

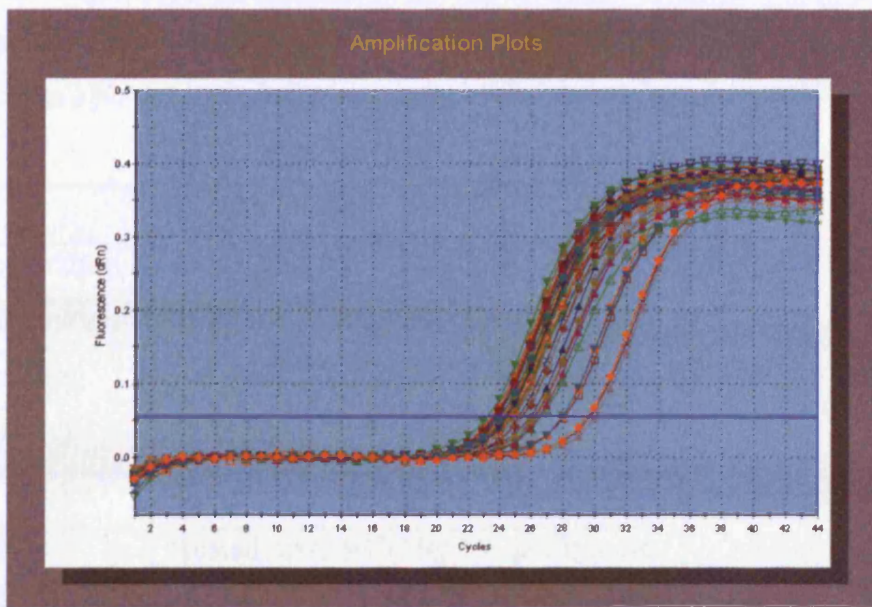
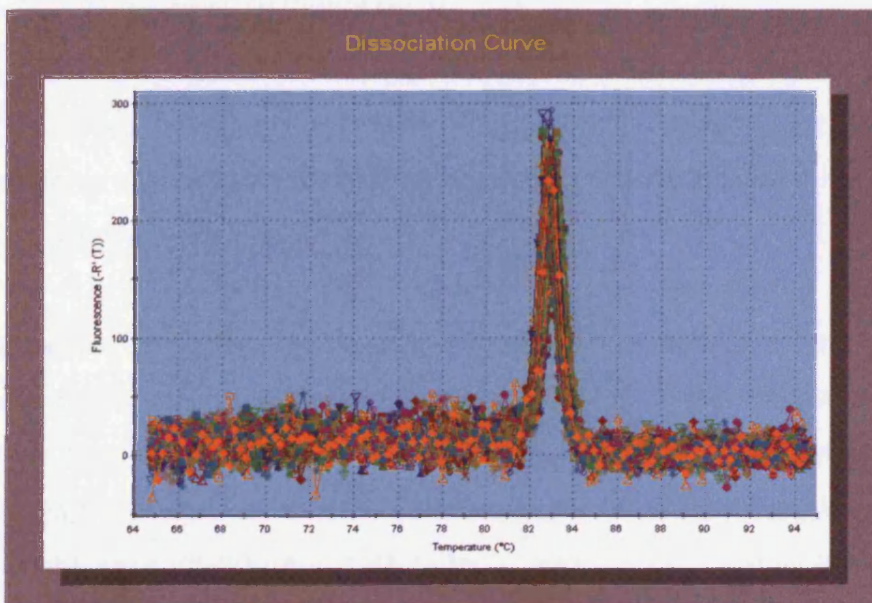
A**B**

Figure 2.9. Real time PCR

A) After each cycle the level of fluorescence emitted is recorded. This represents the amount of PCR product present, which builds up to produce a sigmoidal shaped curve. Each coloured line represents a different cDNA sample. B) Melting curve of samples. After the PCR reaction is complete, samples are heated gradually to determine the melting temperature of the product and to check for primer dimers or other contaminating products. Only one peak should be seen.

For all the mRNAs assayed by RT followed by QPCR, the reaction was run using the protocol outlined below with the only variation being the annealing temperature. The melt was also not included when using molecular probes, rather than SYBR green.

STEP	CONDITIONS
Denaturation	95°C 15mins
Denaturation	95°C 30s
Annealing	χ °C 30s
Extension	72°C 30s
No. Cycles	40
Melt	Heated up to 95°C by 1°C per minute

Table 2.13 Reaction Conditions for Quantitative Real-Time PCR

QPCR - Further reaction optimisation

For each gene the primers used were designed on 'Beacon Designer' computer software, to ensure the utmost efficiency and specificity and the minimum of primer/primer interactions.

The reaction for each mRNA was optimised to determine the optimum MgCl₂ concentration and annealing temperature required to produce the most efficient reaction (as determined by the slope of a standard curve (see below)), but with a clean single product of the correct melting temperature and no primer artefacts. Reactions were always run for 40 cycles to ensure ample product amplification. For real time PCR the actual cycle number used is less important than with semi-quantitative PCR, as analysis of product concentration is done during the exponential phase of the reaction, and not after the reaction has approached the plateau phase. .

Standard Curve

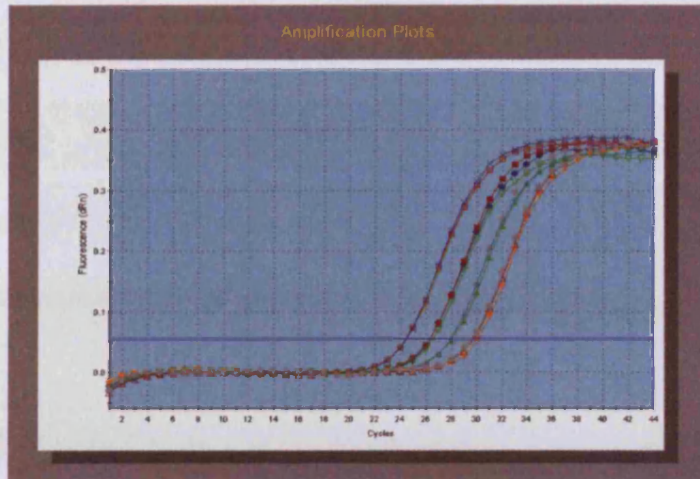
Real-time PCR is a relative quantitative method for measuring gene expression. The reaction is monitored constantly throughout the reaction and the product build-up is illustrated by a sigmoidal curve (see figure 2.10A). The principal behind quantification is due to the inverse correlation between cycle number and product concentration. In general, the lower the cycle number required to amplify the product, the higher the concentration, and vice versa. Some standards, which contain known concentrations of cDNA are therefore required. These samples can then be used to determine the relative concentration of mRNA within an unknown sample

To this end, in each run, samples were included to allow production of a standard curve. These samples contained cDNA diluted 1×, 1/3, 1/9 and 1/27 and were referred to as 27, 9, 3 and 1 arbitrary units respectively. For accuracy, a serial dilution was set up using an RT mix to dilute the samples. This RT mix was produced by carrying out a reverse transcription reaction, as before, with all reagents except the RNA template. If the standard curve was diluted in water, a linear standard curve was not produced. This is because reagents in the RT mix (most notably the RT enzyme and random hexamers) inhibit the QPCR reaction strongly; therefore at each dilution with water the QPCR reaction becomes more efficient.

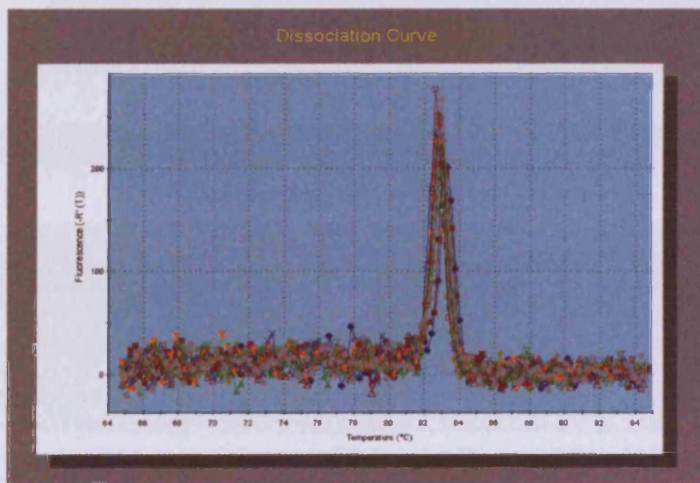
The standards were run alongside the other unknown samples in the PCR reaction. See figure 2.10A for a graphical representation and note that the sigmoidal curves, representing product build up, are equidistant apart for each step up in concentration, resulting in a linear standard curve (figure 2.10A and C). From data like this the computer could produce a standard curve plotting cycle number to threshold vs. arbitrary concentration. An example standard curve is shown in Figure 2.10C. The standard curve should have an RSq value as close to 1 as possible. RSq values of less than 0.95 were deemed unsatisfactory and results were not calculated from these data sets. The efficiency of the PCR reaction was calculated from the slope of the standard curve by the MX300P software. 100% efficiency corresponds to a doubling of product at each PCR cycle. For most mRNAs, efficiencies of 80-100% were

obtained after reaction optimisation. In the case where reactions generated a lot of false products and primer artefacts, as determined by melting curve analysis, the PCR reaction efficiency was often seen to be over 100%. In these cases the data was rejected.

A



B



C

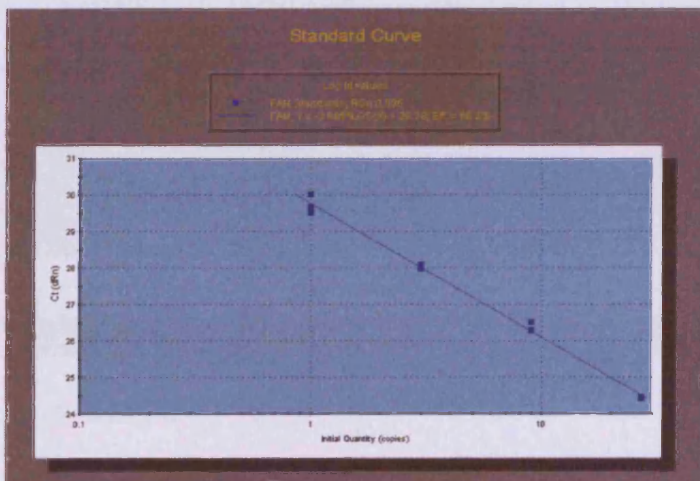


Figure 2.10. Production of standard curve.

Samples of a serial dilution of cDNA are included in each PCR run. 3 replicates of 1×, 1/3, 1/9 and 1/27 are shown as they amplify (A). They all have the correct melting temperature (B) and produce a standard curve with RSq just below 1.

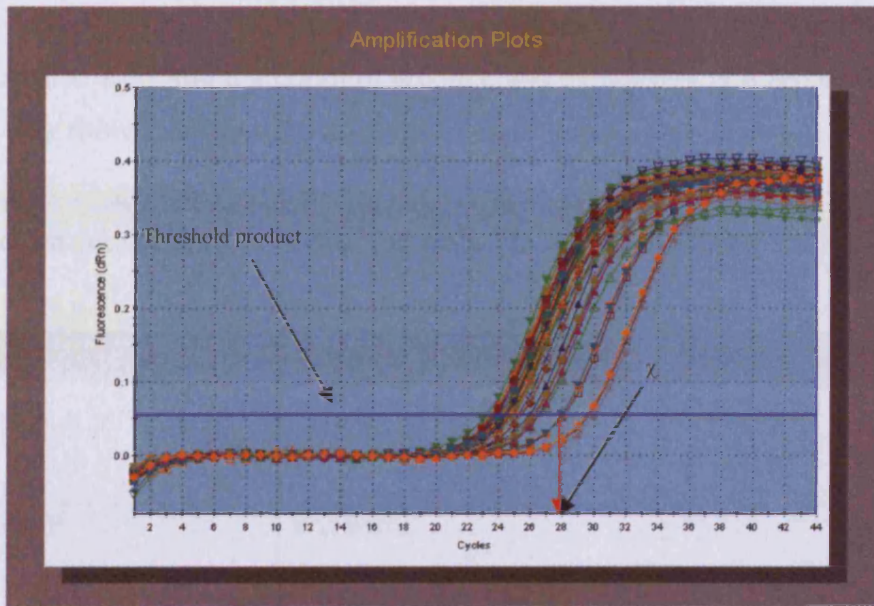
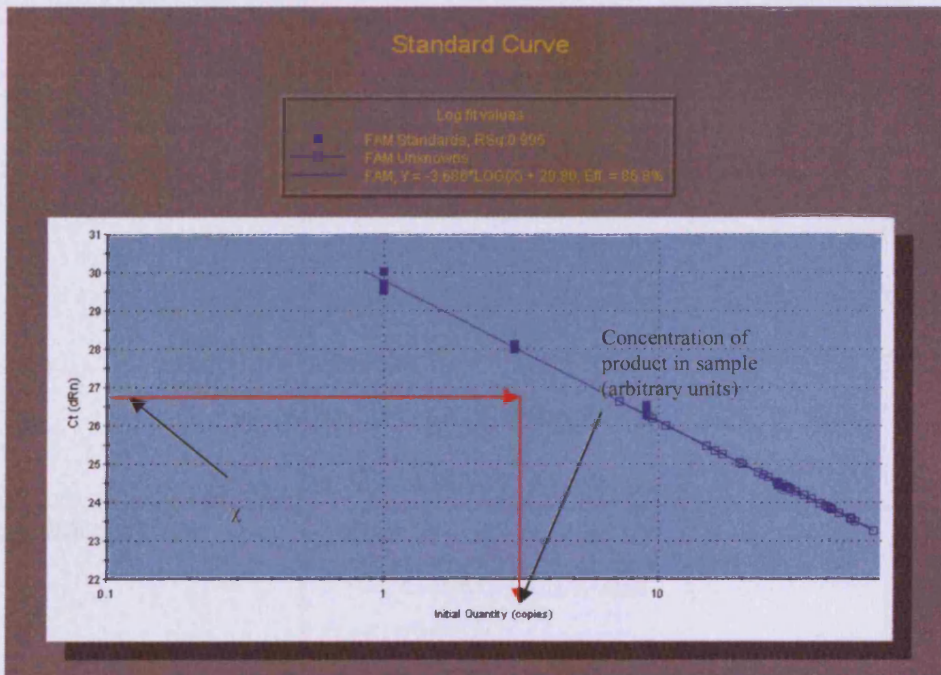
A**B**

Figure 2.11. *Quantification of data.*

A) PCR product builds up exponentially the number of cycles required to produce a threshold amount of product is recorded (χ) and extrapolated onto the standard curve (B) to determine the relative concentration of mRNA in that sample (arbitrary units).

The standard curve generated in each reaction was used to determine the relative level of mRNA in the unknown samples by identifying the number of cycles required to produce a threshold amount of product, and extrapolating it on the calibration curve as shown in figure 2.11.

Reaction conditions and primer sequences for all reactions are outlined as below:

GENE	PRIMER SEQUENCES	MgCl ₂ CONCN. (MM)	ANNEALING TEMP. (°C)
α-CGRP	Forward: 5'-AAG AAA GGC TGA AAG ACA-3' Reverse: 5'-GGA TAC AGA GTC ACA TAC AAC AC-3'	5	51
β-CGRP	Forward: 5'-GAG TTA ATT CTG TGT TTG TTT GC-3' Reverse: 5'-TTG GCT GGA TGG CTC TTG-3'	5	51
Substance P (SP)	Forward: 5'-CCC AAG CCT CAG CAG TTC-3' Reverse: 5'-GCC CAT TAG TCC AAC AAA GG-3'	5	51
Sodium channel Na _v 1.8	Forward: 5'-AGG CTG GAT GGA	4	51

	CAT TAT G-3' Reverse: 5'-TGA AGA CGA AGT ATA GG-3'		
Sodium channel Na _v 1.9	Forward: 5'-CCT TCC GAG TGT TGA GAG-3' Reverse: 5'-AAA GAG AGT GAG GAC CAT C-3'	5	51
Gapdh	Forward: 5'-TCC CAC TCT ACC TTC-3' Reverse: 5'-CTG TAG CCG TAT TCA TTG TC-3'	3	51

Table 2.14 *Primer Sequences, MgCl₂ concentration and annealing temperature for all genes*

α - and β -CGRP mRNAs are almost identical in sequence and are the products of two separate genes. The competitive RT-PCR initially used to analyse CGRP expression could not distinguish between the two different CGRP mRNAs. However, careful primer design generated specific QPCR reactions for α - and β -CGRP.

2.3. Results

2.3.1. NGF/TrkA signalling is not required for initial induction of CGRP mRNA expression in early embryonic mouse sensory neurons.

Initially, the expression of CGRP mRNA was studied in TrkA and NGF knockout mice using a quantitative form of RT-PCR, competitive PCR. In these experiments primers were unable to distinguish between the two isoforms of CGRP.

Trigeminal and DRG were dissected from TrkA^{-/-} and NGF^{-/-} and their wild-type littermates. Ganglia were pooled, as expression of the genes of interest is low at young ages. Total RNA was extracted using a phenol based method and reverse transcribed. Expression of CGRP was quantified using competitive PCR. Primers used here allowed amplification of both transcripts equally.

Competitive PCR involved the use of a synthetic RNA competitor (cRNA) that differed from the amplified product by just 3-4bp. Both competitor and RNA were reverse transcribed and amplified together. Due to their similarities, they compete for primer binding and subsequent amplification. The two amplified products could be distinguished on an acrylamide gel. As the concentration of competitor added to the reactions was known, the relative concentration of native product could be determined from band intensity. The housekeeping gene GAPDH was also amplified within each sample and used to normalise results to account for any differences in starting RNA. Expression of CGRP was studied in both trigeminal and DRG from E13-E15.

In this chapter, data was statistically analysed by use of the t-test. The t-test was chosen as only two sets of data were being compared (knockout vs wildtype) and the data would be expected to fit into the following criteria:

- Both sets of data are normally distributed
- The variances of the samples are similar to each other

This test is also used in the following chapter (chapter 3), as once again only two sets of data are being compared (knockout mouse vs wild-type mouse or 0 hours vs 96 hours). An unpaired t-test was used in all cases since experiments being compared were independent of each other. The t-test compares the mean of each data set, whilst taking into account the variance of the results (the spread of results around the mean). Thus the formula for the t-test (see below) is a ratio - the top part being the difference between the two means or averages, whilst the bottom part illustrates the variance of the results (standard error of difference).

$$t = \frac{\bar{X}_T - \bar{X}_C}{\sqrt{\frac{\text{var}_T}{n_T} + \frac{\text{var}_C}{n_C}}}$$

The significance levels in this thesis are when $p < 0.05$ (*) and when $p < 0.001$ (**). i.e. results are classed as 'significantly different' when confidence levels reach 95% that the results are not different just due to random chance (or there is only 5% chance that you find the results as significantly different when they are not (i.e. by random chance)).

Trigeminal ganglion

In the trigeminal ganglion, the expression of CGRP did not differ between wild-type and $\text{NGF}^{-/-}$ or $\text{TrkA}^{-/-}$, as shown in figure 2.12. This would suggest that in the trigeminal ganglion, the onset of CGRP expression does not require NGF/TrkA signalling.

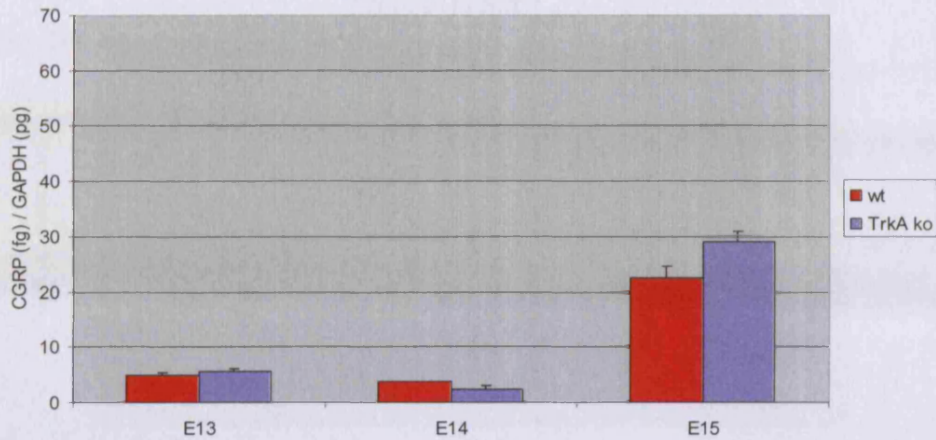
DRG

CGRP mRNA was found to be expressed at lower average levels in DRG than in trigeminal ganglia (figure 2.13, note scale change), suggesting it is either expressed

in a larger sub-population of trigeminal ganglia neurons compared to DRG neurons, the levels of CGRP mRNA per neuron are higher in the trigeminal ganglia than in the DRG or that trigeminal ganglia are developmentally more advanced at these ages than DRG. The expression of CGRP mRNA normalised to the levels of GAPDH mRNA is shown in figure 2.13 for both the $TrkA^{-/-}$ (A) and the $NGF^{-/-}$ (B) mice in comparison to their wild-type littermates at ages between E13 and E15. In $TrkA^{-/-}$ mice CGRP mRNA levels in DRG are comparable to those in wild-type mice (figure 2.13A), as was seen in trigeminal ganglia. Although there is a lower level of CGRP mRNA expression in the null mutant at E15 compared to wild type mice (Figure 2.13B), this reduction in expression is not statistically significant.

It is worth mentioning that NT-3 signalling via TrkA will also be lost in $TrkA^{-/-}$ mice. NT-3 is important during the early embryonic development of sensory neurons (Buchman and Davies, 1993). As well as signalling via its preferred receptor TrkC, NT-3 can also bind to TrkA (Soppet et al., 1991; Klein et al., 1991b; Cordon-Cardo et al., 1991; Glass et al., 1991; Ip et al., 1993) and signal via this receptor. NT-3 was shown to support the survival of cultured trigeminal ganglia from $TrkC^{-/-}$ mice to an extent comparable to NGF (Davies et al., 1995). This suggests that NT-3 can also signal via TrkA. This has been further confirmed through the observation that NT-3 can displace NGF from its preferred receptor (Ernfors et al., 1990). It is quite possible that following the additional loss of NT-3 signalling via TrkA in $TrkA^{-/-}$ mice, neurons that do survive and differentiate have a slightly different phenotype from those of the $NGF^{-/-}$ mice. NT-3 can also regulate PNS neuronal precursors, inducing survival, differentiation and proliferation of a wide range of cell types, including NC precursors, sympathetic neuroblasts and trigeminal progenitor cells (Kalcheim et al., 1992; Pinco et al., 1993; DiCicco-Bloom et al., 1993; Verdi and Anderson, 1994; Karavanov et al., 1995; Memberg and Hall, 1995; Elshamy and Ernfors, 1996; Elshamy et al., 1996). Some of these effects could also be mediated by TrkA and thus neuronal phenotype may be altered in these $TrkA^{-/-}$ mice. Indeed CGRP levels appear lower in the $TrkA^{-/-}$ than in the $NGF^{-/-}$ (figure 2.12 and 2.13) this could be a reflection of the differences in the phenotype of surviving neurons.

Trigeminal TrkA^{-/-} :



Trigeminal NGF^{-/-} :

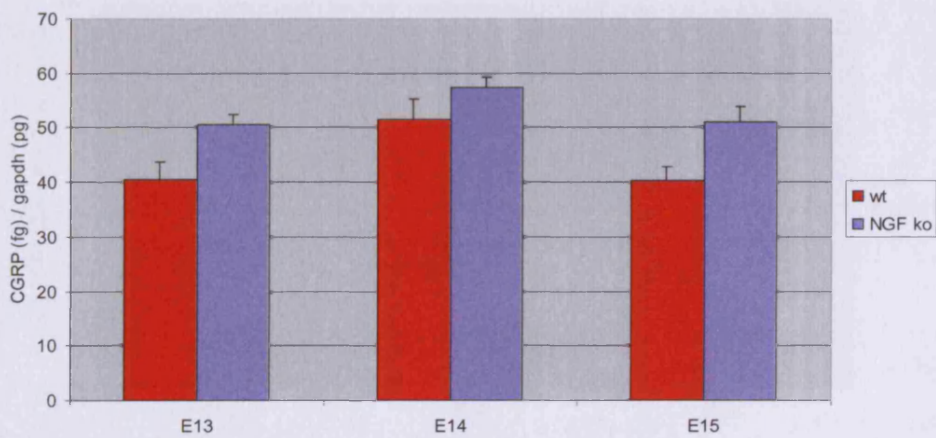


Figure 2.12. Expression of CGRP in trigeminal of *TrkA^{-/-}* and *NGF^{-/-}* mice.

Trigeminal ganglia were dissected from E13 – E15 null mutant and wild-type mice and the expression of CGRP mRNA quantified by competitive RT-PCR. Expression of CGRP in trigeminal of *TrkA^{-/-}* (A) and *NGF^{-/-}* (B) is shown in comparison to their wild-type littermates. All data is normalised with respect to GAPDH mRNA.

Error bars +/- standard error, n = 3-4. * = p = <0.05, ** = p = <0.01 as determined by two-tailed unpaired t-test.

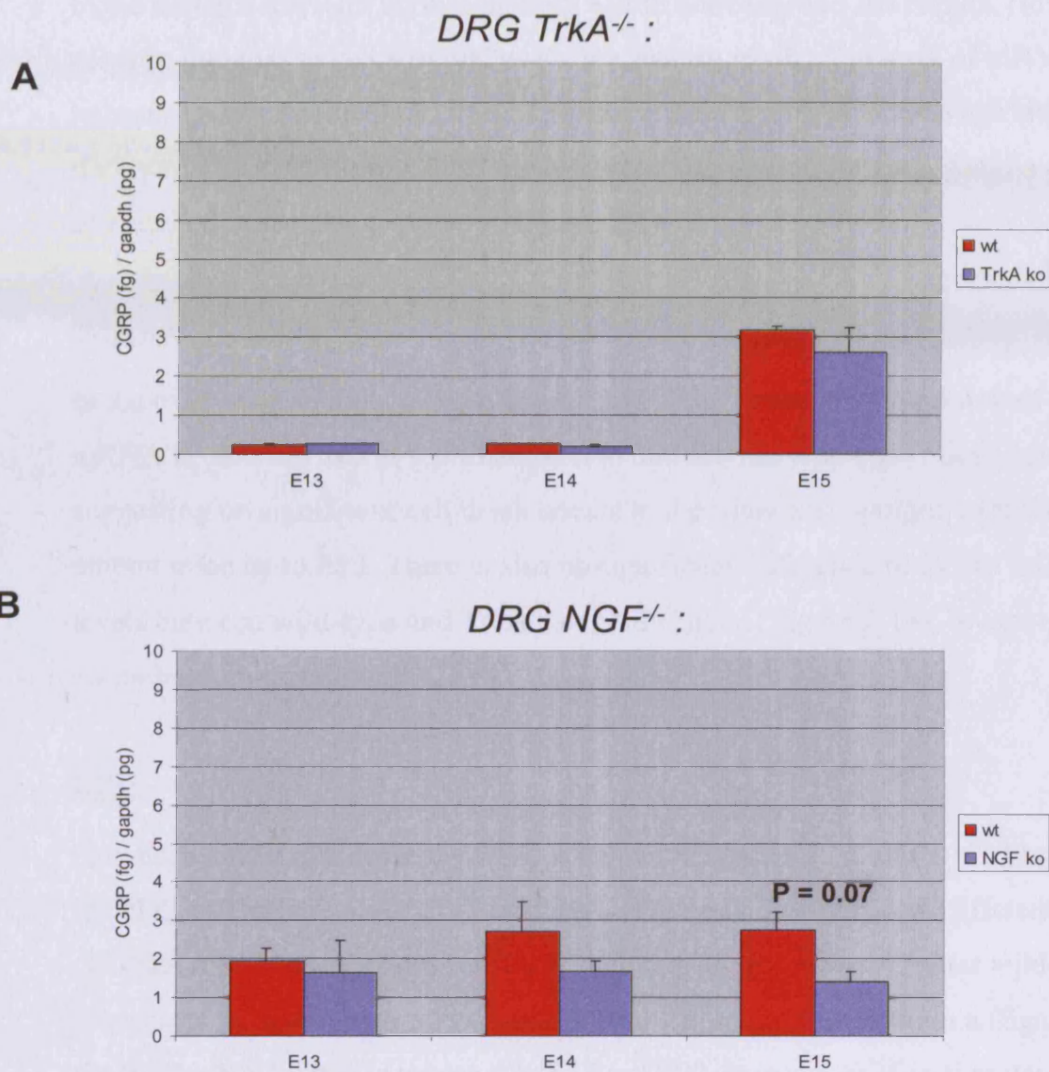


Figure 2.13. Expression of CGRP in DRG of *TrkA^{-/-}* and *NGF^{-/-}* mice.

DRG were dissected from E13 – E15 null mutant and wild-type mice and the expression of CGRP mRNA quantified by competitive RT-PCR. Expression of CGRP in DRG of *TrkA^{-/-}* (A) and *NGF^{-/-}* (B) is shown in comparison to their wild-type littermates. All data is normalised with respect to GAPDH mRNA.

Error bars +/- standard error, n = 3-4. * = p = <0.05, ** = p = <0.01 as determined by two-tailed unpaired t-test.

Non-normalised data

Embryonic day 13-15 mice were chosen specifically for this study so that cell death in the absence of NGF/TrkA signalling would not confound the results. However, to confirm that loss in cell survival was not affecting results, the level of mRNA for the housekeeping protein, GAPDH was plotted for each ganglia at each age studied. The non-normalised levels of CGRP mRNA were also plotted for each ganglia at each age studied. Results are shown in figures 2.14- 2.17

Trigeminal ganglia

In the trigeminal ganglia of both $TrkA^{-/-}$ and $NGF^{-/-}$ mice the expression of GAPDH mRNA is constant and at a similar level to that seen in wild-type mice (figure 2.14), suggesting no significant cell death occurs in the trigeminal ganglia of these null mutant mice up to E15. There is also no significant difference in CGRP mRNA levels between wild-type and $TrkA^{-/-}$ or $NGF^{-/-}$ mice (Figure 2.15), in agreement with normalised data (Figure 2.12).

DRG

Non-normalised data from the DRG is shown in figures 2.16 and 2.17. The levels of GAPDH mRNA are also fairly constant and there is no significant difference in GAPDH mRNA levels between $TrkA^{-/-}$ mice or $NGF^{-/-}$ mice and their wild-type littermates as determined by two-tailed t-test (figure 2.16), although a slightly lower GAPDH mRNA level is apparent at E15 in DRG from the NGF null mutant (2.16B). This data could reflect some cell death, which could possibly account for the difference in expression of CGRP mRNA in the normalised data at E15 (Figure 2.13B). However, as results were normalised against this GAPDH data to take into account such a loss, it is unlikely. On the whole GAPDH mRNA levels are unaffected suggesting little cell death.

Non-normalised CGRP mRNA levels are shown in DRG from $TrkA^{-/-}$ and $NGF^{-/-}$ mice (figure 2.17). The non-normalised data appear to show a significant difference

in CGRP mRNA expression at E15 in DRG from NGF^{-/-} mice compared to wild-type mice, which is not seen in DRG from TrkA^{-/-} mice. This seems to reflect observations in normalised data in which DRG of NGF^{-/-} mice show a decreased expression (figure 2.13).

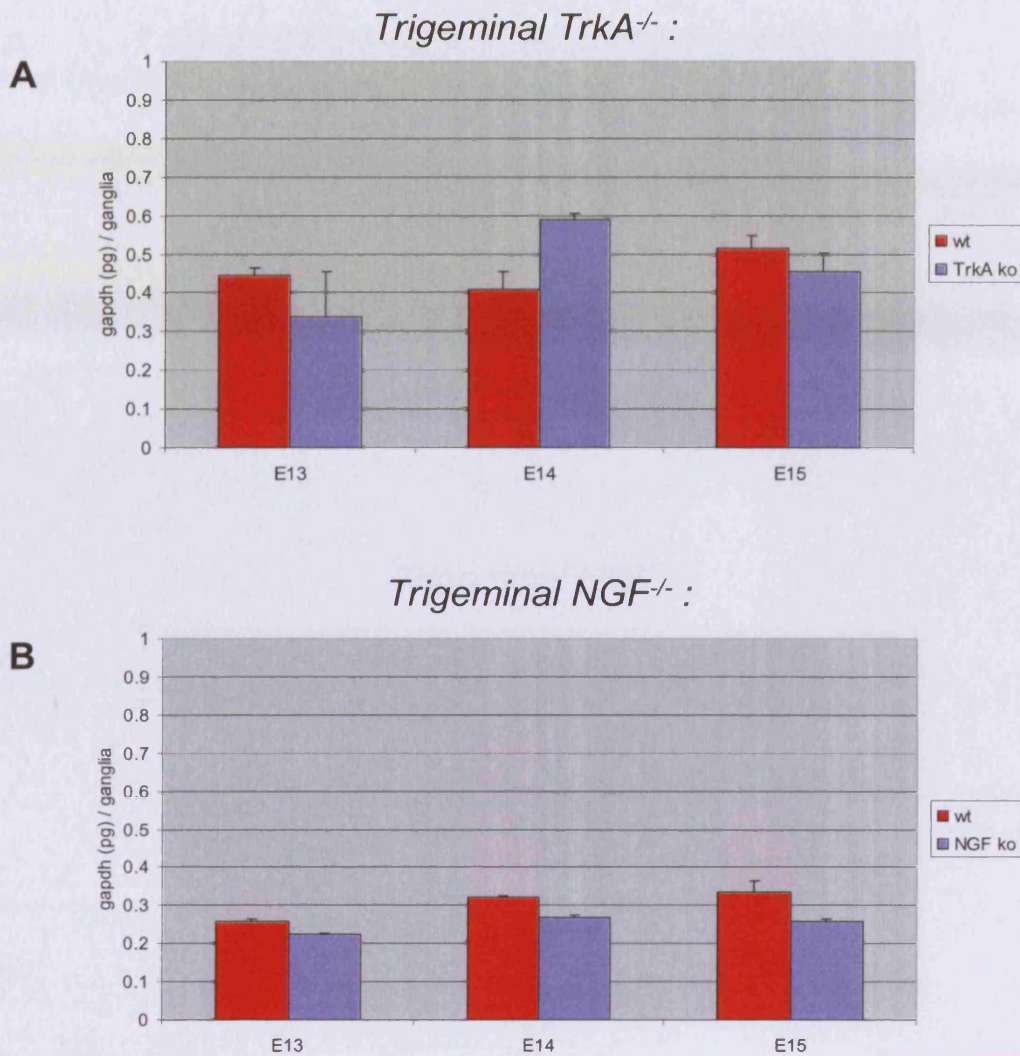
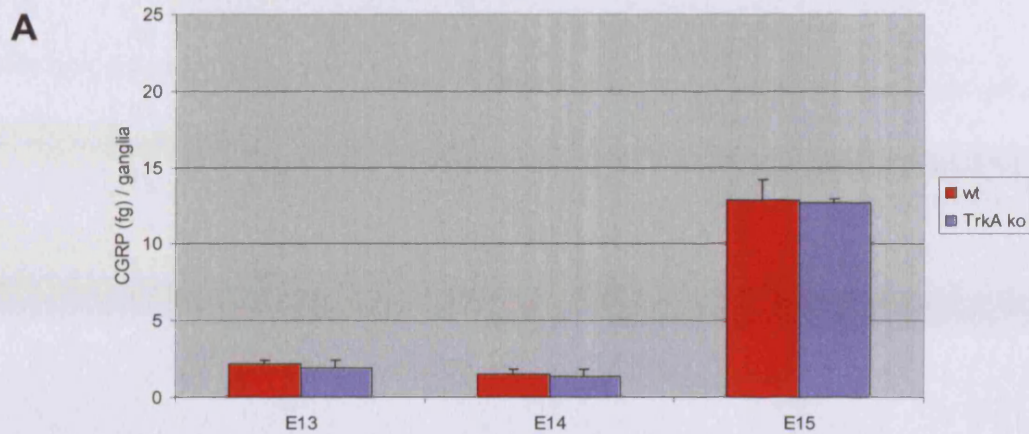


Figure 2.14. Expression of GAPDH per ganglia in trigeminal of *TrkA^{-/-}* and *NGF^{-/-}* mice prior to normalisation..

Trigeminal were dissected from E13 – E15 null mutant and wild-type mice and the expression of GAPDH mRNA quantified by competitive RT-PCR. Expression of GAPDH mRNA per ganglia in *TrkA^{-/-}* (A) and *NGF^{-/-}* (B) is shown in comparison to their wild-type littermates.

Error bars +/- standard error, n = 3-4. * = p = <0.05, ** = p = <0.01 as determined by two-tailed unpaired t-test.

Trigeminal TrkA^{-/-} :



Trigeminal NGF^{-/-} :

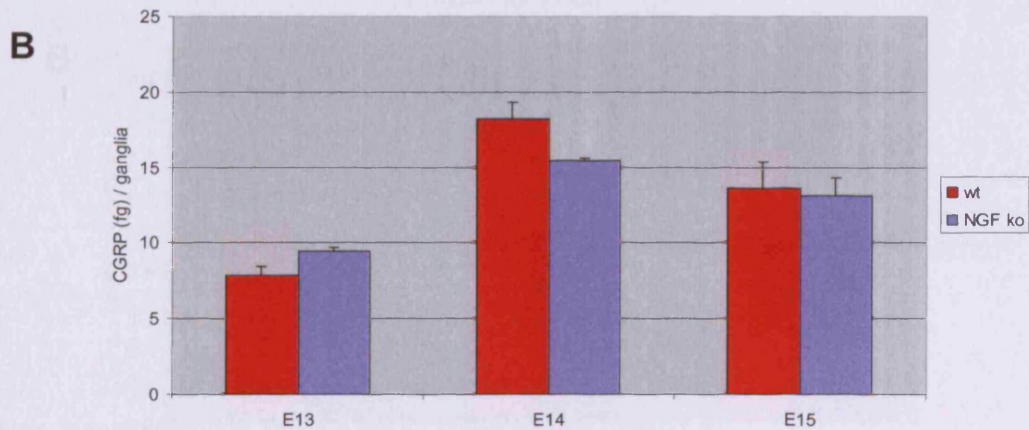


Figure 2.15. Expression of CGRP per ganglia in trigeminal of *TrkA^{-/-}* and *NGF^{-/-}* mice prior to normalisation..

Trigeminal were dissected from E13 – E15 null mutant and wild-type mice and the expression of CGRP mRNA quantified by competitive RT-PCR. Expression of CGRP mRNA per ganglia in *TrkA^{-/-}* (A) and *NGF^{-/-}* (B) is shown in comparison to their wild-type littermates.

Error bars +/- standard error, n = 3-4. * = $p < 0.05$, ** = $p < 0.01$ as determined by two-tailed unpaired t-test.

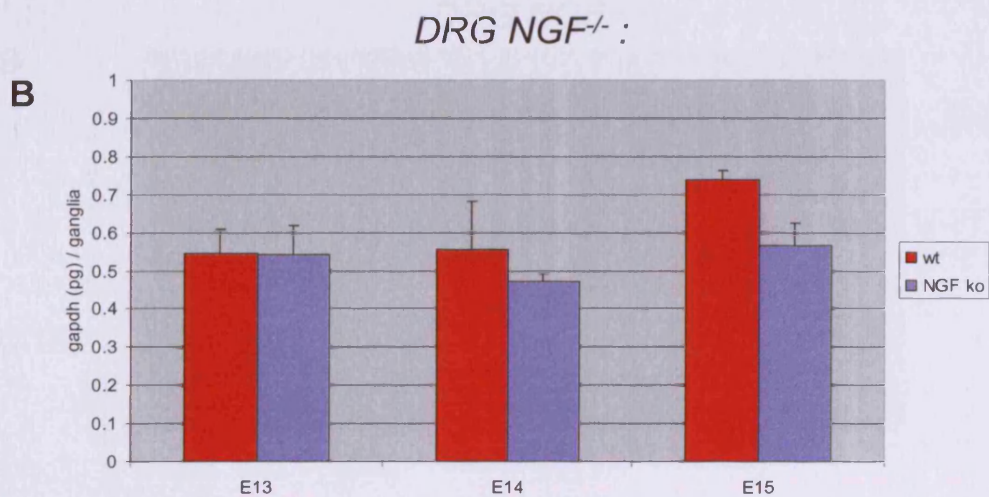
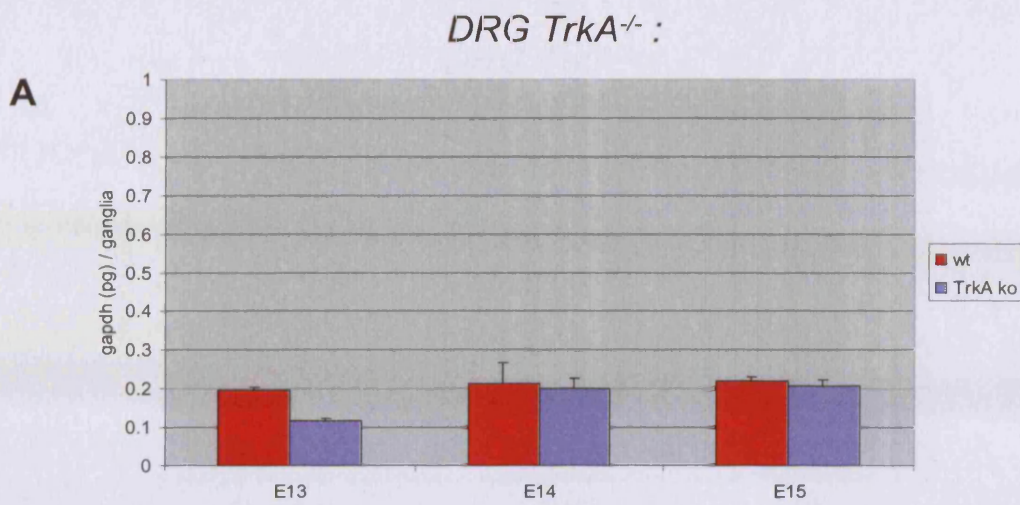


Figure 2.16. Expression of GAPDH per ganglia in DRG of *TrkA^{-/-}* and *NGF^{-/-}* mice prior to normalisation..

DRG were dissected from E13 – E15 null mutant and wild-type mice and the expression of GAPDH mRNA quantified by competitive RT-PCR. Expression of GAPDH mRNA per ganglia in *TrkA^{-/-}* (A) and *NGF^{-/-}* (B) is shown in comparison to their wild-type littermates.

Error bars +/- standard error, n = 3-4. * = $p < 0.05$, ** = $p < 0.01$ as determined by two-tailed unpaired t-test.

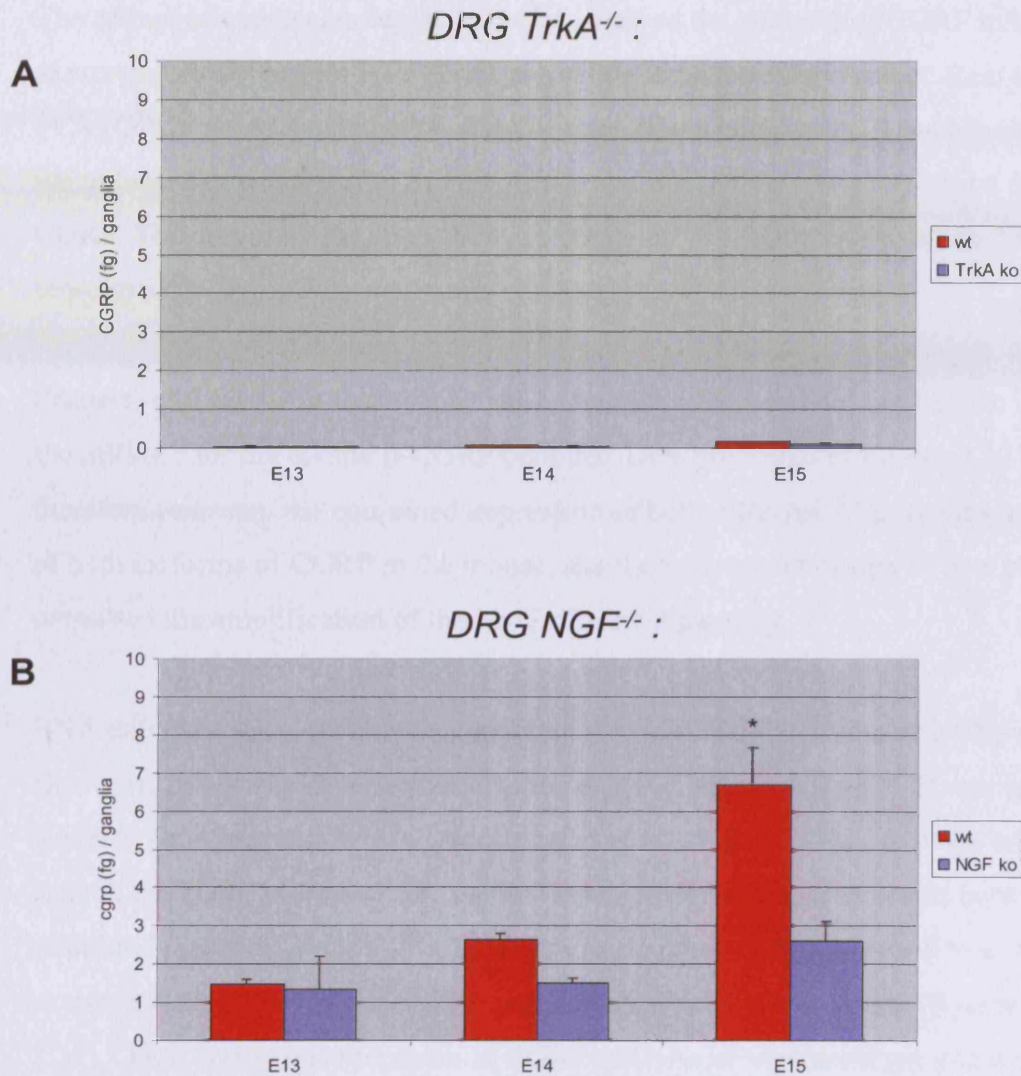


Figure 2.17. Expression of CGRP per ganglia in DRG of *TrkA*^{-/-} and *NGF*^{-/-} mice prior to normalisation..

DRG were dissected from E13 – E15 null mutant and wild-type mice and the expression of CGRP mRNA quantified by competitive RT-PCR. Expression of CGRP mRNA per ganglia in *TrkA*^{-/-} (A) and *NGF*^{-/-} (B) is shown in comparison to their wild-type littermates.

Error bars +/- standard error, n = 3-4. * = p = <0.05, ** = p = <0.01 as determined by two-tailed unpaired t-test.

2.3.2. Alpha and beta transcripts of CGRP may be differentially regulated in the early embryonic mouse

The arrival of new technologies in the lab allowed the analysis of CGRP mRNA expression in absence of NGF/TrkA signalling to be extended further. Real-time PCR (QPCR) is a very sensitive and fast form of quantitative PCR, which allowed me to look at the expression of other genes and also reconfirm competitive data for CGRP. The computer program 'Beacon Designer' also allowed the production of more specific and efficient primers.

Primers used earlier in this chapter for competitive PCR did not distinguish between the mRNAs for the α - and β -CGRP peptides. Data presented in figures 2.12 to 2.17, therefore represent the combined expression of both mRNAs. The recent sequencing of both isoforms of CGRP in the mouse, and the subsequent design of new primers permitted the amplification of the two isoforms separately.

RNA extracted previously for competitive RT-PCR studies was used in this study. However, Stratscript reverse transcription enzyme, and real-time PCR was used to quantify gene expression (See materials and methods 2.2.5). Results were normalised against GAPDH, to account for any discrepancies in initial RNA levels between samples. Transcripts for both CGRP isoforms appear to be expressed to a similar extent in both DRG and trigeminal ganglia of the developing mouse (figures 2.18 – 2.21). Once again, the expression of CGRP mRNAs in trigeminal ganglia was visibly higher than in DRG.

α -CGRP

In the DRG, as was indicated by the competitive data, there was a marked decrease in α -CGRP mRNA expression in NGF^{-/-} mice compared to NGF^{+/+} mice, becoming significant by E15 (figure 2.18B). This was once again not reflected in the TrkA^{-/-} mouse (figure 2.18A). This could suggest a role for NGF signalling via p75 rather

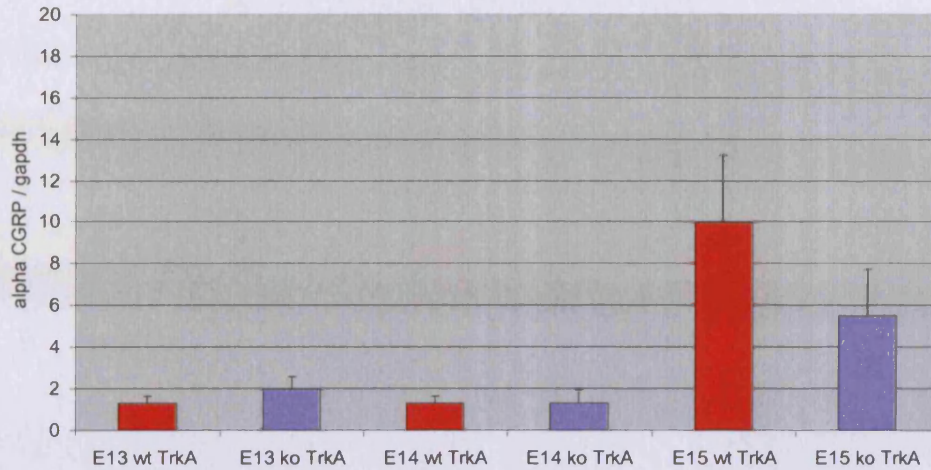
than TrkA in the onset of α -CGRP expression or it could be a reflection of the loss of NT-3/TrkA signalling that could affect the phenotype of neurons in these TrkA^{-/-} mice.

In the trigeminal ganglia the expression of α -CGRP mRNA was not diminished in either NGF^{-/-} or TrkA^{-/-} mice compared to wild type mice (figure 2.19A and B), suggesting that in the trigeminal ganglia NGF and TrkA signalling are not required for the onset of expression of the α isoform.

β -CGRP

Unlike α -CGRP mRNA, there is no significant difference in β -CGRP mRNA levels in TrkA^{-/-} or NGF^{-/-} DRG in comparison to wild-type (figure 2.20.A and B). In the trigeminal ganglia, the same result as for α -CGRP mRNA is observed, with no difference in expression of β -CGRP between either null mutants in comparison to wild-type (figure 2.21.A and B)

A. DRG – *TrkA*^{-/-} :



B. DRG – *NGF*^{-/-} :

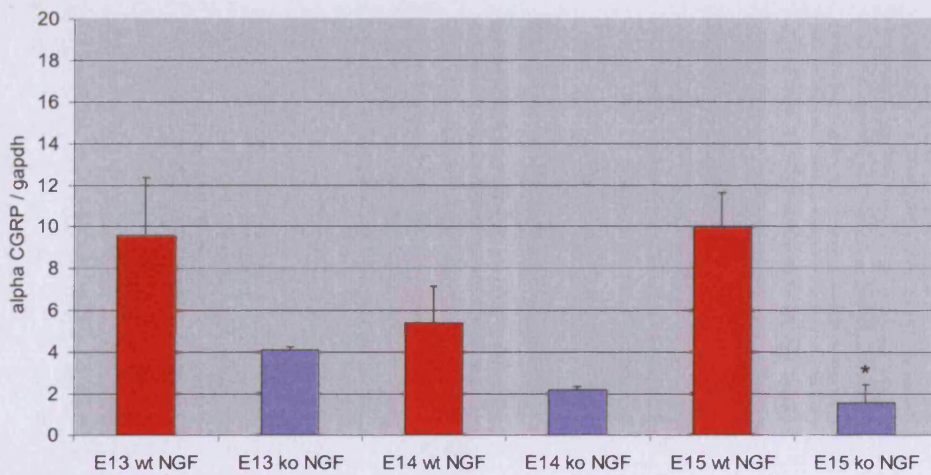
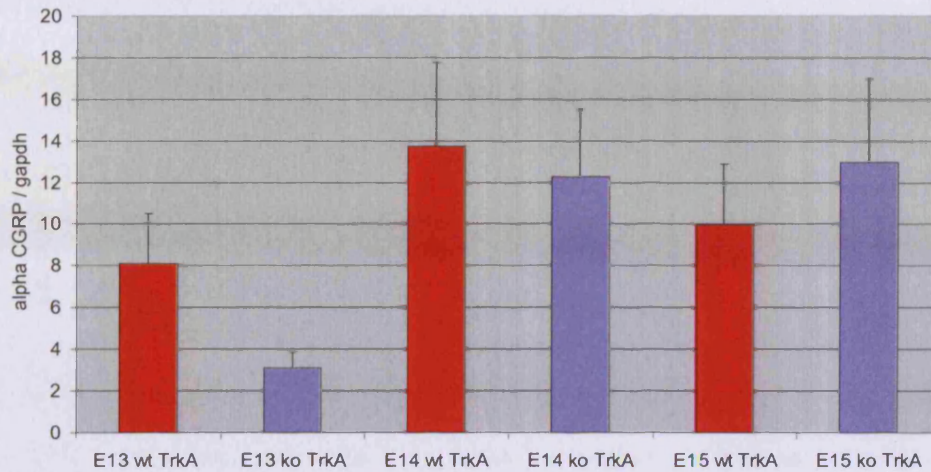


Figure 2.18. Expression of alpha CGRP mRNA in DRG of *TrkA*^{-/-} and *NGF*^{-/-} mice.

DRG were dissected from E13-E15 null mutant and wild-type mice and the expression of alpha CGRP mRNA quantified via real-time QPCR. Expression of alpha CGRP mRNA in the DRG of *TrkA*^{-/-}(A) and *NGF*^{-/-}(B) mice is shown in comparison to their wild-type litter mates.

Error bars +/- standard error. * = $p < 0.05$, ** = $p < 0.01$ as determined by two-tailed unpaired t-test, $n = 3-4$

A. Trigeminal ganglia – *TrkA*^{-/-} :



B. Trigeminal ganglia – *NGF*^{-/-} :

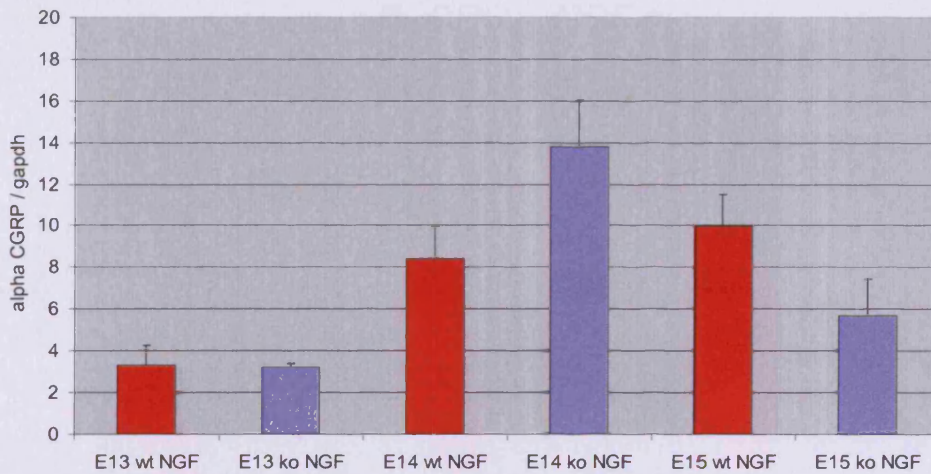


Figure 2.19. Expression of alpha CGRP mRNA in trigeminal ganglia of *TrkA*^{-/-} and *NGF*^{-/-} mice. Trigeminal ganglia were dissected from E13-E15 null mutant and wild-type mice and the expression of alpha CGRP mRNA quantified via real-time QPCR. Expression of alpha CGRP mRNA in the trigeminal ganglia of *TrkA*^{-/-}(A) and *NGF*^{-/-}(B) mice is shown in comparison to their wild-type litter mates. Error bars +/- standard error. * = $p < 0.05$, ** = $p < 0.001$ as determined by two-tailed unpaired t-test, n = 3-4

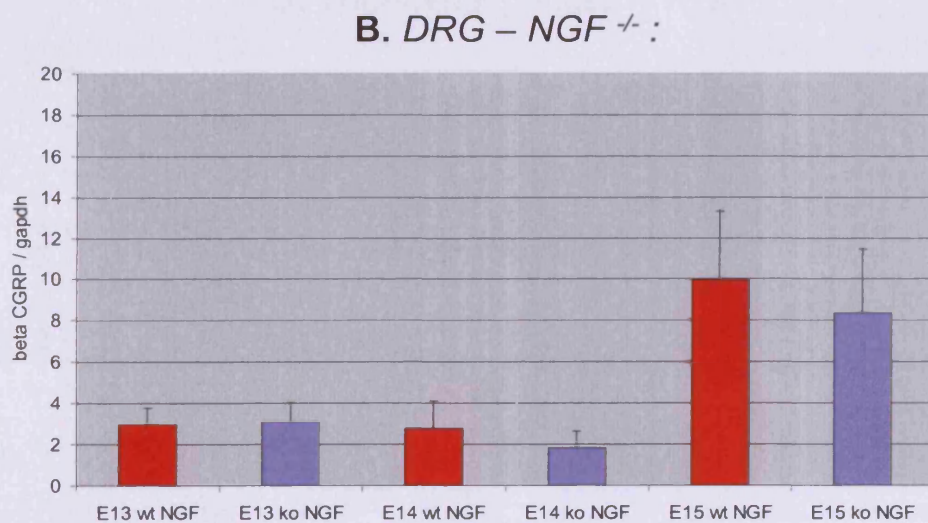
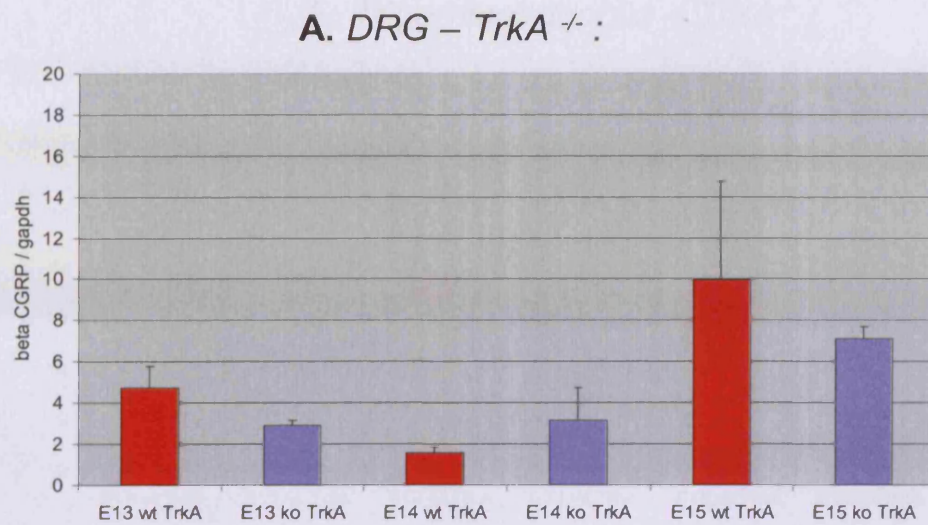
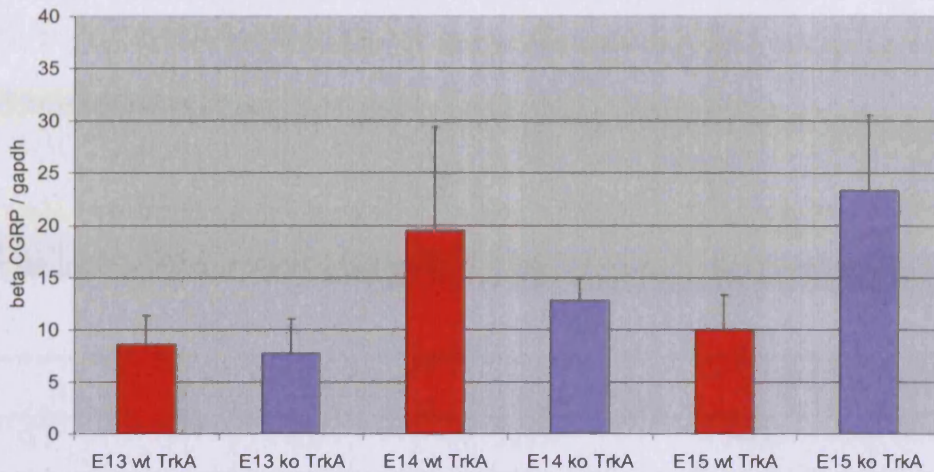


Figure 2.20. Expression of beta-CGRP mRNA in DRG of *TrkA*^{-/-} and *NGF*^{-/-} mice.

DRG were dissected from E13-E15 null mutant and wild-type mice and the expression of beta-CGRP mRNA quantified via real-time QPCR. Expression of beta CGRP mRNA in the DRG of *TrkA*^{-/-}(A) and *NGF*^{-/-}(B) mice is shown, in comparison to their wild-type litter mates.

Error bars +/- standard error. * = p < 0.05, ** = P < 0.01 as determined by two-tailed unpaired t-test, n = 3-4

A. Trigeminal ganglia – $TrkA^{-/-}$:



B. Trigeminal ganglia – $NGF^{-/-}$:

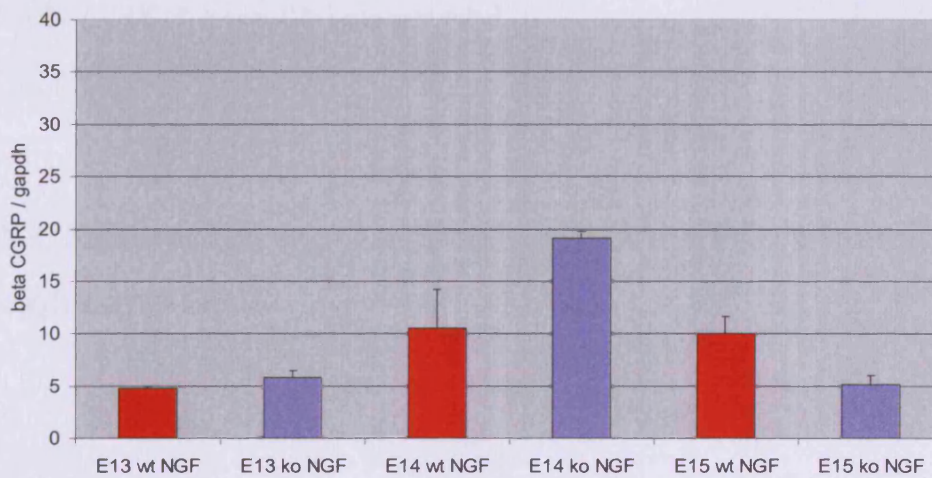


Figure 2.21. Expression of beta CGRP mRNA in trigeminal ganglia of $TrkA^{-/-}$ and $NGF^{-/-}$. Trigeminal ganglia were dissected from E13-E15 null mutant and wild-type mice and the expression of beta CGRP mRNA quantified via real-time QPCR. Expression of beta CGRP mRNA in the trigeminal ganglia of $TrkA^{-/-}$ (A) and $NGF^{-/-}$ (B) mice is shown in comparison to their wild-type litter mates. Error bars +/- standard error. * = $p < 0.05$, ** = $p < 0.001$ as determined by two-tailed unpaired t-test, $n = 3-4$

These results parallel those obtained by competitive RT-PCR where a decrease in CGRP mRNA in the DRG of NGF^{-/-} mice was observed, but was not significant, in comparison to wild type mice. This lack of significance could be due to the additional detection of the β - isoform by the non-specific competitive primers. β -CGRP mRNA was not found to be down-regulated in NGF^{-/-} mice (figure 2.20B), so any real decrease in α -CGRP mRNA may have been masked.

A table to summarise the regulation of both CGRP mRNA isoforms in DRG and trigeminal ganglia is shown below.

	<u>ALPHA CGRP</u>		BETA CGRP	
	NGF	TrkA	NGF	TrkA
DRG	✓	×	×	×
Trigeminal	×	×	×	×

Table 2.10 Summary of NGF/TrkA signalling required for expression of α and β CGRP transcripts, in embryonic (E13-E15) mouse DRG and trigeminal.

✓ = required, × = not required.

2.3.3. Differential regulation of substance P in trigeminal ganglia and DRG

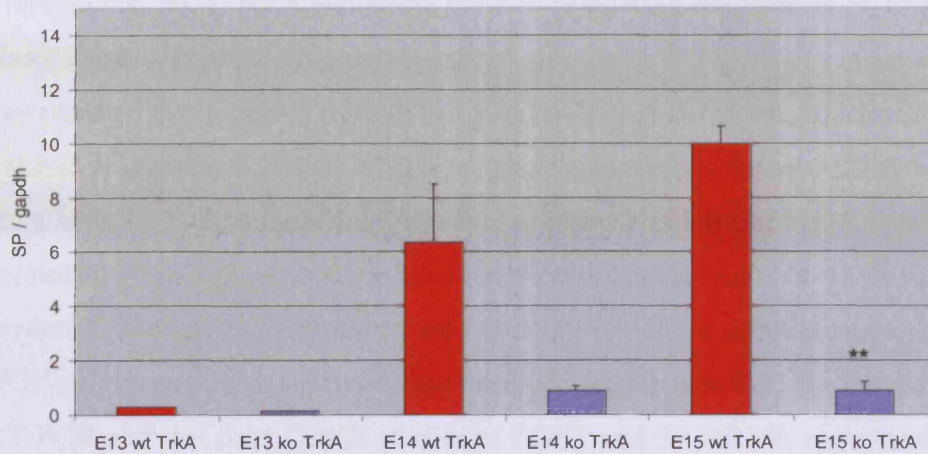
SP is largely co-expressed with CGRP in the same subpopulation of sensory neurons in the adult (Lee et al., 1985; Skofitsch et al., 1985; Lundberg et al., 1985). To investigate whether the expression pattern of SP parallels that of CGRP throughout development and if there is comparable neurotrophic factor regulation during development, the expression of the SP precursor mRNA was quantified in embryonic trigeminal ganglia in TrkA^{-/-} and NGF^{-/-} mice and their wild type littermates using

reverse transcription followed by real-time PCR. SP is only 12 amino acids long, and because of its small size was difficult to detect via PCR. Instead the mRNA for the PPTA precursor was reverse transcribed and amplified at the portion that is cleaved to produce SP (see figure 2.1.). Because the expression levels of SP precursor mRNA at early ages is low and very difficult to detect in DRG, since they are smaller than trigeminal ganglia and yield less purified RNA, only results for the trigeminal ganglia were obtained (figure 2.22).

In trigeminal ganglia of both $TrkA^{-/-}$ and $NGF^{-/-}$ mice there is a decrease in the expression of SP precursor mRNA in comparison to wild-type mice at both E14 and E15. This reduction is significant at E15 in both null mutants and also at E14 in $NGF^{-/-}$ mice. This would suggest that NGF and TrkA expression are both required at least in part for the onset of SP precursor mRNA expression in developing trigeminal ganglia. The expression of SP precursor mRNA at E13 was very low, but still detectable, suggesting that the onset of SP precursor mRNA expression may occur at this age.

These data for SP differ from those of both isoforms of CGRP mRNAs. Neither α - nor β -CGRP mRNAs showed an apparent requirement for NGF/TrkA signalling for initial induction of expression in the trigeminal ganglia (figures 2.19 and 2.21).

A. Trigeminal ganglia – *TrkA*^{-/-}



B. Trigeminal ganglia – *NGF*^{-/-}

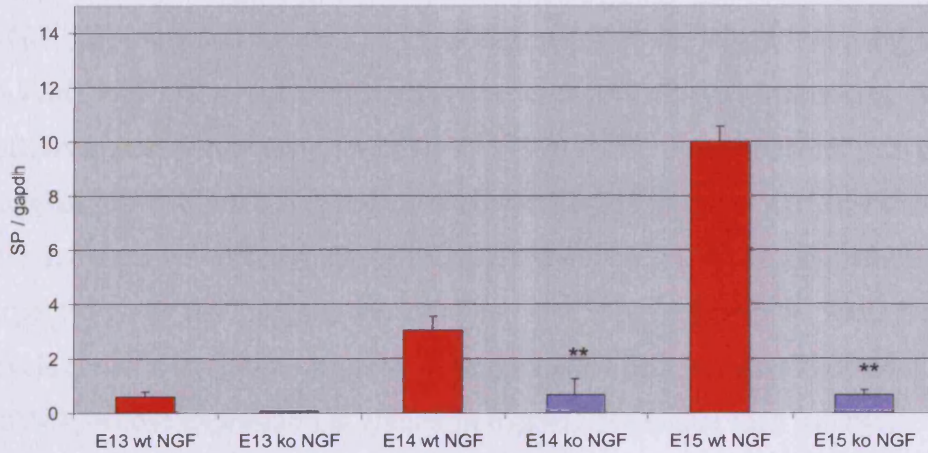


Figure 2.22. Expression of SP mRNA in sensory neurons of *TrkA*^{-/-} and *NGF*^{-/-}.

Trigeminal ganglia were dissected from E13-E15 null mutant and wild-type mice and the expression of SP mRNA quantified via real-time QPCR. Expression of SP mRNA in the trigeminal ganglia of *TrkA*^{-/-}(A) and *NGF*^{-/-}(B) mice is shown in comparison to their wild-type litter mates.

Error bars +/- standard error. * = $p < 0.05$, ** = $p < 0.01$ as determined by two-tailed unpaired t-test, $n = 3-4$

2.3.4. Initial induction of mRNA expression of the Tetrodotoxin (TTX) resistant sodium channels Nav1.8 and Nav1.9 is reliant upon NGF/TrkA signalling

The role that NGF/TrkA signalling plays in regulating the expression of Nav1.8 and Nav1.9 mRNAs in developing trigeminal ganglia and DRG was examined by measuring the expression of both mRNAs by RT-QPCR in ganglia from NGF and TrkA null mutants and their wild type littermates. The two genes *Scn10a* and *Scn11a* are transcribed to produce the functional proteins Nav1.8 and Nav1.9 respectively. Primers were designed to allow amplification of Nav1.8 and Nav1.9 mRNAs using reverse transcription followed by real time Q-PCR RNA samples from TrkA and NGF null mutants that had been collected previously and used for the competitive RT-PCR and real time-QPCR studies of CGRP and SP mRNA expression were used to determine the expression of sodium channel mRNAs.

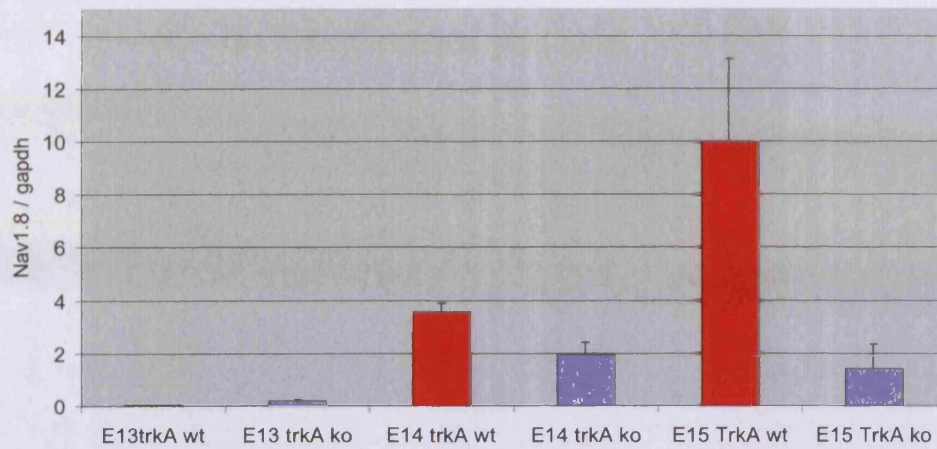
Figure 2.23 shows the expression levels of Nav1.8 mRNA in DRG of *TrkA*^{-/-} (A) and *NGF*^{-/-} (B) mice and figure 2.24 show expression in the trigeminal ganglia of *TrkA*^{-/-} (A) and *NGF*^{-/-} (B) mice compared to that of their wild-type littermates. Nav 1.8 mRNA expression is just detectable at E13 in DRG, more noticeably so in trigeminal ganglia. Nav 1.8 mRNA is therefore detectable at a younger age than Nav1.8 protein, which cannot be detected until E15 (Benn et al., 2001), as would probably be expected given the high sensitivity of this method of RT-QPCR. Nav1.8 mRNA levels are similar in both trigeminal ganglia and DRG, unlike CGRP isoforms mRNA, whose expression is greater in trigeminal ganglia than in DRG .

The data in figures 2.23 and 2.24 suggest that the initial induction of expression of Nav1.8 mRNA is regulated to some extent by TrkA/NGF signalling. In both DRG and trigeminal ganglia there is a large, often significant reduction in expression of Nav1.8 mRNA in both *TrkA*^{-/-} and *NGF*^{-/-} mice compared to wild type mice (figures 2.23 and 2.24). This is observed from E14 in DRG and from E13 in trigeminal

ganglia, suggesting that TrkA/NGF signalling is required for the initial onset of Nav1.8 expression.

Data showing the expression of Nav1.9 mRNA in DRG and trigeminal ganglia from E13 to E15 is plotted in figures 2.215 and 2.26. Nav1.9 mRNA was not detectable at E13, but was apparent by E14. This later expression of Nav1.9 than Nav1.8 follows the expression pattern at the protein level where Nav1.8 is detectable at E15, whereas Nav1.9, is below the limits of detection until E17 (Benn et al., 2001). Nav1.9 mRNA is expressed at similar levels in DRG and trigeminal ganglia. Like Nav1.8, NGF/TrkA signalling seems to be required for the onset of Nav1.9 mRNA expression in both ganglia, with mRNA levels being significantly diminished in DRG and trigeminal ganglia from both null mutants at E15 compared to ganglia from wild type mice (figures 2.25 and 2.26).

A. DRG – *TrkA*^{-/-} :



B. DRG – *NGF*^{-/-} :

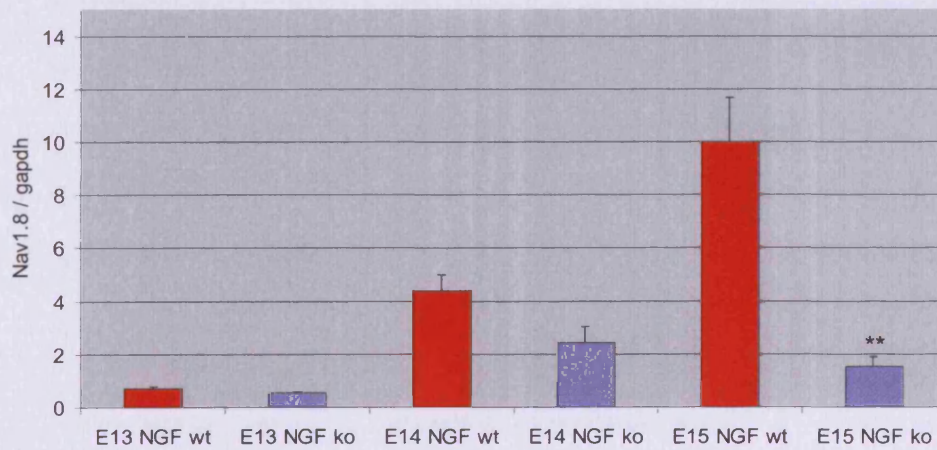
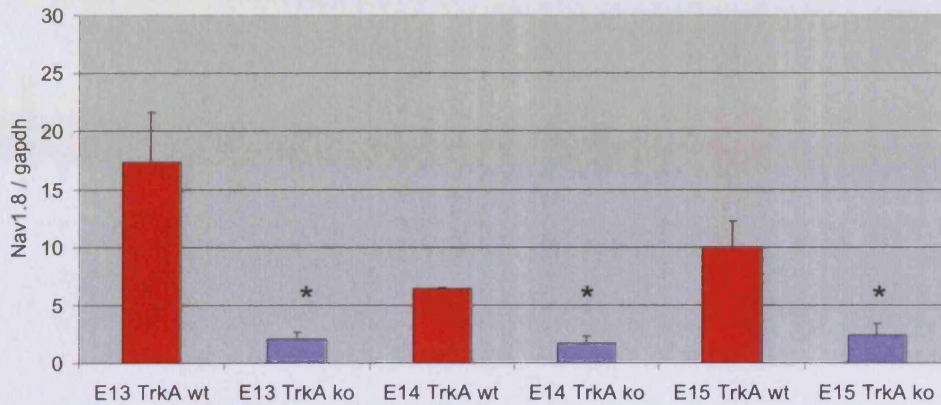


Figure 2.23. Expression of *Nav1.8* mRNA in DRG of *TrkA*^{-/-} and *NGF*^{-/-}.

DRG were dissected from E13-E15 null mutant and wild-type mice and the expression of *Nav1.8* mRNA quantified via real-time QPCR. Expression of *Nav1.8* mRNA in the DRG of *TrkA*^{-/-}(A) and *NGF*^{-/-}(B) mice is shown, in comparison to their wild-type littermates.

Error bars = +/- standard error. * = $p < 0.05$ and ** = $p < 0.01$ as determined by two-tailed, unpaired t-test, $n = 3-4$

B. Trigeminal ganglia – *TrkA*^{-/-}:



A. Trigeminal ganglia – *NGF*^{-/-}:

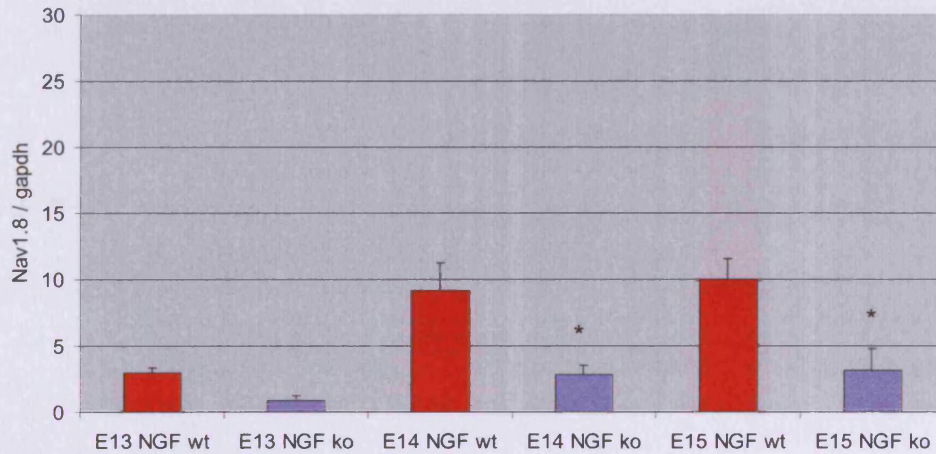
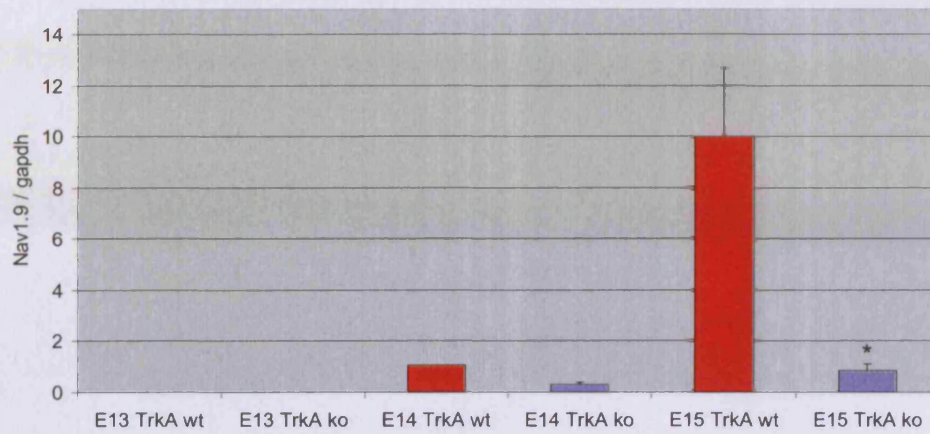


Figure 2.24. Expression of *Nav1.8* mRNA in trigeminal ganglia of *TrkA*^{-/-} and *NGF*^{-/-}.

Trigeminal ganglia were dissected from E13-E15 null mutant and wild-type mice and the expression of *Nav1.8* mRNA quantified via real-time QPCR. Expression of *Nav1.8* mRNA in the trigeminal ganglia of *TrkA*^{-/-} (A) and *NGF*^{-/-} (B) mice is shown in comparison to their wild-type litter mates.

Error bars +/- standard error. * = $p < 0.05$, ** = $p < 0.01$ as determined by two-tailed unpaired t-test, $n = 3-4$

A. DRG -- *TrkA*^{-/-} :



B. DRG -- *NGF*^{-/-} :

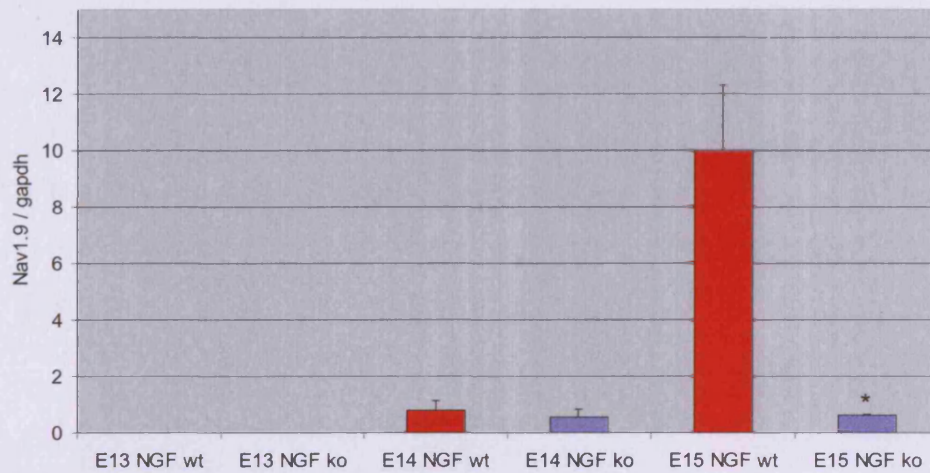
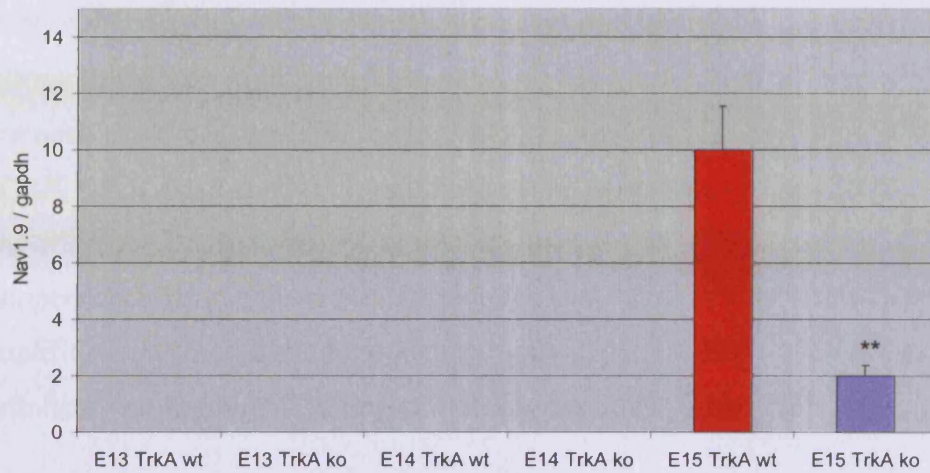


Figure 2.25. Expression of *Nav1.9* mRNA in DRG of *TrkA*^{-/-} and *NGF*^{-/-}.

DRG were dissected from E13-E15 null mutant and wild-type mice and the expression of *Nav1.9* mRNA quantified via real-time QPCR. Expression of *Nav1.9* mRNA in the DRG of *TrkA*^{-/-}(A) and *NGF*^{-/-}(B) mice is shown, in comparison to their wild-type litter-mates.

Error bars +/- standard error. * = $p < 0.05$ and ** = $p < 0.01$ as determined by two-tailed unpaired t-test, $n = 3-4$

A: Trigeminal ganglia – *TrkA*^{-/-} :



B. Trigeminal ganglia – *NGF*^{-/-} :

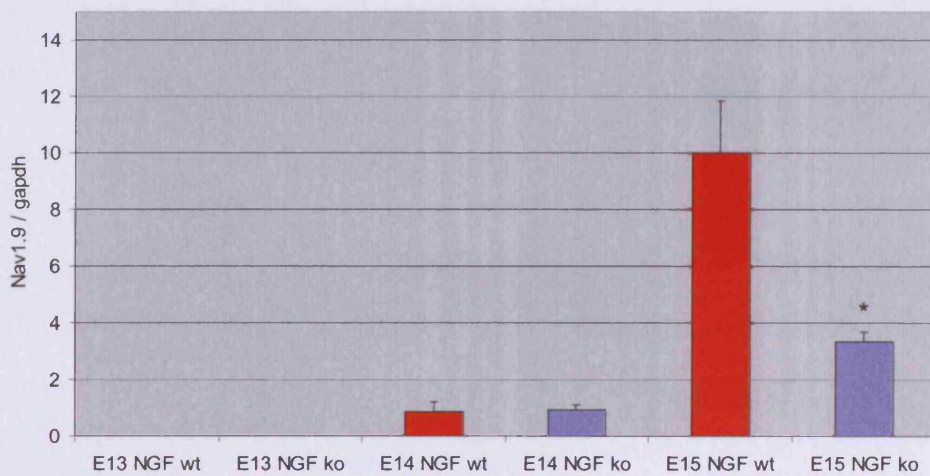


Figure 2.26. Expression of *Nav1.9* mRNA in trigeminal ganglia of *TrkA*^{-/-} and *NGF*^{-/-}.

DRG and Trigeminal ganglia were dissected from E13-E15 null mutant and wild-type mice and the expression of *Nav1.9* mRNA quantified via real-time QPCR. Expression of *Nav1.9* mRNA in the trigeminal ganglia of *TrkA*^{-/-}(A) and *NGF*^{-/-}(B) mice is shown, in comparison to their wild-type litter-mates.

Error bars +/- standard error. * = $p < 0.05$ and ** = $p < 0.01$ as determined by two-tailed unpaired t-test, $n = 3-4$

2.3.5 All PCR reactions are highly efficient

To check the reliability and specificity of the QPCR reactions for α - and β -CGRP, SP, Nav1.8 and Nav1.9, RT-QPCR reactions were run using RNA extracted from tissue that should not express any of these mRNAs mouse liver was chosen for this purpose. Mouse Liver RNA (Ambion) was reverse transcribed, as described previously. Mouse trigeminal RNA was also transcribed alongside liver RNA, to act as a positive control and ensure efficient transcription. No expression of α -CGRP, β -CGRP, SP, Nav1.8 and Nav1.9 mRNAs could be detected in liver RNA. Any amplification (as observed by increasing fluorescence with increasing PCR cycle number) occurring in these QPCR reactions was due to primer artefacts or amplification of non-specific products (figure 2.27). GAPDH mRNA was present in both liver and trigeminal, acting as a positive control (figure 2.27).

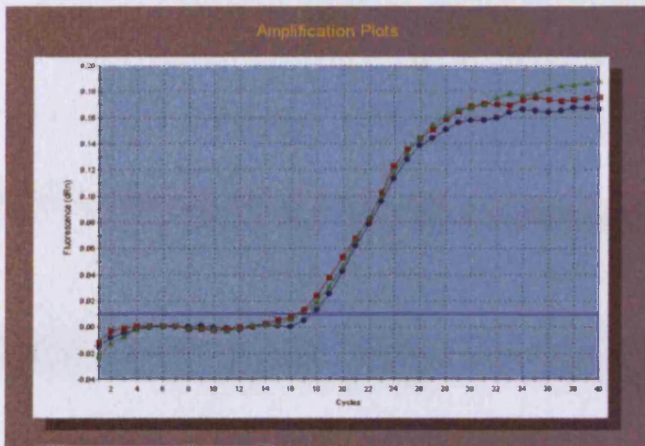
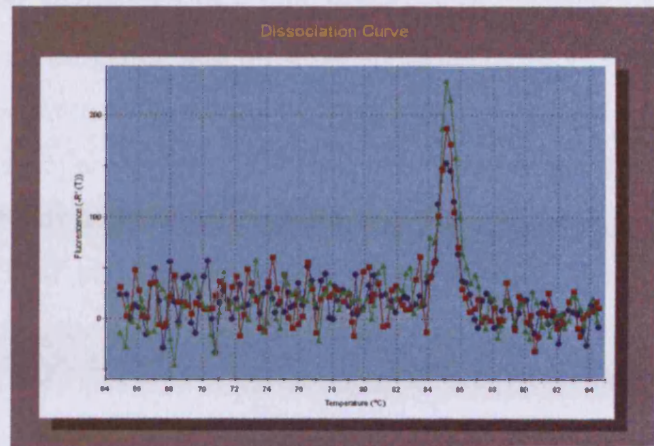
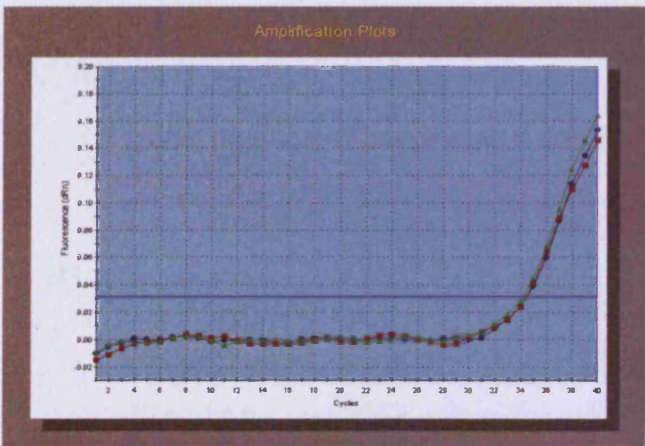
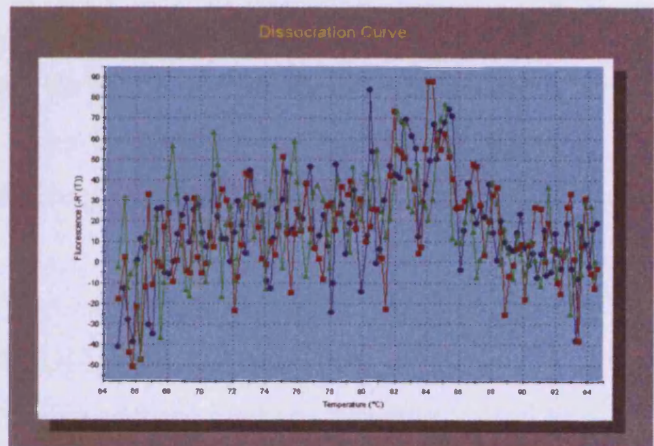
A**B****C****D**

Figure 2.27. Expression of genes of interest in Liver RNA.

To check the 'Leakiness' of the PCR reactions, reactions were run for all genes using RNA from Liver. No gene expression of any genes under test was observed in liver, with the exception of GAPDH.

A and B show expression of GAPDH clearly present. C and D show the absence of α CGRP mRNA expression. N.B. Although some amplification is seen late on (C) the product produced is not the correct one, as illustrated by the melting curve (D).

2.4. Discussion

The highly sensitive, quantitative methods of competitive RT-PCR and, more recently, real time-QPCR, have allowed investigation into the developmental expression of several genes that are important in determining the functional characteristics of sensory neurons. The use of embryonic transgenic mice allowed the effects of NGF and TrkA signalling on the developmental expression of these genes to be studied. This helped to ascertain if NGF and TrkA signalling are required for the initial induction of transcripts for these genes in the different sensory ganglia of the embryonic mouse.

Whilst interpreting results, one important caveat should be born in mind. The Real time Q-PCR technique used in this thesis allows relative quantification of mRNA levels i.e. comparisons are made between samples after *normalising* for differences in sample concentration and loading. Normalisation is necessary to control for the numerous errors associated with such experiments, including the inherent variability of RNA, the variability of extraction protocols and the differences in efficiencies between reverse transcription and PCR. However, despite its necessity, normalisation remains one of the main problems and drawbacks of real-time QPCR at present.

Several methods of normalisation exist. Two such techniques are: a) Ensuring similar sample sizes are initially obtained prior to RNA extraction and b) Normalising results to total RNA within the sample. Neither of these methods however control for errors that occur at the reverse transcription or PCR stages. To this end, the most popular method of normalisation employed tends to be the use of a reference gene. In this method an internal control is amplified, in the PCR reaction, alongside the gene of interest. This method is particularly advantageous as the internal control is subject to the same conditions as the mRNA of interest, and it was this method that was used in this thesis.

The identity of the most reliable internal control is still a controversial and much debated topic. The ideal RNA species for an 'internal control' should be expressed at a constant level throughout the tissue in question and should not be regulated by the experimental conditions. Unfortunately, there is no single RNA which can fulfil such criteria at present, with most being altered through the course of the experiment. The most popular internal controls used experimentally include β actin, Glyceraldehyde - 3-phosphate-dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase (HPRT) and 18s ribosomal RNA. Despite all being used frequently, as illustrated in numerous published papers, they all have drawbacks. Levels of GAPDH and β -actin have been shown to vary over certain conditions, however because of its abundance the use of 18S rRNA impedes the detection of the PCR product of rare messages.

Throughout this thesis Glyceraldehyde -3-phosphate-dehydrogenase (GAPDH) was chosen as internal control. The mRNA encoding GAPDH is moderately expressed in many tissues including neuronal cells, and has been used as an internal control in many similar experiments. As mentioned briefly, GAPDH levels can vary with developmental stage and so its use may not provide the accurate results sought. This possibility should be borne in mind when interpreting results.

A round-table debate on the limitations of the use of such internal controls was published in the journal of leukaemia in 1997-1998, and is still of debate to this day. There is a thought that perhaps an 'alien RNA' molecule that could be synthesised and added in accurate quantities prior to RNA extraction could solve most problems, however until such a molecule is created researchers must continue to debate the pros and cons of the 'classic' housekeeping genes to make their, albeit not 100% foolproof, decision as to which will be the gene of choice in their laboratory.

Table 2.11 summarises findings.

	ALPHA CGRP		BETA CGRP		SP		NAV1.8		NAV1.9	
	NGF	TrkA	NGF	TrkA	NGF	TrkA	NGF	TrkA	NGF	TrkA
DRG	✓	×	×	×	-	-	✓	✓	✓	✓
Trigeminal	×	×	×	×	✓	✓	✓	✓	✓	✓

Table 2.11 Summary of NGF/TrkA signalling required for expression of sensory neuron predominant genes, in embryonic (E13-E15) mouse DRG and trigeminal.

✓ = required, × = not required. - = not carried out

Initially, the investigation of the neuropeptide CGRP was studied using competitive PCR to quantify mRNA expression in DRG and trigeminal ganglia of TrkA^{-/-} and NGF^{-/-} mice in comparison to wild-type littermates. The development of real-time QPCR in the laboratory allowed me to extend this analysis. This method of PCR was favoured due to its efficiency and flexibility. In particular, time did not have to be spent in the manufacture of competitors for each gene, gels did not need to be run and reactions could be set-up in a high-throughput 96-well format. In addition, QPCR has the advantage that it has a much wider dynamic range than competitive RT-PCR, allowing the direct comparison of samples that have greatly differing levels of target mRNA expression. Although QPCR is not as accurate or reproducible as competitive PCR, performing more replicates can compensate for this drawback and the advantages of the system far outweigh the disadvantages.

The introduction of QPCR and a more efficient primer design package, allowed me to design primers and set up reactions that allowed both isoforms of CGRP to be studied separately. Little research thus far has been done on the mouse β -CGRP isoform for several reasons. First, high sequence homology between the two isoforms has led to a practical difficulty in discriminating between them. Secondly, little has been done in the mouse, as until recently both genes were not sequenced in this

species (Thomas et al., 2001). Finally the α - transcript is usually considered the more abundant of the two, at least in the adult (Amara et al. 1985, Gibson et al., 1988), so research has tended to focus on this isoform. Throughout development, however, β -CGRP has been shown to be the predominant form in many areas of the nervous system, suggesting a strong developmental role (Terrado et al., 1999). My results do show that β -CGRP is indeed expressed to a similar extent to the α isoform in both trigeminal and DRG at these early stages of development. Although no statistical comparisons have been made, the pattern of β -CGRP mRNA expression in trigeminal ganglia of wildtype mice (figure 2.21) is indeed comparable to that of α -CGRP mRNA (figure 2.19). In trigeminal ganglia of wild-type mice from $TrkA^{-/-}$ litters i.e. $TrkA^{+/+}$ mice, expression of *both* α and β CGRP mRNA is similar at E13. Levels of both then double from E13 – E14 before dropping back to approximately E13 levels by E15 (figures 2.19A and 2.21A). In trigeminal ganglia of wildtype mice from $NGF^{-/-}$ litters i.e. $NGF^{+/+}$, a similar mRNA level for α and β CGRP is found once again at E13. A two-fold increase in expression is then observed in *both* isoforms between E13 and E14 (figure 2.19B and 2.21B), and this increase is maintained until E15. This maintenance in expression between E14-E15 in trigeminal of $NGF^{+/+}$ mice is in contrast to the dip observed in $TrkA^{+/+}$ mice. Such discrepancies between $TrkA^{+/+}$ and $NGF^{+/+}$ are odd since both of these phenotypes are essentially wild-type. However it could be due to experimental issues, or down to differences in the strain or background of the mice. In the DRG, α -CGRP mRNA was also expressed to a similar extent to β -CGRP mRNA. In $TrkA^{+/+}$ mice expression of *both* isoforms increased in DRG from E13 – E15 (figure 2.18A and 2.20A). In DRG of $NGF^{+/+}$ mice, levels of α -CGRP mRNA were initially higher than those of the β isoform, however by E15 both isoforms were detectable at comparable levels (figure 2.18B and 2.20B). These results would indicate that β -CGRP is expressed as abundantly as the α - isoform, at least in some sensory neuronal populations of the developing mouse. A possible role for β -CGRP in development of such neuronal subpopulations is therefore indicated.

Despite a high sequence homology between the CGRP isoforms, the two peptides are transcribed from separate genes that most probably arose as a result of gene duplication. The difference in expression patterns in development, and their production from distinct genes would therefore suggest functionally different roles for α - and β -CGRP in the young embryo, and possible differential regulation by trophic factors. My results showed that mRNAs for both α - and β - transcripts are present at a similar level in both sensory ganglia studied from E13 up to E15. Both transcripts are expressed more highly in the trigeminal ganglia than in the DRG. In embryonic trigeminal ganglia, no significant difference between the expression of the α - and β - transcripts was observed between wild-type and TrkA and NGF null mutant mice from E13 to E15 (figures 2.19 and 2.21). This suggests no regulation of expression of either α - or β - CGRP mRNA by NGF or TrkA signalling in the embryonic trigeminal ganglia at these ages. This demonstrates that NGF/TrkA signalling is not required for the induction of α - and β - CGRP expression in trigeminal neurons. In the DRG, however, NGF may play a role in regulating the expression of α -CGRP mRNA, but not the mRNA for the β - isoform. No difference was seen in the expression of α -CGRP mRNA in the DRG of TrkA^{-/-} mice compared to TrkA^{+/+} mice, however, in NGF^{-/-} mice there was a notable decrease in the expression of the α transcript compared to wild-type mice. This was seen in real-time data (figure 2.18) and also suggested in competitive data (figure 2.13.) β -CGRP mRNA was conversely expressed at similar levels in DRG from TrkA and NGF null mutants and wild type mice (figure 2.20). It is therefore apparent that despite high sequence homology, the two CGRP transcripts are regulated differentially in the DRG of the developing mouse. This suggests that not all subpopulations of sensory neurons require NGF/TrkA signalling for the induction of CGRP expression, as has been thought previously from a much less extensive study of developing sensory neurons (Patel et al., 2000).

It is curious that α -CGRP mRNA induction and maintenance of expression between E14 to E15 is independent of TrkA signalling in developing DRG, but appears to require NGF signalling. One possibility is that NGF regulated induction and initial up-regulation of α -CGRP mRNA expression is affected entirely through signalling

through the common p75 neurotrophin receptor. Analysis of the expression of α -CGRP mRNA in the DRG of p75 knockout mice would address this possibility. Another possibility is that the TrkA and NGF mouse colonies show subtle inherent strain differences in the timing of sensory neuron loss in the absence of functional TrkA/NGF signalling. The neurotrophin NT-3 can also signal through TrkA (Soppet et al., 1991; Klein et al., 1991b; Cordon-Cardo et al., 1991; Glass et al., 1991; Ip et al., 1993). NT-3 is required for the correct timing of neuroblast differentiation (Kalcheim et al., 1992; Pinco et al., 1993; Elshamy and Ernfors, 1996), and mice deficient in NT-3 show a perturbation in sensory neuron differentiation and a subsequent loss of post-mitotic neurons (Liebl et al., 1997). Much of the effects of NT-3 on survival and differentiation of sensory neurons are proposed to be via receptors other than its preferred receptor TrkC, since NT-3^{-/-} mice show 70% loss in sensory neurons, a much greater proportion than the 30% loss observed in TrkC^{-/-} mice (Liebl et al., 1997). It is possible that TrkA^{-/-} mice suffer a loss of functional NT-3/TrkA signalling as well as NGF/TrkA signalling during the period E13 to E15 with concomitant derangement of normal neuronal differentiation that may lead to DRG with different proportions of different neuronal sub-types (CGRP mRNA expressing or non-expressing) and different numbers of neurons at E15 compared to wild-type mice or NGF^{-/-} mice.

It is noteworthy that figure 2.13 shows that the timing of CGRP mRNA up-regulation from basal levels is different in DRG of wild type mice from the TrkA and NGF colonies. In the TrkA colony, CGRP mRNA increases in the DRG of wild type mice from E14 to E15, but this is not the case in wild-type mice from the NGF colony. Similar differences between strains are also shown for α -CGRP mRNA in figure in 2.18, and in trigeminal ganglion in figure 2.12. Perhaps both strains show subtle differences in the timing of the induction and up-regulation of CGRP mRNA and/or the requirement for NGF/TrkA signalling to up-regulate CGRP mRNA from the basal levels expressed immediately after initial induction. This could be responsible for the data, suggesting that NGF but not TrkA signalling is required for the up-regulation of α -CGRP mRNA expression between E14 and E15 in DRG. This hypothesis is supported by the observation that both TrkA and NGF are necessary for

the up-regulation of CGRP expression in the late embryonic period and the maintenance of expression in the neonatal period (Patel et al, 2000).

The neuropeptide SP is expressed in the same subpopulation of neurons as CGRP. However, as revealed by my studies, its regulation by NGF/TrkA signalling is different from that of CGRP. In particular, SP does require TrkA/NGF signalling for the onset of expression in the trigeminal ganglion, unlike α - or β - CGRP. In both null mutants there was a decreased expression of SP mRNA in comparison to wild-type (figure 2.22). This decrease was significant at E14 in NGF^{-/-} mice and at E15 in both TrkA^{-/-} and NGF^{-/-} mice. SP mRNA was expressed at lower levels than CGRP mRNA in trigeminal ganglia at all ages studied but could not be detected in the early DRG, possibly because it is not expressed at this young age or because levels are too low to detect because the smaller size of the DRG compared to the trigeminal ganglion may result in a lower concentration of total RNA in DRG samples. Taken together, these results indicate that the induction of expression and up-regulation of SP mRNA from E13 to E15 is largely dependent on NGF acting via its receptor tyrosine kinase TrkA in trigeminal sensory neurons.

The expression of the two TTX resistant sodium channels Nav1.8 and Nav1.9 was also investigated. Previous research has identified that NGF and GDNF regulate the expression of both channels in adult DRG (Fjell et al., 1999), but the regulation of these sodium channel mRNAs in sensory neurons during embryonic development has not been previously addressed. Throughout embryonic development GDNF will no doubt have little effect on the expression of either channel as the majority of sensory neurons don't respond to this factor until after birth, when approximately half of small diameter neurons down-regulate TrkA and up-regulate receptors for members of the GDNF family of neurotrophic factors (Molliver and Snider, 1997; Molliver et al., 1997; Baudet et al., 2000). My results have shown that NGF does play a role in onset of expression and initial developmental up-regulation of these channels in sensory neurons during embryonic development. In both trigeminal ganglia and DRG of TrkA and NGF null mutant mice there was a large reduction in Nav1.8 mRNA levels in comparison to wild-type mice, at all ages studied. this

suggests a critical role for NGF/TrkA signalling in regulating the expression of this sodium channel in embryonic sensory neurons. The onset of expression of Nav1.9 mRNA in sensory neurons was later than that for Nav1.8, as Nav1.9 mRNA was not detectable in E13 mice in either DRG or trigeminal ganglia. This is in accordance with sodium channel protein expression patterns. Benn et al. (Benn et al., 2001) showed Nav1.8 protein was detectable at E15 whilst Nav1.9 protein was not detectable until E17 in mouse DRG. At E15 the induction and developmental up-regulation of Nav1.9 mRNA, like Nav1.8 mRNA, showed a distinct requirement for NGF/TrkA signalling with a significant reduction in Nav 1.9 mRNA in both DRG and trigeminal ganglia of NGF^{-/-} and TrkA^{-/-} mice in comparison to wild-type mice.

Summary

In this chapter the expression of several genes that are required for a number of key functional properties of subsets of sensory neurons have been studied in the DRG and trigeminal ganglia during the early stages of sensory neuron development. Previous work on the expression of these genes has largely focused on the adult and postnatal DRG. In contrast to these earlier studies, which have concluded that NGF positively regulates the expression of all of these genes, my results have revealed that the expression of only some of these genes is regulated by endogenous NGF and TrkA signalling during the earlier stages of development. This was true for Nav1.8 and Nav1.9. mRNAs whose levels were significantly decreased in sensory neurons of TrkA^{-/-} and NGF^{-/-} mice in comparison to wild type mice. Likewise, the expression of SP is highly dependent on NGF/TrkA signalling for initial induction and up-regulation in the trigeminal ganglia. Despite the co-expression of CGRP and SP in subsets of sensory neurons and the previous demonstration of co-regulation by NGF (Lundberg et al., 1985; Donaldson et al., 1992; Donnerer et al., 1992; Woolfe et al., 1994; Jiang and Smith, 1995; Verge et al., 1995; Shadiack et al., 2001; Gardell et al., 2003), the expression of CGRP is more complex than was previously thought. It seems that mRNAs for the two isoforms of CGRP, α - and β -, are regulated differentially in the DRG developmentally, but neither have a requirement for NGF/TrkA signalling in the trigeminal ganglia. This contradicts the previous

hypothesis that NGF and TrkA signalling was imperative for the induction and initial up-regulation of expression of CGRP mRNA in sensory neurons of the developing mouse (Patel et al., 2000). In the DRG, β -CGRP mRNA expression also showed no requirement for NGF/TrkA signalling. Of interest was the finding that α -CGRP mRNA expression does seem to be dependent upon NGF signalling within the DRG, but not on TrkA signalling. This suggests either that NGF may regulate the expression of the α isoform solely through the common neurotrophin receptor p75 at these early stages of DRG development or that subtle strain differences exist between the timing of CGRP mRNA expression and the requirement for NGF/TrkA signalling for its up-regulation between the TrkA mouse line and the NGF mouse line. Alternatively, the timing of neuronal cell death in sensory ganglia in the absence of functional TrkA signalling may be different to that in the absence of NGF, since the neurotrophin NT-3 can also signal through TrkA.

2.5. Results in Brief

As discussed, this chapter explored the expression of several mRNAs of interest, and their regulation by neurotrophic factors, in embryonic mice (E13-E15). Results and findings can be summarised as below:

CGRP:

- α - and β -CGRP mRNAs appear to show differing regulation in embryonic DRG. Results suggest that NGF signalling (through TrkA/p75?) may be required for initial induction of α CGRP mRNA expression, but not for β CGRP mRNA.
- Expression of both α and β CGRP mRNA seems to be independent of TrkA/NGF signalling within the trigeminal ganglia.
- Differences in results in the DRG vs the trigeminal ganglia show that not all genes are regulated in a similar fashion in all sensory neuronal ganglia. α -CGRP mRNA seems dependent upon NGF/TrkA signalling within the DRG, but not in the trigeminal ganglia.

SP:

- In trigeminal ganglia, initial induction of SP mRNA requires NGF/TrkA signalling, despite the finding that α and β CGRP mRNAs do not. This is of interest since SP and CGRP are predominantly co-localised and usually regulated in a similar fashion.

Nav1.8 and Nav1.9:

- NGF/TrkA signalling is required for initial induction of expression of both Nav1.8 and Nav1.9 mRNAs in both trigeminal and dorsal root ganglia at embryonic ages.

Chapter 3

The role of NGF and NT-3 in the regulation of gene expression in sensory neurons of the early postnatal mouse

3.1 Introduction

In the previous chapter the effects of NGF/TrkA signalling on sensory neuron gene expression were explored in the mouse embryo. The results showed that NGF signalling, via TrkA, has distinctive effects on the expression of SP, CGRP, Nav1.8 and Nav1.9 mRNAs in embryonic DRG and trigeminal sensory ganglia. In the current chapter, the previous study was extended to analyse the role that neurotrophin signalling plays in regulating the mRNA expression of these genes in sensory neurons later in development. Expression was additionally studied in another sensory neuronal ganglion, the nodose ganglion (or inferior vagal ganglia). The majority of nodose neurons express TrkB rather than TrkA, making them predominantly dependent upon BDNF for survival during development. However, since a small population of nodose neurons are NGF dependent during the late embryonic period (Forgie et al., 1999), the effect of NGF/TrkA signalling on nodose neuron gene expression was investigated. In addition, the effects of NGF on the expression of some other important sensory neuron genes was studied alongside those mRNAs investigated in chapter 2.

In the current chapter, the effects of NT-3 on sensory neuron gene expression were also investigated. NT-3, like NGF, is highly important throughout development both with regards to neuronal survival and determination of neuronal phenotype. Embryonic sensory neuron survival is influenced by both factors, as is illustrated through study of transgenic mice with null mutations in the genes encoding these neurotrophic factors and their preferred receptors. Both TrkA^{-/-} and NGF^{-/-} mice die shortly after birth and show a massive loss of small diameter sensory neurons of the

DRG and trigeminal ganglia (Smeyne et al., 1994; Crowley et al., 1994). The small diameter sensory neurons lost are responsible for transmission of nociceptive information. This is reflected by a failure of both NGF^{-/-} and TrkA^{-/-} mice to respond to noxious stimuli (Smeyne et al., 1994; Crowley et al., 1994). Mice with a null mutation in the gene encoding NT-3 die within a few weeks of birth and suffer from abnormal limb movement, most likely due to a lack of muscle afferent projections to the spinal cord (Farinas et al., 1993; Ernfors et al., 1994). Disruption of the TrkC gene to remove the catalytic portion of the expressed receptor also produces mice with severe proprioceptive sensory neuron loss (Klein et al., 1994). These results highlight the essential role for NGF in the development of small nociceptive neurons and NT-3 in the development and maintenance of large proprioceptive neurons.

TrkA is the preferred receptor for NGF and TrkC the preferred receptor for NT-3, and both ligands additionally bind to the common neurotrophin receptor p75. Although NGF specifically binds to TrkA (Cordon-Cardo et al., 1991; Kaplan et al., 1991, 1991b; Klein et al., 1991a), NT-3 can also signal through other Trk receptors, binding to TrkA and TrkB, as well as its preferred receptor, TrkC (Lamballe et al., 1991; Squinto et al., 1991; Klein et al., 1991b; Soppet et al., 1991). Because of this, NT-3 may not only exert effects on gene expression in large TrkC expressing neurons, but also upon genes present in the small, peptidergic, TrkA-positive and low threshold mechanoreceptor, TrkB-positive sub-populations of trigeminal and dorsal root ganglia neurons. In addition, NT-3 may possibly regulate gene expression in TrkB responsive nodose neurons as well as the small sub-population of NGF responsive nodose neurons. Indeed, both mid-embryonic nodose and trigeminal neurons from TrkC kinase domain knockout mice survive *in-vitro* in the presence of NT-3, despite the lack of its preferred receptor (Davies et al., 1995). This TrkC knockout shows a decrease in 6-22% of trigeminal neurons (Pinon et al., 1996), which is significantly less than the 70% neuronal deficit in the NT-3 knockout (Wilkinson et al., 1996; Elshamy and Ernfors, 1996; Liebl et al., 1997), a clear indication that NT-3 activation of TrkA and TrkB is highly important throughout development. Indeed, few mid-embryonic trigeminal neurons survive with NT-3 when they lack functional TrkA expression. Likewise, loss of functional TrkB

prevents NT-3 from promoting the survival of a large proportion of mid-embryonic nodose neurons (Davies et al., 1995).

Interestingly, NT-3 has also been shown to regulate expression of TrkA, in the adult. Exogenous NT-3 down-regulates the expression of TrkA at the protein and mRNA level in nociceptive adult DRG neurons *in-vivo* (Gratto and Verge, 2003). Such regulation of TrkA receptors could have effects on gene expression and the nociceptive phenotype associated with TrkA expression in small nociceptive neurons (Gratto and Verge, 2003; Wilson-Gerwing et al., 2005). It is proposed therefore, that NT-3 may not only have effects on gene expression through activation of TrkC receptors present on large proprioceptive neurons. It may also have direct actions on non-preferred Trk receptors, and the subsequent alteration of mRNA levels in small peptidergic nociceptive sensory neurons and medium sized mechanoreceptive sensory neurons. Moreover, NT-3 may modulate NGF mediated gene expression through alterations in TrkA receptor availability.

The effects of both NT-3 and NGF on the expression of sensory neuron genes is therefore of interest. This will be addressed in this chapter. Real time QPCR was used to quantify changes in relative mRNA levels, both *in-vitro* using neuronal cultures and *in-vivo* using transgenic mice with null mutations in genes encoding neurotrophic factors and their receptors.

TrkA null mutant mice suffer a dramatic loss of sensory neurons, an attenuated response to pain and die shortly after birth (Smeyne et al., 1994). NT-3 null mutant mice display severe movement defects of the limbs due to the loss of proprioceptive sensory and sympathetic neurons and also die shortly after birth (Ernfors et al., 1994). To overcome this loss of neurons, mice heterozygous for a null mutation of the pro-apoptotic protein Bax were crossed with TrkA and NT-3 heterozygous mice to create TrkA^{-/-}/Bax^{-/-} and NT-3^{-/-}/Bax^{-/-} double knockout mice, respectively. Bax is a pro-apoptotic member of the bcl-2 family and is required for apoptotic cell death in many tissues. In these double knockouts, the large decrease in cell death normally seen in the single knockouts is prevented. In TrkA^{-/-}/Bax^{-/-} mice, the cells that survive

show a phenotype similar to those that are normally lost in the single knockout. In the DRG they have been shown to express TrkA and also the neuropeptides CGRP and SP suggesting that, *in-vivo*, a deficiency of Bax permits the survival of TrkA peptidergic neurons in the absence of NGF/TrkA signalling (Patel et al., 2000). Similarly, in NT-3^{-/-}/Bax^{-/-} mice, NT-3 dependent sensory neurons survive and express the proprioceptive neuronal marker, parvalbumin (Patel et al., 2003).

The NGF and NT-3 mediated regulation of all the mRNAs studied in Chapter 2 were included in this study (α - and β - CGRP, SP, Nav1.8, Nav1.9) alongside some additional primary sensory neuron expressed genes: Galanin, Pituitary Adenylate Cyclase-Activating Peptide (PACAP) and Vanilloid Receptor 1 (VR1). These genes are not exclusively expressed in primary sensory neurons, but are expressed significantly within these subpopulations. These additional genes will be discussed further here with regards to physiological relevance, expression patterns and regulation by neurotrophic factors.

3.1.1. Pituitary adenylate cyclase-activating peptide (PACAP)

PACAP is a neuropeptide belonging to the glucagon/secretin/vasoactive intestinal peptide (VIP) family, and shares 70% homology with VIP. Originally isolated from ovine hypothalamic tissues (Miyata et al., 1989), its name reflects its ability to stimulate production of adenylate cyclase (AC) in cultured rat anterior pituitary cells (Miyata A et al., 1998). A single gene product is alternatively spliced to give rise to two isoforms of the peptide. The predominant form is a 38 amino acid peptide, PACAP₁₋₃₈, but it also exists as PACAP₁₋₂₇, which consists of the 27 NH₂ terminal residues of PACAP₁₋₃₈ (Miyata et al., 1999).

Receptors

Ligand binding studies revealed two proposed binding sites for PACAP, one with a much higher affinity binding for PACAP than the other, which could bind both

PACAP and VIP equally (Shivers et al., 1991). This led to the subsequent discovery of two receptor types, PAC₁ and VPAC (reviewed in Harmar et al., 1995). PAC₁ was discovered by 6 different laboratories (Hashimoto et al., 1993; Hoysoya et al., 1993; Morrow et al., 1993; Pisegna and Wank, 1993; Spengler et al., 1993; Svoboda et al., 1993) and was found to have a high affinity for PACAP and a very low affinity for VIP. It is located predominantly in the CNS, most abundantly in the olfactory bulb, thalamus, hypothalamus, the dentate gyrus of the hippocampus; granule cells of the cerebellum and in the adrenal medulla (Spengler et al., 1993; Hashimoto et al., 1996). PAC₁ expression has not been found in adult DRG neurons, but it is expressed by dorsal horn second order sensory neurons (Jongsma et al., 2000; Vaudry et al., 2000). Six splice variants of the PAC₁ receptor have been isolated in the rodent. They differ with binding affinities and potency of second messenger stimulation. They can all act via G_s to activate AC and subsequently stimulate the production of cAMP (Spengler et al., 1993). Phospholipase C (PLC) accumulation is also triggered to varying degrees via G_q, although this is dependent on the splice variant receptor activated and the ligand form of PACAP (38 or 27) (Spengler et al., 1993; for review see Sherwood et al., 2005). In addition to activating AC and PLC, PACAP also appears to modulate Ca²⁺ signalling pathways by signalling through PAC₁ (Spengler et al., 1993; Chatterjee et al., 1996).

VPAC has equal binding affinity for both PACAP and VIP (Ishihara et al., 1992; Lutz et al., 1993; Couvineau et al., 1994; Rawlings and Hezareh, 1996; Cai et al., 1997) and exists in two isoforms: VPAC₁ and VPAC₂ (reviewed in Harmar et al., 1998). VPAC₁ was isolated from a rat lung cDNA library (Ishihara et al., 1992) and VPAC₂ was cloned from the rat olfactory bulb (Lutz et al., 1993) and later published independently by Usdin et al. (Usdin et al., 1994). VPAC₁ and VPAC₂ have very different distribution patterns. VPAC₁ mRNA is widely distributed in the CNS, most abundantly in cerebral cortex and hippocampus (Ishihara et al., 1992; Usdin et al., 1994) and in peripheral tissues including liver, lung and intestine (Ishihara et al., 1992; Usdin et al., 1994). VPAC₂ mRNA is abundant in the thalamus and superchiasmatic nucleus (SCN) and at lower levels in the hippocampus, brain stem, spinal cord and DRG (Usdin et al., 1994; Sheward et al., 1995). This receptor

subtype is also found in many peripheral tissues including pancreas, skeletal muscle, heart, kidney, adipose tissue, testis and stomach (Adamou et al., 1995; Krempels et al., 1995; Usdin et al., 1994).

VPAC₁ couples with G_s to stimulate an increase in cAMP via AC. Originally VPAC₁ activation was not thought to stimulate the inositol phosphate/phospholipase C (IP/PLC) system (Ishihara et al., 1992; Couvineau et al., 1994), however following transfection into hamster Chinese hamster ovarian (CHO) cells, VPAC₁-receptor couples with G_{i/o} to stimulate inositol phosphate (IP) production. IP production can then regulate intracellular calcium, affecting a number of pathways and signalling proteins. The VPAC₂ receptor also stimulates the production of cAMP via activation of AC (Lutz et al., 1993; Usdin et al., 1994). Effects of VPAC₂ activation on calcium regulation are suggested by the PACAP-induced increase in inositol trisphosphate (IP₃) production in rat strial cells (Cai et al., 1997). Furthermore, effects on calcium-activated chloride currents mediated by the VPAC₂ receptor have been observed in *Xenopus* oocytes (Inagaki et al., 1994).

PACAP expression

In the CNS, PACAP and PACAP mRNA are most abundantly expressed in the hypothalamus, with lower levels in other brain tissues (Ghatei et al., 1993). They are also present in peripheral tissues including: gastro-intestinal (GI) tract, adrenal gland and testis (Ghatei et al., 1993; reviewed in Arimura and Shioda, 1995). The expression patterns of PACAP suggest involvement in various bodily functions. PACAP expression within adult sensory neurons is primarily localised to small and medium sized neurons. The distribution pattern of PACAP is similar to that of CGRP and SP, but less widespread, PACAP being expressed by 10% of neurons and CGRP by 46% of neurons within adult DRG (Moller et al., 1993; Mulder et al., 1994). Developmentally, PACAP expression is detectable within sensory neurons at early embryonic stages. Both the peptide and PAC1 receptor mRNAs can be detected in E11.5 mice in abundance in both DRG and trigeminal using *in-situ* hybridisation (Sheward WJ et al., 1998). Levels of PACAP mRNA and protein decrease with age, with less PACAP immunoreactive neurons being present in the adult mouse and fish

(Shuto et al., 1996; Waschek et al., 1998; Jiang et al., 2003), indicative of an important role for PACAP in sensory neuron development.

Physiological functions of PACAP

PACAP has been shown to have a wide array of effects within the body. Roles in cell cycle and development, the cardiovascular system, the immune system, bone metabolism and as a mediator of endocrine, paracrine and exocrine secretions have all been suggested. I will briefly discuss some of these effects here.

Evolutionary conservation of PACAP combined with high level of expression in the embryo (Sheward et al., 1998) suggests a strong role for PACAP in embryonic development. Reglodi et al., found that injection of PACAP into young rat pups accelerated development with regards to facial features and sensory and motor neurological signs (Reglodi et al., 2003). Anti-PACAP treatment had the opposite effect, retarding ear folding, eye opening and hindlimb placing.

Sixty-percent of mice, with a null mutation in the VPAC₁ gene, die within the first four weeks of birth (Jamen et al., 2000). Surviving VPAC₁^{-/-} mice have an impaired insulintropic response to glucose, showing reduction in glucose-stimulated insulin secretion both *in-vitro* and *in-vivo*. This suggests that PACAP, acting via VPAC₁, has an important role in the normal insulin secretory response to glucose. Such a role has been suggested previously by findings that PACAP can potentiate glucose stimulated insulin secretion both *in-vitro*, in isolated perfused rat pancreas (Kawai et al., 1992; Bertrand et al., 1996) and in rat and mouse islets (Yada et al., 1994; Filipsson et al., 1999), and *in-vivo* in the mouse (Filipsson et al., 1998). Both isoforms of PACAP (PACAP₃₈ and PACAP₂₇) can stimulate insulin secretion with similar efficacies in mouse and rat islets (Yada et al., 1994; Filipsson et al., 1998).

As well as insulin secretion, PACAP has also been shown to have effects on catecholamine secretion, potentiating the release of noradrenaline and adrenaline from chromaffin cells of the rat adrenal medulla (Chowdhury et al., 1994; Guo et al.,

1994; Watanabe et al., 1994). Both PACAP₂₇ and PACAP₃₈ also have a mitogenic effect on such cells, stimulating the proliferation of cultured adult chromaffin cells via a PKA mediated signalling pathway (Tischler et al., 1995). Conversely mitogenic concentrations of PACAP inhibit mitogenic effects of NGF or FGF (unpublished observation in Frodin et al., 1994) indicating both mitogenic and anti-mitogenic roles for PACAP in adult rat chromaffin cell cultures (Tischler et al., 1995).

Within the cardiac system, intravenous or intra-arterial injection of PACAP into rats has been shown to cause a decrease in blood pressure (Nandha et al., 1991; Minkes et al., 1992). Such effects are mediated by the vasorelaxant activity of PACAP on arterial segments (Warren et al., 1991; Huang et al., 1993; Cardell et al., 1997), mediated via AC/cAMP signalling (Absood et al., 1992; Warren et al., 1991).

A role for PACAP as a daytime regulator of the biological clock has also been suggested. The suprachiasmatic nucleus (SCN), located in the hypothalamus acts as an endogenous pacemaker generating circadian rhythms. It regulates the production of melatonin from the pineal gland, in response to environmental cues such as light/dark cycles. PACAP immunoreactivity has been found in axons from the retinal ganglion cells, which transmit information on light/dark cues to the SCN (Hannibal et al., 1997). Levels of PACAP are low during the day, and high at night, suggestive of circadian rhythm signalling. Within the SCN, PACAP has been shown to regulate phosphorylation of CREB, which mediates melatonin synthesis (Kopp et al., 1997). This increase in melatonin is then thought to negatively feedback and inhibits the PACAP induced phosphorylation of CREB (Kopp et al., 1997). Evidence suggests that these effects of PACAP are mediated via the VPAC₁ receptor subtype. Agonists of this receptor potently stimulate melatonin production and likewise, antagonists prevent the action of PACAP (Simonneaux et al., 1998).

A role for PACAP in the immune system has been suggested by its widespread location in central and peripheral lymphoid tissues (Gaytan et al., 1994; Abad et al., 2002). PACAP₃₈ and PACAP₂₇ can protect CD4⁺ CD8⁺ thymus lymphocytes from glucocorticoid-induced apoptosis in the rat (Delgado et al., 1996). Suggesting a role

for PACAP in T-cell maturation. Furthermore, an indirect role for PACAP in lymphocyte maturation is suggested through the stimulation of interleukin-6 (IL-6) release from folliculostellate cells in the pituitary gland (Tatsuno et al., 1991), via a PACAP mediated increase in intracellular Ca^{2+} levels (Yada et al., 1993). The subsequent release of IL-6 stimulates the growth and maturation of B cells (Tatsuno et al., 1991; Yada et al., 1993).

Within the CNS and PNS, PACAP has been shown to promote the *in vitro* survival of several populations of neurons. It can prevent NMDA-induced cell death in rat cortical cultures (Frenchilla et al., 2001) and it can also promote survival and neurite outgrowth in rat cerebellar neuroblasts (Cavallaro et al., 1996; Gonzalez et al., 1997; Kienlen Campard et al., 1997; Vaudry et al., 1998), PC12 cells (Hernandez et al., 1995; Tanaka et al., 1997; Lazarovici et al., 1999) and rat basal forebrain cholinergic neurons (Takei et al., 2000) by signalling through the cAMP/PKA pathway. The same survival and neurite outgrowth promoting effects were observed in cultured embryonic and neonatal DRG neurons, alongside an increased immunoreactivity for the neuropeptide CGRP (Lioudyno et al., 1998). Such effects of PACAP on neurite outgrowth and gene expression suggest a role for PACAP in modulating sensory neuron differentiation as well as survival. Nielsen et al., (Nielsen et al., 2004) provided further evidence for this. They found that exogenous PACAP could enhance neuronal differentiation of cultured embryonic chick DRG. Furthermore, the addition of PAC1 receptor antagonists' reduced neuronal differentiation in cultured DRG, suggesting that endogenously produced PACAP or a similar peptide acts via VPAC1 to produce neuronal differentiation. Of further interest was the discovery that both NT-3 and CNTF can block this effect. It has also been suggested that the survival promoting effects of PACAP are mediated through cross-talk with Trk neurotrophin receptors (Lee et al., 2002; Rajagopal et al., 2004). Such regulatory effects of neurotrophic factors on PACAP will be discussed later.

Further to its neurotrophic effects on sensory neuron survival, a role for PACAP in nerve regeneration has been suggested following facial nerve injury. PACAP treatment was shown to restore the latency of compound muscle action potentials

after facial nerve transection in guinea pigs (Kimura et al., 2003; Kimura et al., 2004). Numerous pieces of experimental data suggest that PACAP plays a role in nociception. For example, inflammation, that is associated with thermal and tactile hyperalgesia, causes an NGF-mediated increase in PACAP mRNA expression in TrkA expressing nociceptive neurons that can be blocked by administration of an anti-NGF antibody (Zhang et al., 1998; Jongsma-Wallin et al., 2003). Following both sciatic nerve transection and sciatic nerve compression (procedures that also lead to tactile and thermal hyperalgesia), a large up-regulation of PACAP mRNA and peptide is observed in DRG neurons. In the case of nerve transection this up-regulation predominantly occurs in medium and large neurons, which normally display negligible PACAP expression, and it is partially antagonised by exogenous NT-3 (Jongsma-Wallin et al., 2001). Nerve compression leads to an increase in PACAP expression in both large and small DRG neurons (Pettersson et al., 2004). This data may suggest a role for PACAP in the generation of inflammatory and neuropathic pain.

PACAP's role in nociception is somewhat conflicting, with PACAP both inhibiting (Zhang et al., 1993; Zhang et al., 1996; Yamamoto and Tatsuno, 1995), and potentiating pain (Narita et al., 1996; Xu and Wiesenfeld-Hallin, 1996; Jongsma et al., 2001, Mabuchi et al., 2004). For example, PACAP administered intrathecally could produce anti-nociceptive effects following formalin-induced pain in adult rats (Yamamoto and Tatsuno, 1995). Conversely, VPAC1^{-/-} mice have a 75% decrease in nociceptive response; and Mabuchi et al., showed that PACAP^{-/-} mice do not exhibit inflammatory pain induced by carrageenan injection, or neuropathic pain following nerve transection.

Neurotrophic factor regulation of PACAP and PACAP receptor/Trk cross-talk

In adult rat DRG, different neurotrophic factors have been shown to have differing effects on PACAP expression in inflammation and also following nerve injury, in both injured and intact neurons. Expression of PACAP mRNA is up-regulated in small-medium neurons in response to inflammation (Zhang et al., 1998; Jongsma-

Wallin et al., 2003. The inflammation associated increase in NGF has been implicated in producing the observed alterations in neuropeptide levels (Reviewed in McMahon et al., 1996). Systemic administration of anti-NGF antibodies prevented the inflammation associated increase in PACAP mRNA (Jongsma-Wallin et al., 2003).

Neurotrophic factor regulation of PACAP expression has also been observed in models of nerve damage and axotomy. In intact sensory neurons of the DRG, PACAP protein and mRNA is predominantly expressed by small/medium-sized neurons that also express TrkA. Following sciatic nerve transection, however, there is a large increase in the expression of PACAP mRNA in medium to large, predominantly TrkC expressing neurons (Zhang et al., 1995; Zhang et al., 1996; Jongsma Wallin et al., 2001). Infusion of NT-3 resulted in a decrease in the injury-induced increase in PACAP protein and mRNA expression in TrkC-positive neurons. In non-lesioned animals, NT-3 also decreased PACAP expression selectively in TrkA-expressing nociceptive neurons (Jongsma Wallin et al., 2001), an effect possibly mediated by NT-3 induced down-regulation of high affinity NGF receptors (Gratto and Verge, 1997). Whilst NGF increased the expression of PACAP in small, TrkA-expressing, nociceptive neurons in both lesioned and intact animals, it also selectively reduced the expression of PACAP in large proprioceptive neurons after nerve transection, but not in intact animals (Jongsma Wallin et al., 2001). How endogenously produced neurotrophic factors affect expression of this neuropeptide, is of interest with regards to the ability of PACAP peptide to provide its own trophic effects, possibly indirectly via effects on Trk receptors.

PACAP has been shown to have effects on differentiation in late embryonic and neonatal rat DRG neurons, alongside survival effects similar to that of NGF (Lioudyno et al., 1998). Recent work suggests that such effects could be mediated through GPCR cross-talk with the neurotrophin Trk receptors (Lee et al., 2002; Rajagopal et al., 2004). PACAP treatment has been shown to cause an increase in TrkA tyrosine kinase activity in PC12 cells and TrkB activity in rat hippocampal cells (Lazarovici et al., 1999; Lee et al., 2002; Rajagopal et al., 2004). Furthermore, this VPAC₁ mediated Trk activation led to an up-regulation of phosphorylated Akt

and subsequent cell survival. Blockade of Trk receptors with K252a prevented PACAP-induced phosphorylation of Akt and neuronal survival confirming a Trk mediated pathway of cell survival (Lee et al., 2002; Rajagopal et al., 2004). More recently, however, Nielsen et al. (2004) found no such effect of Trk receptor blockade on PACAP induced neuronal differentiation in chick DRG, rather they demonstrated that differentiation was effected via a VPAC1/MAPK pathway. It therefore seems that the many actions of PACAP are mediated through different pathways, which may interact with those of the neurotrophins.

The ability of PACAP to regulate the expression of neurotrophins is of further interest. In rat cortical cultures, presence of PACAP prevented cell death induced by NMDA and also attenuated serum-induced apoptosis (Frenchilla et al., 2001). BDNF protein expression was reduced by NMDA in both cellular injury conditions, but presence of PACAP prevented this decrease in BDNF and prevented cell death. Furthermore pre-incubation with anti-BDNF prevented the neuroprotective effects of PACAP₃₈, suggesting PACAP-induced survival is mediated by preventing the suppressed expression of a neurotrophin essential for cortical neurotrophin survival. The ability of PACAP to regulate expression of neurotrophic factors, alongside the receptor cross-talk that occurs between its GPCRs and the Trk receptors adds complexity to the regulation of PACAP expression by neurotrophic factors investigated in this chapter.

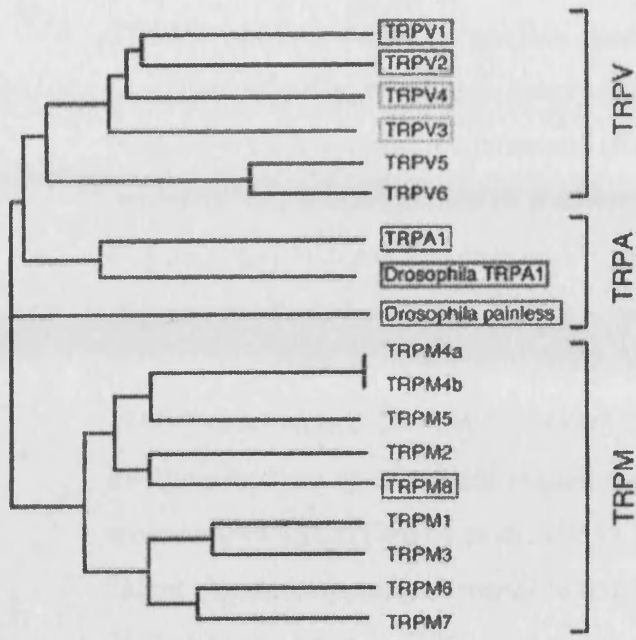
3.1.2. Vanilloid receptor 1 (VR1)

Capsaicin, a component of chilli peppers, has been widely studied for its effects upon primary afferent neurons. It depolarises subsets of primary sensory neurons (Oh et al., 1996) to evoke pain. The associated release of the neuropeptides SP and CGRP contribute to inflammation and subsequent hyperalgesia (Malmberg and Yash, 1992; Hingtgen et al., 1995; Traub et al., 1996; Kilo et al., 1997). Conversely, capsaicin has been shown to act as an anti-inflammatory and anti-nociceptive agent for the treatment of painful disorders (e.g. bladder hyperreflexia), through nociceptor desensitisation (Maggi et al., 1987; reviewed in Szallasi and Blumberg, 1996). Studies found that capsaicin, alongside other molecules such as resiniferatoxin

(RTX), produce their effects by activating a sensory neuron specific calcium channel (Wood et al., 1988).

The cloned VR1 receptor, also known as TRPV1, encodes an 838 amino acid protein with a molecular weight of 92,000 Da that is a non-selective cation channel with high calcium permeability (Caterina et al., 1997; Szallasi and Blumberg, 1999). In addition to capsaicin, numerous other molecules can activate VR1. These include the vanilloids, zingerone and piperine, and non-vanilloids like the endocannabinoid, anandamide (Hwang et al., 2000; Reviewed in Sterner et al., 1999). Thermal stimuli in the noxious range, can also directly open the channel, further suggestive of a role for VR1 as a transducer of painful stimuli (Caterina et al., 1997; Helliwell et al., 1998). Protons have been shown to act as modulators of VR1 gating, with low concentrations (moderate acidification) facilitating channel opening and lowering heat thresholds (Tominaga et al., 1998) and high concentrations blocking VR1 activation (Baumann et al., 2000). VR1 appears also to be a sensor of noxious hypertonicity and can be directly gated by divalent and monovalent cations (Ahern et al., 2005).

Following the discovery of VR1, three more homologous receptors (VRL-1 (TRPV2) VRL-2 (TRPV4) and VRL-3 (TRPV3)) have been isolated and, together with VR1, these compose the transient receptor potential vanilloid (TRPV) family, a subgroup of the transient receptor potential (TRP) family of ion channels. See figure 3.1. for details of phylogenetic relationship among mammalian TRP channels and details of TRPV channel nomenclature.



Vanilloid receptor family nomenclature	TRPV nomenclature	Alternative nomenclature	Full cDNA available (for vertebrates)	Predicted protein size (human)	Human chromosomal localization
VR1	TRPV1	OTRPC1	Human, mouse, rat, chicken	95 kDa	17p13
VRL-1	TRPV2	GRC, OTRPC2	Human, mouse, rat	86 kDa	17p11
VRL-2	TRPV4	OTRPC4, VR-OAC, TRP12	Human, mouse, rat, chicken	98 kDa	12q24.1
VRL-3	TRPV3?	-	-	-	17p13
ECAC1	TRPV5	CaT2, ECaC, OTRPC3	Human, rat, rabbit	83 kDa	7q35
ECAC2	TRPV6	CaT, CaT1, CaT-like	Human, mouse, rat	83 kDa	7q35

Figure 3.1. Phylogenetic relationship among the mammalian TRP channels with further detail on the nomenclature of the TRPV channels. Taken from Tominaga and Caterina, 2004 and Gunthorpe et al., 2002.

VRL-1 was identified through a search of genomic databases for TRPV1 homologues (Caterina et al., 1999; Reviewed in Gunthorpe et al., 2002, Benham et al., 2003 and Tominaga and Caterina, 2004). VRL-1 is not activated by ligands of TRPV1, but is activated by noxious heat ($>53^{\circ}\text{C}$). It has been suggested that VRL-1 mediates responses to extreme noxious temperatures, whilst VR-1 responds to medium – high noxious temperatures (Xu et al., 2002). VRL-1 is predominantly expressed in a subpopulation of medium sized thinly myelinated sensory neurons (A δ neurons), but it is also present at low levels in the CNS and non-neuronal tissues, suggestive of another role and other possible ligands.

VRL-2 (also named TRPV4, VR-OAC, OTRPC4 and TRP12) was discovered using in-silico analysis of expressed sequence tag (EST) databases and conventional molecular cloning (Delany et al., 2001). It appears to be an osmotically regulated cation channel, opening in response to hypotonic swelling of the cell (Liedtke et al., 2000; Strotmann et al., 2000; Delany et al., 2001). Like VR1 and VRL-1, VRL-2 is expressed in sensory neurons and their peripheral fibres (Liedtke et al., 2000; Guler et al., 2002; Alessandri-Haber et al., 2003). VRL-2 is also expressed in sympathetic and parasympathetic nerves, on airway and kidney epithelia, sweat glands, intestine and blood vessels (Delany et al., 2001). Additionally, VRL2 expression has been observed in keratinocytes where it appears to mediate warmth evoked currents in conjunction with VRL-3 (Chung et al., 2004; Tominaga and Caterina, 2004).

The virtual completion of the human genome project allowed identification of VRL-3 (TRPV3) through a search of genomic sequences (Smith et al., 2002; Xu et al., 2002, reviewed in Benham et al., 2003). In humans it is expressed mainly in CNS and sensory neurons (Smith et al., 2002; Xu et al., 2002), but also in skin and keratinocytes (Peier et al., 2002). Like VR1 and VRL-1, VRL-3 is sensitive to heat, although the reported temperature of activation varies from 23°C to 39°C (Smith et al., 2002; Xu et al., 2002; Peier et al., 2002). This discrepancy between the activation temperatures determined by different groups is thought due to the fact that the

channel becomes sensitised to heat, opening at lower temperatures, once it has been heat-gated for the first time. The result of this is that the recorded temperature of activation in any one experimental manipulation is dependent on the “heat history” of the VR-3 expressing cells under investigation (Benham et al., 2003). The recent production of TRPV3^{-/-} mice has confirmed a role for this receptor in heat sensation. Mice with a mutation in this receptor have strong deficits in responses to innocuous and noxious heat, but not in other sensory modalities (Moqrich et al., 2005). Recently non-thermal stimuli have also been determined for this receptor. 2-Aminoethoxydiphenyl (2-APB) was shown to activate the receptor in HEK cells (Chung et al., 2004). The natural compound camphor, known for its heat sensation-producing effects in humans, was also shown to produce effects in cultured mouse primary keratinocytes, an effect that was abolished in TRPV3^{-/-} mice (Moqrich et al., 2005). Other more distant cousins of VR1 include two epithelial Ca²⁺ channels, TRPV5 (ECAC1) and TRPV6 (ECAC2). I will not discuss these further here, but for a review see Gunthorpe et al., 2002.

Expression profile of VR1

VR1 is located predominantly in primary sensory neurons, but is also found in various brain nuclei and spinal cord (Mezey et al., 2000). Non-neuronal cells have also been shown to express VR1 (TRPV1) including: pancreatic B cells (Akiba et al., 2004), liver epithelial cells (Reilly et al., 2003), astrocytes from the rat spinal cord (Doly et al., 2004), endothelial cells (Yamaji et al., 2003), polymorphonuclear granulocytes (Heiner et al., 2003), macrophages (Chen et al., 2003), oral epithelial cells (Kido et al., 2003) and thymocytes (Amantini et al., 2004).

In DRG, VR1 is expressed by small-medium sized neurons (Caterina et al., 1997) that have either non-myelinated (C-fibre) or thinly myelinated (A δ -fibre) axonal processes (Michael and Priestly, 1999; Guo et al., 1999; Tominaga et al., 1998). In rat DRG, VR1 is expressed by both TrkA- positive, peptidergic nociceptive neurons, and GDNF responsive *Griffonia simplicifolia* isolectin B4 (IB4) positive c-fibre nociceptors (Michael and Priestly 1999; Guo et al., 1999). In contrast, in mouse DRG,

only a small (2-3%) population of neurons are both IB4 and VR1 positive (Zwick et al., 2002), the majority of functional VR1 receptors being expressed by the peptidergic TrkA expressing neurons. It is also worth noting that approximately 10% of VR1 positive rat lumbar DRG neurons are SP and IB4 negative (Tominga et al., 1998). A heterogeneous population of cells therefore express VR1 within the DRG. In nodose ganglia, expression of VR1 mRNA is co-localised with that of TrkB rather than TrkA (Michael and Priestly, 1999).

Roles of VR1

The apparent pro-nociceptive and anti-nociceptive effects of capsaicin mentioned above are thought to be mediated via VR1. Further evidence for such apparent contradictory roles of VR1 was provided by the production of a VR1 null mutant mouse (Caterina et al., 2000; Bolcskei et al., 2005). Mice lacking the capsaicin receptor exhibited impaired detection of painful heat and, unlike wild type mice, failed to develop thermal hyperalgesia after mustard oil, CFA or acid application to the hindpaw or a mild burn injury, showing that VR1 is necessary for normal thermal nociception and plays a role in the generation of thermal hyperalgesia in response to noxious chemical and heat insults. (Caterina et al., 2000; Bolcskei et al., 2005). In contrast, VR1 knockout mice were identical to wild type mice developed mechanical hyperalgesia in response to formalin and carrageenan treatment and partial sciatic nerve lesion in an identical manner to wild type mice. VR1 is therefore not apparently necessary for normal mechanical nociception or the development of mechanical hyperalgesia. The data from VR1 knockout mice have been confirmed by experiments that specifically blocked the peripheral activation of VR1 in sensory fibres of the sciatic nerve by intra-plantar injection of the potent VR1 antagonist iodo-resiniferatoxin (IRTX). Low dose IRTX blocked the increase in excitatory electrical responses evoked by noxious heat in wide dynamic range (WDR) c-fibre nociceptive neurons that typically occurs after carrageenan application and spinal nerve ligation (SNL). In contrast, IRTX failed to inhibit the increased response of WDR neurons to mechanical stimuli that occur following carrageenan treatment and SNL. In addition, higher doses of IRTX reduced the heat response but not the

mechanical response of naive WDR neurons that were not subjected to inflammatory insult or SNL (Jhaveri et al., 2005). Interestingly, anti-nociceptive effects of VR1 on mechanical thresholds have been suggested by examination of VR1 null mice subjected to agents that induce polyneuropathy. Chronic mechanical hyperalgesia evoked by streptozotocin-induced diabetes and cisplatin-evoked toxic polyneuropathy occurred earlier and was of greater magnitude in VR1 knockout mice compared to wild-type mice (Bolcskei et al., 2005).

Post-translational modification of VR1 in response to inflammation and chemical insult has been postulated to regulate onset of hyperalgesia and enhance VR1 activity. In the absence of any other agonist, PKC activation has been shown to phosphorylate VR1, inducing its activity and enhancing the response of the receptor to capsaicin (Crandall et al., 2002; Premkumar et al., 2000). cAMP can similarly phosphorylate VR1, leading to an enhanced response to capsaicin through reduced desensitisation of the receptor (Bhave et al., 2002). This has also been illustrated in a mouse model of diabetic neuropathy (Hong and Wiley, 2005). Hong and Wiley (Hong and Wiley, 2005) show that painful diabetic neuropathy is associated with not only alterations in VR1 levels, but also by PKC-mediated phosphorylation, which blunts VR1 desensitisation, allowing enhanced flow of receptor currents. NGF rapidly potentiates TRPV1 channel activity in cultured DRG neurons treated with capsaicin (Shu and Mendell, 1999; Bonnington and McNaughton, 2003). Studies using pharmacological inhibitors in these neurons suggest that the PI3K pathway is crucial step in mediating NGF sensitization, with both calcium/calmodulin-dependent kinase II (CaMK II) and PKC involved downstream of PI3K (Bonnington and McNaughton, 2003; Zhuang et al., 2004). PKC ϵ sensitizes TRPV1 by directly phosphorylating this channel, leading to increased channel activity (Cesare et al., 1999; Premkumar and Ahern, 2000; Numazaki et al., 2002) and translocation of the channel to the cell surface (Morenilla-Palao et al., 2004). Consistent with these findings, NGF-induced hyperalgesia is inhibited by a PKC ϵ -selective peptide inhibitor (Khasar et al., 1999).

Aside from an obvious role in peripheral nociception, VR1 also appears to play a role in visceral nociception. A role for VR1 in the gastrointestinal (GI) tract is suggested by the demonstration of VR1-like immunoreactivity on sensory fibres that enter myenteric ganglia and surround enteric neurons and fibres that run in the interganglionic fibre tracts throughout the GI tract. These sensory fibres are largely CGRP positive and originate from thoracic and lumbar DRG, although a small number appear to originate from the nodose ganglion (Ward et al., 2003).

Interestingly, the expression of VR1 is up-regulated in colonic nerve fibres of patients with active inflammatory bowel disease (IBD) compared to control subjects, suggesting a role for VR1 in the aetiology of this condition (Yiangou et al., 2001; Facer et al., 2001). More recently, it has been demonstrated that activating VR1 in nerve fibres innervating the mucosa, blood vessels and smooth muscles of the stomach by the intragastric administration of specific VR1 agonists in rats reduces the severity of gastric lesions due to the intragastric administration of 0.6 N HCl in a dose dependent manner. Moreover, the protective effect of VR1 activation is blocked by specific VR1 antagonists, again in a dose dependent manner (Horie et al., 2004).

Recently, evidence has emerged suggesting that VR1 also plays a role in regulating urine production. Activation of VR1 in rat sensory afferents innervating the kidney by perfusion of capsaicin into the pelvis leads to increases in urine flow rate and urinary sodium excretion (Zhu et al., 2005). VR1 is expressed in bladder neuroepithelial cells and sensory fibres innervating the bladder. Up-regulation of VR1 in both these cell types is associated with neurogenic detrusor overactivity (NDO) and treatment with low doses of the VR1 agonist resiniferatoxin ameliorates the symptoms of this condition, possibly by causing a sustained slow depolarization of VR1 positive afferents (in the absence of action potentials) that prevents action potential generation by other physical and chemical VR1 agonists (Apostolidis et al., 2005; Raisinghani et al., 2005). Recently it has been demonstrated TRPV1 is functionally expressed in rat islet β cells where it may play a role in insulin secretion as a calcium channel. This finding may account for the effect of capsaicin on food intake and energy consumption as well as on the pathophysiological regulation of pancreatic endocrine functions (Akiba et al., 2004)

VR1 also appears to play a role in regulating blood pressure and blood flow in the cardiovascular system. Anandamide has been shown to act via the VR1 receptor to produce vasodilation, most likely through the accompanied release of the vasodilator, CGRP (Zygmunt et al., 1999; Vaishnava et al., 2003). Additionally capsaicin, and also to a lesser extent anandamide, can produce a dose dependent decrease in heart-rate and blood pressure, an effect that can be reversed by the addition of the VR1-specific antagonist, capsazepine (Malinowska et al., 2001).

Regulation of VR1 expression by neurotrophic factors following nerve damage, axotomy and inflammation

Local inflammation, produced by application of the local irritant, Freuds Complete Adjuvant (CFA) causes a p38 MAP kinase dependent increase in VR1 protein expression in adult rat DRG neurons (Amaya et al., 2003; Ji et al., 2002). This increase in VR1 expression appears to play a role in the generation of thermal hyperalgesia, as mice lacking VR1 no longer show hypersensitivity to heat following inflammation (Caterina et al, 2000). Studies using anti-sera against NGF and GDNF in the adult rat have shown that both neurotrophic factors have a role in the onset of such thermal hyperalgesia following inflammation through up-regulation of VR1 (TRPV1) expression. CFA induces an up-regulation of NGF and GDNF protein in adult rat DRG, and anti-serum to both trophic factors prevents the up-regulation of VR1 in TrkA expressing and IB4 positive nociceptors, respectively, and also ameliorates the thermal hyperalgesia that develops in response to CFA (Amaya et al., 2004; Ji et al., 2002; Woolf et al., 1994). Up-regulation of VR1 by NGF and GDNF is not likely to be the only way these trophic factors contribute to the generation of thermal hyperalgesia in adult rodents. For example, NGF has been shown to regulate the expression of a number of other genes in DRG neurons, to increase the release of neuropeptides (possibly via increased VR1 activation) and lead to the release of a number of inflammatory mediators from mast-cells (reviewed in Hefti et al., 2006).

VR1 mRNA and protein are down-regulated in injured adult rat DRG neurons following sciatic nerve or spinal nerve transection (Michael and Priestley, 1999; Michael and Priestley, 2002; Fukuoka T et al., 2002; Wendland et al., 2003). This effect is proposed to be due to the limited availability of endogenous neurotrophic factor support in such conditions. Much evidence supports this hypothesis. Administration of NGF and GDNF can prevent the axotomy-induced down-regulation of VR1 mRNA observed in cultured adult rat DRG (Winston et al., 2001; Ogun-Muyiwa et al., 1999 Wendland et al., 2003). Furthermore, *in-vivo* sequestration of NGF using neutralising anti-sera also reduces sensitivity of neurons to capsaicin through a loss of VR1 (McMahon et al., 1995). Following total or partial sciatic nerve transection (Hudson et al., 2001) or spinal nerve ligation (Hudson et al., 2001; Fukuoka et al., 2002) in rats, expression of VR1 in intact DRG neurons increased (Hudson et al., 2001; Fukuoka et al., 2002). This increase in undamaged nerves may be due to increased availability of growth factors from target tissues because of an overall reduction in the amounts of growth factors taken up from the tissue by retrograde transport.

Nodose ganglia

Previous studies on the expression of VR1 in nodose ganglia have revealed expression and regulation patterns that differ from those observed in the DRG. In nodose ganglia, the majority of VR1 positive cells also express TrkB, with very few being TrkA immuno-reactive (Michael and Priestly, 1999). This co-expression of VR1 and TrkB agrees with the finding that BDNF, but not NGF, can regulate the capsaicin sensitivity of cultured adult rat nodose neurons (Winter, 1998). It is worth noting however that the use of BDNF in these experiments was very high at 1µg/ml, suggesting that at physiological levels such effects may not be observed. This difference in the regulation of VR1 expression between nodose ganglia and DRG demonstrates that a particular determinant of nociceptive thresholds is not regulated in the same manner in all types of rat sensory neurons. A study of the regulation of VR1 expression in the trigeminal ganglion would therefore be of interest to

determine whether VR1 is regulated by NGF or BDNF in these cranial sensory neurons.

Summary

VR1 (TRPV1) functions in many systems within the body, including the cardiovascular system, GI tract and nervous system, although most work has focused on the role of VR1 in pain. Several neurotrophic factors have been implicated in the regulation of VR1 expression and the gating properties of this ion channel. From a therapeutic perspective, it is important to understand fully the regulation of VR1 expression as this is an established drug target in painful conditions.

To date there is little experimental data concerning the regulation of VR1 expression in neonatal sensory neurons. One of the objectives of the studies reported in this chapter was to explore how neurotrophic factors regulate the expression of VR1 in post-natal sensory neurons

3.1.3. Galanin

Background

The neuropeptide galanin was isolated from porcine intestine in 1983 by Tatemoto et al. (Tatemoto et al., 1983). It is a 29 amino acid peptide that is widely expressed in the nervous system and present in a small population of peptidergic, small-diameter adult DRG neurons (Chang et al., 1985; Skofitsch and Jacobowitz 1985). Galanin mRNA is expressed in the majority of embryonic sensory neurons, peaking at E15-E17 in DRG and trigeminal ganglia, but decreasing after birth so that only 2% of adult DRG neurons are galanin mRNA-positive (Xu et al., 1996; Ma et al., 1999). Such high levels of expression in embryonic sensory neurons suggest an important role for galanin in sensory neuron development (discussed below). Although galanin expression is predominantly in small/medium sized peptidergic neurons in the adult, some expression in large diameter neurons has been observed (Xu et al., 2000; Ma et

al., 1999). Whilst galanin expression levels are low in adult sensory ganglia, expression is dramatically increased in large diameter neurons following nerve injury or axotomy. This contrasts with many neuropeptides, for example, SP and CGRP, whose expression decreases following axotomy. Up-regulation of galanin following injury raises the possibility that this neuropeptide may play a role in promoting neuron survival and/or regeneration following nerve trauma and may also play a role in the modulation of nociceptive thresholds, since these can change following nerve trauma. These aspects of galanin function are discussed below.

Receptors

Galanin mediates its effects via three G protein coupled receptors; GAL-R1 (Habert-Ortoli et al., 1994), GAL-R2 (Smith et al., 1997) and GAL-R3 (Wang et al., 1997; Smith et al., 1998). There is a region of 83 amino acids conserved in all three rat galanin receptor subtypes, whilst outside this conserved region, homology is only approximately 23% (Branchek et al., 2000). GAL-R1 mRNA is found in approximately 50% of rat DRG neurons, predominantly in those that are of medium and large size (O'Donnell et al., 1999; Xu et al., 1996). In contrast, GAL-R2 mRNA is expressed in approximately 80% of rat and mouse DRG neurons, with 60% of these being small CGRP-positive neurons (O'Donnell et al., 1999; Shi et al., 1997; Liu and Hokfelt, 2002). Only low levels of GAL-R3 can be detected in DRG neurons (Waters and Kraus 2000; reviewed in Branchek et al., 2000).

The expression patterns and the downstream signal transduction pathways recruited following receptor activation differ for the three galanin receptors (reviewed in Bartfai et al., 1993 and Branchek et al., 2000). GAL-R1 and GAL-R3 mediate their effects through $G_{i/o}$ type proteins, leading to an inhibition of adenylate cyclase (Habert-Orli et al., 1994; Smith et al., 1998; Wang et al., 1997). Conversely GAL-R2 acts via G_q to activate phospholipase C that in turn causes a mobilisation of calcium and activation of protein kinase C (PKC) (Fathi et al., 1998; Howard et al., 1997; Waters et al., 2000). Signalling via GAL-R2 can also activate MAP kinase pathways (Wang et al., 1998).

Physiological function

Because of its widespread distribution and interactions with multiple signalling pathways, a role for galanin in a wide variety of physiological processes is to be expected. The effects of galanin are commonly mediated through modulation of hormone and neurotransmitter secretion. For example, galanin inhibits the release of noradrenaline, serotonin, acetylcholine and glutamate (Seutin et al., 1989; Pieribone et al., 1995; reviewed in Bartfai et al., 1993). These molecules have a variety of actions within the body, hence the multiple effects of galanin throughout the nervous system. A role for galanin in a number of important bodily functions has been implicated. Effects on; cognition (Chan-Palay, 1988; Mastropaolo et al., 1988; Ogren et al., 1996; Robinson and Crawley, 1993; Malin et al., 1992; Reviewed in Wrenn and Crawley, 2001; Wrenn et al., 2004; Rustay et al., 2005), stress and anxiety (Ceresini et al., 1998; Reviewed in Branchek et al., 2000; Khoshbouei et al., 2002; Holmes et al., 2003), endocrine modulation (Bartfai et al., 1993; Carey et al., 1993; Pieribone et al., 1995), nociception (Reviewed in Xu et al., 2000; Reviewed in Liu and Hokfelt, 2002, Hygge-Blakeman et al., 2004; Wiesenfeld-Hallin et al., 2005), feeding (Crawley et al., 1990; Dube et al., 1994; Crawley, 1999) and in nerve regeneration following injury (Wynick et al., 2001; Holmes et al., 2005) have all been reported.

Co-localisation of galanin with acetylcholine (ACh) and choline acetyltransferase (ChAT) in medial septal neurons projecting to the rodent hippocampus, suggest a role for galanin in the modulation of cholinergic transmission and thus cognition (Melander et al., 1995, 1996). Indeed, negative effects of galanin over-expression on learning and memory were demonstrated by several studies in the rat in which central injection of galanin produced performance deficits in working memory, fear conditioning and spatial tasks (Mastropaolo et al., 1988; Malin et al., 1992; Robinson and Crawley, 1993; Ogren et al., 1996). These findings, combined with the discovery that galanin is over-expressed in the basal forebrain during the onset of Alzheimer's

disease (AD) (Chan-Palay, 1988; Beal et al., 1990), has led to the conclusion that galanin may be the cause of the cognitive dysfunction characteristic of AD (Chan-Palay, 1988; Hokfelt et al., 1997; Wrenn and Crawley, 2001). The production of mice that over-express galanin has further supported this hypothesis. Although galanin over-expressing mice, display normal general health, they are impaired in cognitive tasks including the Morris water-maze probe trial and trace fear conditioning (Steiner et al., 2000; Kinney et al., 2002). They also displayed neurochemical deficits characteristic of AD, with reduced levels of ChAT-containing cells in the horizontal diagonal band (HDB) (Steiner et al., 2000). The signalling pathways galanin utilises to produce these detrimental effects are unclear. Of note, however, is that mice with a null-mutation in the GAL-R1 gene were found to behave normally on most memory tasks, implicating other receptor subtypes in such spatial learning and memory (Wrenn et al., 2004; Rustay et al., 2005).

Although GAL-R1 null mice show no deficits in memory-related tasks, they do display increased anxiety-like behaviour, suggesting a role for GAL-R1 and hence galanin in stress and anxiety (Holmes et al., 2003). This hypothesis is reinforced by the expression of galanin in neural systems that utilise noradrenaline and serotonin as neurotransmitters. Further studies have noted increased levels of prepro-galanin mRNA in the locus coeruleus (LC) in situations of high stress that produce high levels of noradrenaline release, e.g. repeated exercise (O'Neal et al., 2001) and chronic social stress (Holmes et al., 1995). However, low stress tasks such as swimming (Austin et al., 1990) or wheel running (Soares et al., 1999) show no such increase in prepro-galanin mRNA. These results suggest galanin gene expression is modulated in response to repeated stress, but not to low stress tasks. Its up-regulation in the LC in situations of high stress, and the increase in anxiety in GAL-R1 knockout mice suggest galanin may attenuate noradrenergic neuron firing here to produce anxiolytic effects. Indeed an inhibitory effect of galanin on the firing rate of LC neurons has previously been observed (Seutin et al., 1989; Pieribone et al., 1995). Such a role for galanin in modulating stress responses is further supported by studies in the galanin over-expressing (OE) mouse. Such mice were normal with regards to general health, however showed no anxiety-like phenotype to three separate anxiety

tests in comparison to wild-type mice (Holmes et al., 2002). Furthermore, stimulation of noradrenergic cells with the alpha 2 adrenoreceptor antagonist, yohimbine produced pro-anxiety effects in wild-type mice in the light-dark exploration test, but no such effects were observed in galanin over expressing mice (Holmes et al., 2002). Taken together, these data suggest that galanin and its receptors, particularly GAL-R1, are potential therapeutic targets for the development of novel anxiolytic treatments.

Epilepsy is a diverse neurological disorder caused by a misbalance between excitatory (glutamatergic) and inhibitory (GABAergic) neurotransmission leading to hyperexcitability in neuronal circuits. A potential role for galanin as an anticonvulsant is suggested following the discovery that galanin antagonises glutamatergic neurotransmission in the hippocampus (Zini et al., 1993, 1993b; reviewed in Mazarati et al., 2001). Such effects of galanin on glutamate are mediated by K^+ channels, as effects were abolished by the K^+ blocker, glibenclamide (Zini et al., 1993). The production of transgenic mice has further supported these hypotheses. Mice that over-express galanin have been shown to exhibit a marked suppression of seizure development in an animal model of human complex partial seizures (Kokai et al., 2001). Furthermore, mice with a null mutation in the GAL-R1 gene show an enhanced susceptibility to seizures in limbic status epilepticus (SE), suggesting that the ability of galanin to reduce seizures is mediated through GAL-R1 (Mazarati et al., 2004). A role for GAL-R2 has also been implicated in mediating the anticonvulsant effects of galanin (Mazarati et al., 2004a, Lu et al., 2005). Taken together, these results suggest that agonists for both GAL-R1 and GAL-R2 would be of therapeutic use in the treatment of seizures. However, since GAL-R2, but not GAL-R1, protein and mRNA levels are decreased following pilocarpine induced SE (Lu et al., 2005), GAL-R1 would seem to be the better therapeutic target.

The distribution of galanin in brain areas that regulate ingestive behaviours led to a number of studies on the role of galanin in feeding (reviewed in Crawley, 1999). It was shown that centrally administered galanin in the rat induced a dramatic increase in food consumption (Crawley et al., 1990; Dube et al., 1994), an effect that could

be attenuated by injection of the galanin antagonist, M40 (Bartfai et al., 1993), into the hypothalamus or amygdala prior to injection of galanin (Bartfai et al., 1993; Corwin et al., 1993). No effect of M40 alone on food intake was observed (Bartfai et al., 1993; Corwin et al., 1993; reviewed in Crawley, 1999).

A role for galanin in nociception is well established. Following peripheral nerve damage or axotomy in adult rodents, a dramatic up-regulation in galanin mRNA and protein expression is observed in sensory neurons (Hokfelt et al., 1987; Villar et al., 1989; Wiesenfeld-Hallin, 1992; Zhang et al., 1998; Holmes et al., 2005). This up-regulation of galanin expression is accompanied by a down-regulation in GAL-R1 and GAL-R2 expression (Xu et al., 1996; Shi et al., 1997; Reviewed in Wiesenfeld-Hallin, 2001). Following nerve injury, intrathecal application of exogenous galanin produces an enhanced inhibitory effect on nociceptive behaviours (Hao et al., 1999; Yu et al., 1999; Liu and Hokfelt, 2000), whilst application of the high affinity galanin receptor antagonist, M35, increases pain-like behaviour (Wiesenfeld-Hallin et al., 1992, Liu and Hokfelt., 2000).

Transgenic mice have provided further evidence for an inhibitory role for galanin in nociception. Mice over-expressing galanin show significant elevation of nociceptive threshold to thermal stimulation in both the tail-flick and Hargreaves test (Blakeman et al., 2001) and a decrease in the development of pain-like behaviours following partial sciatic nerve injury (Hygge-Blakeman et al., 2004; reviewed in Wiesenfeld-Hallin et al., 2005). Interestingly, GAL-R1 knockout mice show no differences compared to wild type mice in either the tail flick test, or in paw withdrawal latency to radiant heat stimulation. There was a slight, but significant reduction in the latency to jumping/hind-paw licking in the hotplate test in GAL-R1 knockout mice versus wild type mice and also an increase in response scores to cold stimulation (Hygge-Blakeman et al., 2003; Reviewed in Wiesenfeld-Hallin et al., 2005). Such GAL-R1^{-/-} mice therefore only have a subtle nociceptive phenotype, suggesting a possible role for other galanin receptor subtypes in mediating the inhibitory effects of galanin on nociception. As mentioned above, GAL-R2 is expressed predominantly on small

nociceptive neurons, unlike GAL-R1, and hence may have a more prominent role than GAL-R1 in mediating galanin's effects on nociceptive thresholds..

Alongside these physiological roles, galanin has also been shown to act as a survival factor for a population of developing sensory neurons. Adult galanin null mutant mice contain 13% less DRG neurons than wild type mice due to increased cell death neonatally. Analysis of neuronal properties determined that the neurons lost were those specifically <200microns in diameter (Holmes et al., 2000). This would suggest that galanin expression is important for the development of a specific subpopulation of small primary sensory neurons within the DRG. A further trophic role for galanin is suggested in the adult, since mice with a null mutation in the galanin gene showed a reduced rate of regeneration following nerve injury (Holmes et al., 2000). Additionally, a 30% reduction in neurite outgrowth was observed in cultures of adult DRG from these mice. (Holmes et al., 2000; reviewed in Holmes et al., 2005 and Wynick et al., 2001). This apparent neurotrophic effect of galanin will need to be considered when interpreting the effects of other neurotrophic factors on modulating galanin expression

Regulation of galanin mRNA expression by neurotrophic factors

Although some studies of the regulation of galanin mRNA expression by neurotrophic factors has been undertaken in the adult, little is known about the roles of neurotrophic factors in regulating galanin expression in developing sensory neurons. The role of NGF in strongly up-regulating galanin mRNA is suggested by several observations. NGF up-regulates galanin mRNA in cultured DRG (Kerekes et al., 1997; Ozturk and Tonge, 2001). Injection of anti-NGF antibody into mice produced an increase in galanin mRNA that mimicked the effects seen in axotomy (Shadiack et al., 2001). Infusion of NGF following nerve damage produced a decrease in galanin immunoreactivity (Verge et al., 1995). Interestingly, no effect of exogenous NGF on galanin mRNA or protein levels is observed in intact neurons (Verge et al., 1995), suggesting an inhibitory role for NGF on galanin expression *in-vivo* only following nerve damage.

Aside from NGF, other neurotrophic factors have also been shown to regulate galanin expression. BDNF treatment decreases galanin mRNA levels in cultured adult DRG (Kerekes et al., 1997). Additionally, following nerve injury, GDNF given intrathecally was found to inhibit several of the nerve injury-related alterations in gene expression, including the up-regulation of galanin in large diameter neurons (Wang et al., 2003).

3.2. Material and Methods

3.2.1. Transgenic mice

3.2.1.1. Bax double knockout mice

To produce the data in this chapter, several different transgenic mouse strains were used to study the effects of growth factors and their receptors on gene expression *in-vivo*.

Since neurons that are dependent on NGF for embryonic survival undergo extensive cell death in TrkA^{-/-} and NGF^{-/-} fetuses (Crowley et al 1994, Smeyne et al, 1994), it was necessary to produce double-transgenic mice that were homozygous for a null mutation in the gene encoding the pro-apoptotic protein BAX in order to study the *in-vivo* regulation of gene expression by NGF/TrkA signalling. Both NGF^{-/-}/Bax^{-/-} and TrkA^{-/-}/Bax^{-/-} have been generated previously (Patel et al., 2000). In both double mutants the large decrease in cell death seen in TrkA or NGF knockout mice does not occur. In fact in DRG of TrkA^{+/+}/Bax^{-/-} mice at P0, neuron counts are actually higher than those in DRG of TrkA^{+/+}/Bax^{+/+}, most likely attributable to the elimination of naturally occurring cell death (Patel et al., 2000). Furthermore similar increases in neuronal number are found in DRG of TrkA^{-/-}/Bax^{-/-} mice at P0 in comparison to DRG of TrkA^{+/+}/Bax^{+/+} indicating that the absence of Bax allows the rescue/survival of all neurons requiring NGF/TrkA signalling during embryonic life (Patel et al., 2000). The sensory neurons that survive in these double knockout mice are small and atrophic (Patel et al., 2000), however such “rescued” neurons have been shown to express peripherin and the neuropeptides CGRP and SP. DRG neurons of NGF^{-/-}/Bax^{-/-} mice have also have been shown to express TrkA. These findings suggest that an *in-vivo* deficiency of Bax permits the survival of peptidergic neurons in the absence of NGF/TrkA signalling (Patel et al., 2000).

In this chapter $\text{TrkA}^{-/-}/\text{Bax}^{-/-}$ mice were used to explore the regulation of gene expression by NGF/TrkA signalling. It would have also been informative to use $\text{NGF}^{-/-}/\text{Bax}^{-/-}$ mice, however time constraints hindered this investigation.

$\text{TrkA}^{-/-}/\text{Bax}^{-/-}$ mice were generated by breeding $\text{TrkA}^{+/-}/\text{Bax}^{+/-}$. The pups produced were therefore one of nine possible genotypes:

$\text{TrkA}^{-/-}/\text{Bax}^{-/-}$,
 $\text{TrkA}^{-/-}/\text{Bax}^{+/-}$,
 $\text{TrkA}^{-/-}/\text{Bax}^{+/+}$,
 $\text{TrkA}^{+/-}/\text{Bax}^{-/-}$,
 $\text{TrkA}^{+/-}/\text{Bax}^{+/-}$,
 $\text{TrkA}^{+/-}/\text{Bax}^{+/+}$,
 $\text{TrkA}^{+/+}/\text{Bax}^{+/-}$,
 $\text{TrkA}^{+/+}/\text{Bax}^{-/-}$,
 $\text{TrkA}^{+/+}/\text{Bax}^{+/+}$

On the day of birth, the whole litter of mice were killed by decapitation just above the neck. Tail tips were kept for genotyping and each pup was individually dissected to remove trigeminal ganglia, nodose ganglia and DRG. Ganglia were stored at -80°C until genotyping was complete.

Following genotyping ganglia from mice that contained null mutations in both genes ($\text{TrkA}^{-/-}/\text{Bax}^{-/-}$) were kept alongside ganglia from wild-type litter mates ($\text{TrkA}^{+/+}/\text{Bax}^{+/+}$) for further investigation. In addition, as a control, to ensure that the deletion of functional Bax did not have profound effects on sensory neuron gene expression, sensory ganglia were also collected from Bax single knockout mice ($\text{TrkA}^{+/+}/\text{Bax}^{-/-}$). All other ganglia were discarded.

NT-3 null mutant mice also have a severe phenotype, displaying pronounced limb movement defects, due to loss of sensory and sympathetic neurons, and perinatal mortality (Ernfors et al., 1994). In order to study the effects of NT-3 in regulating

gene expression in sensory neurons *in vivo*, NT-3^{-/-}/Bax^{-/-} mice were therefore generated. In these double null mutant mice, NT-3 dependent neurons can now survive, and express the proprioceptive marker parvalbumin, suggesting these “rescued” neurons display characteristics of proprioceptive neurons from normal wild-type mice (Patel et al., 2003).

NT-3^{-/-}/Bax^{-/-} mice were generated by breeding NT-3^{+/-}/Bax^{+/-} with NT-3^{+/-}/Bax^{+/-}.

Each pup from these parents will therefore be one of nine possible genotypes:

NT-3^{-/-}/Bax^{-/-},

NT-3^{-/-}/Bax^{+/-},

NT-3^{-/-}/Bax^{+/+},

NT-3^{+/-}/Bax^{-/-},

NT-3^{+/-}/Bax^{+/-},

NT-3^{+/-}/Bax^{+/+},

NT-3^{+/+}/Bax^{+/-},

NT-3^{+/+}/Bax^{-/-},

NT-3^{+/+}/Bax^{+/+}

On the day of birth, the whole litter of mice were killed by decapitation just above the neck. Tail tips were kept for genotyping and each pup was individually dissected to remove trigeminal ganglia, nodose ganglia and DRG. Ganglia were stored at – 80°C until genotyping was complete.

Following genotyping, ganglia from mice that contained null mutations in both genes (NT-3^{-/-}/Bax^{-/-}) were kept alongside ganglia from their wild-type litter mates (NT-3^{+/+}/Bax^{+/+}). In addition, as a control, to ensure that the deletion of functional Bax did not have profound effects on sensory neuron gene expression, sensory ganglia were also collected from Bax single knockout mice (NT-3^{+/+}/Bax^{-/-}).

3.2.1.2. Dissections

Trigeminal ganglia

At P0, this dissection is very similar to that of the late embryonic mouse (see 2.2.1). Serrated scissors are used to cut off the top of the skull in a plane just above the eyes and whisker pads. The brain was gently removed to reveal the two trigeminal ganglia lying at the base of the skull, sitting in two niches either side of the mid-bone (as seen previously in figure 2.2). The ganglia were removed with forceps and adherent connective tissue removed with tungsten needles.

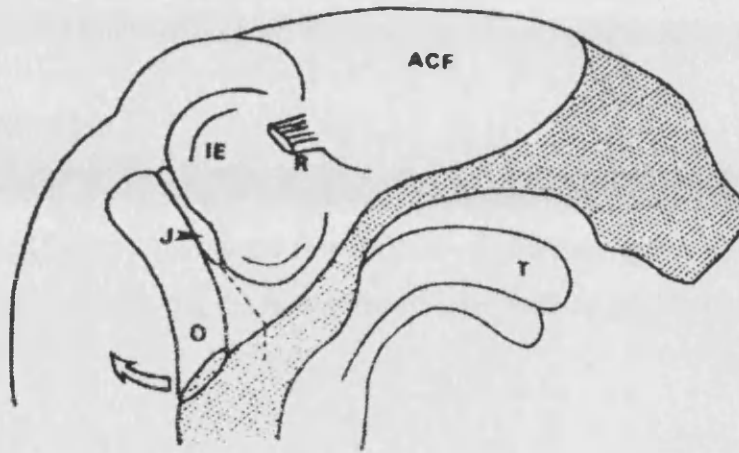
Nodose ganglia

The two murine nodose ganglia, also known as the inferior vagal ganglia, are situated just below the base of the skull, close to the jugular foramen. The top of the skull and underlying brain were removed and the head bisected along the sagittal plane. The hindbrain was removed from each half of the head, and the slit-like jugular foramen was now visible. By opening up the mouth of the foramen, the nodose ganglion was revealed lying just above the superior cervical ganglia (SCG). The SCG is composed of sympathetic neurons and is distinct from the nodose ganglion by its oval, elongated structure and its attachment to the thin sympathetic chain. The nodose ganglion is a clearly defined spherical structure, which has the prominent vagus nerve projecting from its distal aspect. The nodose ganglion was removed from each side of the head using forceps to gently tease it from surrounding tissue, and once again 'cleaned' of any adherent connective tissue using tungsten needles (figure 3.2)

DRG

For the DRG, the dissection was very similar to that described in section 2.2.1. First, skin was cut away from the back of the mouse, and a portion of the lumbar spinal column was removed using serrated scissors. As with the embryo, small scissors were used to cut the length of this section of spinal column along the ventral aspect

A



B

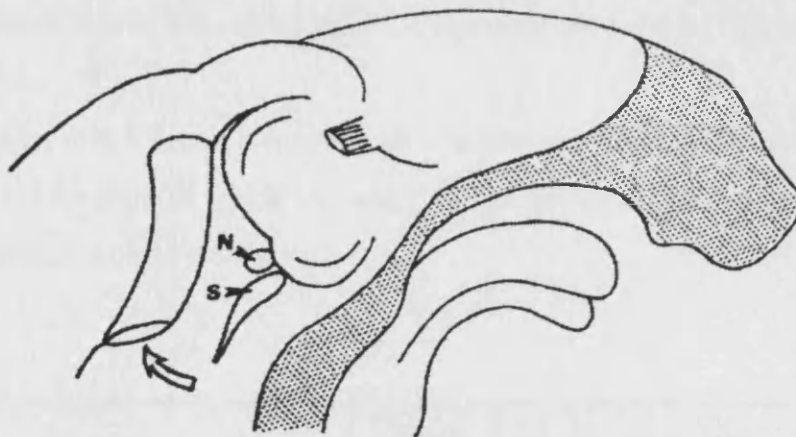


Figure 3.2: *Dissection of the nodose ganglia*

The medial aspect of half of the head of an E14 mouse embryo. A: An incision is made from the jugular foramen (J) to the midline (dotted line) and the occipital bone is reflected in the direction of the arrow. B: The jugular foramen is now open to reveal the nodose (N) and superior cervical ganglion (s) just beneath. (T - tongue, ACF - anterior cranial fossa, IE - inner ear, R - root of the trigeminal nerve). Taken from Davies, 1995.

3.2.2. Genotyping

Genotyping was performed in a similar manner to that outlined in section 2.2. DNA was extracted and the presence or absence of the knockout allele identified by PCR.

DNA Extraction

Genomic DNA was extracted from tails of all mice using the Nucleospin DNA extraction kit following the manufacturers protocol as detailed in section 2.2.

PCR

PCR reactions were carried out to determine the genotype of each mouse dissected. Details of primer sequences and PCR reactions are outlined below

A master mix solution containing all reagents was made up for each reaction, 19 μ l (or 15 μ l for Bax) of which was added to 1 μ l (or 5 μ l for Bax) of genomic DNA.

Mastermix recipes are shown below:

	TRK A	NT-3	BAX
10 \times hot taq buffer	2 μ l	2 μ l	2 μ l
5mM dNTPs	1 μ l	1 μ l	1 μ l
WT primers (10pmol/ μ l)	0.2 μ l	0.4 μ l	0.45 μ l
KO Primers (10pmol/ μ l)	0.2 μ l	0.4 μ l	0.3 μ l
Common Primer (10pmol/ μ l)	0.23 μ l	0.4 μ l	0.525 μ l
Hot Start Taq (GeneSys Ltd.)	0.2 μ l	0.15 μ l	0.2 μ l
25mM MgCl ₂	1 μ l	0.4 μ l	2 μ l

Mr solution	-	3.8 μ l	3.8 μ l
dH ₂ O	14.17 μ l	10.45 μ l	4.725 μ l
DNA	1 μ l	1 μ l	5 μ l

Table 3.1. *Mastermix reagents for genotyping reactions*

Primer sequences are outlined below:

TrkA:

P095-4 : 5'-CGG ACC TCA GTG TTG GAG AGC TGG-3'
P096-0: 5'-GAC CCT GCA CTG TCG AGT TTG C-3'
P097-0: 5'-GCT CCC GAT TCG CAG CGC ATC G-3'

Bax:

IN5R 5'-TTG ACC AGA GTG GCG TAG-3'
EX5F 5'-GCT GAT CAG AAC CAT CAT G-3'
NeoR 5'-GCT TCC ATT GCT CAG CG-3'

NT-3:

N3 (wild-type) 5'-CCT GGC TTC TTT ACA TCT CG-3'
N4 (mutant) 5'-TGG AGG ATT ATG TGG GCA AC-3'
P1 (common) 5'-GGG AAC TTC CTG ACT AGG GG-3'

Two drops of mineral oil were layered on top of each PCR mix to prevent evaporation of reagents during thermocycling.

Initially samples were heated to 95°C for 15 minutes to activate taq. This was followed by a series of heating and cooling steps to allow denaturation of secondary structure, annealing of primers and elongation of product. Finally samples were heated to 72°C for 10mins for final extension. The length and temperature of these steps are specific to each reaction and are repeated in a cycling fashion to build up the PCR product. The details of each reaction are outlined in table 3.2.

	TRKA	NT-3	BAX
Denaturation	95°C 15mins	95°C 15mins	95°C 15mins
Denaturation	95°C 40s	95°C 40s	95°C 40s
Annealing	65°C 1min 10s	65°C 1min 10s	50°C 1min 20s
Elongation	72°C 1min 40s	72°C 1min 40s	72°C 2min 20s
No. of cycles	38	40	38
Final Extension	72°C 10min	72°C 10min	72°C 10min
Hold	4°C	4°C	4°C

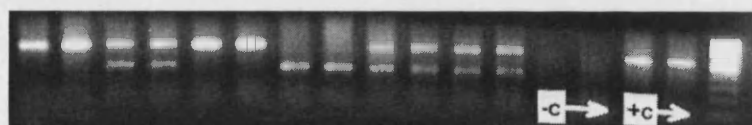
Table 3.2. Reaction conditions for genotyping PCRs

Products were run on agarose gel cast with 1 X TAE and containing ethidium bromide (1µg/ml) (Sigma). PCR product DNA bands were then visualised using a UV gel documentation system (Biogene). An example gel for Bax genotyping is shown in Figure 3.3.

	TRKA	NT-3	BAX
Wt band size	400	250	307
KO band size	800	350	507

Table 3.3. Band sizes for genotyping gels

Bax genotyping



1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.3: Genotyping gel showing the three possible Bax genotypes.

Lanes 1,2,5 and 6 show the knock-out DNA, lanes 3,4,9,10,11 and 12 show products from heterozygote DNA and lanes 7 and 8 show products from the wild-type DNA.

For the TrkA/Bax and NT-3/Bax mice, double-knockout pups needed to have both copies of both genes knocked out, only a 1 in 16 chance. Similarly, double

homozygous wild-type mice only occurred with a frequency of 1 in 16. Therefore, many litters of mice had to be dissected in order to obtain enough ganglia of each genotype to allow sufficient replicates for RT-QPCR. Following genotyping, ganglia from double knockouts, wild type and single Bax knockouts, were identified and RNA was extracted, as described later in this chapter. At least 4 replicates were collected for each ganglia for each of the desired genotypes.

Time-course

To study the expression of all genes of interest over a period of time in wild-type mice, ganglia were also dissected from E16, P0, P5 and P60 mice. Six replicates of each ganglia at each age were collected. RNA was extracted and used to produce a time-course of mRNA expression for all genes.

3.2.3. Neuronal cultures

To confirm that changes in mRNA expression observed in knockout neonates were due to the absence of neurotrophic factors or receptors and not cell death, neuronal cultures of nodose and trigeminal ganglia neurons were set up to study the effects of the neurotrophic factors, NT-3, NGF and artemin on gene expression in primary sensory neurons *in-vitro*. DRG cultures were not set up since trigeminal neurons are functionally and biochemically similar to DRG neurons at neonatal ages and *in vivo* results showed gene expression to be similarly regulated in these two ganglia. At these early postnatal stages, wild-type sensory neurons do not survive in culture without the addition of neurotrophic factors (NGF for trigeminal neurons and BDNF for nodose neurons). For this reason, caspase inhibitors were used to prevent the normal apoptosis that would occur in the absence of trophic support, thus allowing a true no neurotrophic factors control to be set up.

Overnight matings of CD-1 mice were set up. Pups were collected on the day of birth (P0) and killed by decapitation. All dissections and subsequent preparations were

carried out in a laminar flow hood using standard sterile techniques. Dissections of trigeminal and nodose ganglia were performed (as described earlier in this chapter, 3.2.1.1) under stereomicroscope with fibre-optic light source to illuminate specimens but prevent overheating of tissue. Dissections were carried out in sterile plastic 60mm petri dishes (Greiner) in Liebowitz (L-15, Invitrogen) medium, pH 7.3, (supplemented with streptomycin (100mg/l) and penicillin (60mg/l)), that had been filtered and pre-heated to 37°C. All dissection tools were sterilised by flaming in 70% ethanol prior to dissection.

Ganglia were dissected and collected in Calcium and Magnesium free Hank's Balanced Salt Solution (CMF-HBSS) in 15ml falcon tubes. Approximately 30 ganglia were used per experiment with approximately 10 ganglia per tube. Ganglia were incubated in 0.05% trypsin (Worthington), at 37°C for 30 minutes, to allow enzymatic break down of the ganglia. Ham's F12 medium, supplemented with 10% heat inactivated horse serum (HIHS), was then used to wash cells twice and to arrest trypsin action. The ganglia were transferred into defined, serum free, F14 medium (Imperial), supplemented with Albumax I and 2mM glutamine (both from Invitrogen). A flame-polished, sterile, siliconised glass Pasteur pipette was used to mechanically dissociate ganglia and to produce a single cell suspension.

High-density cultures were required in order to obtain the concentrated RNA required for RT-QPCR analysis of rare genes. Cells were plated at approximately 3000 - 5000 neurons per 35mm diameter plastic tissue culture dish (Greiner). The dishes had been prepared in advance by coating with a substratum of poly-DL-ornithine (0.5mg/ml in 0.15M borate buffer, pH 8.4) overnight at room temperature. The following day they were washed three times with sterile water and left to dry. Prior to culture, dishes were treated with laminin (20µg/ml in CMF-HBSS, Sigma), for 4 hours, at 37°C.

After 4 hours, laminin was washed from dishes using F12 + HIHS, before adding 1ml of F14 medium supplemented with glutamine and Albumax I. It was important to ensure that dishes were not allowed to dry at any point during this washing step.

1ml of the dissociated cell suspension was added to each dish to give a final volume of 2ml. After plating the cells, the appropriate neurotrophic factors were added to each dish. Additionally at this point four tubes of this single cell suspension were collected as a 'time 0' sample. These four tubes of cells (1ml per tube) were spun down, the supernatant was removed and the cell pellets were re-suspended in 350µl RLT lysis buffer, containing 1% β mercaptoethanol (Qiagen) to lyse the cells and release RNA.

Cells were grown with no factors, or in the presence of 10ng/ml NGF or NT-3 (these concentrations had been determined previously in the Davies lab to be saturating concentrations for promoting survival and neurite outgrowth of P0 neurons). 3 or 4 replicates per condition were set up. Caspase Inhibitors (caspase inhibitor 1 (Calbiochem) to a final concentration of 50µM.) were added to all dishes so that some cells could be grown in the absence of all neurotrophic support as a true control. Representative photomicrographs of cultured sensory neurons are shown in Figure 3.3a

Plated cells were grown in 5.5% CO₂ in a humidified incubator, at 37°C, for 24 hours (nodose and trigeminal ganglia) or 48 hours (just trigeminal ganglia), after which RNA was extracted from them by gently removing the culture media and replacing it with 350µl RLT lysis buffer supplemented with 1% β mercaptoethanol. Dishes were left at room temperature for 10-15 minutes, to allow cell lysis, and then the lysis buffer containing extracted RNA was transferred to RNase free 1.5ml tubes and stored at 4°C until all samples had been collected. It was only possible to extract RNA from nodose neurons at one time point, since the small nodose ganglia contain too few neurons to seed enough tissue culture dishes for two time points.

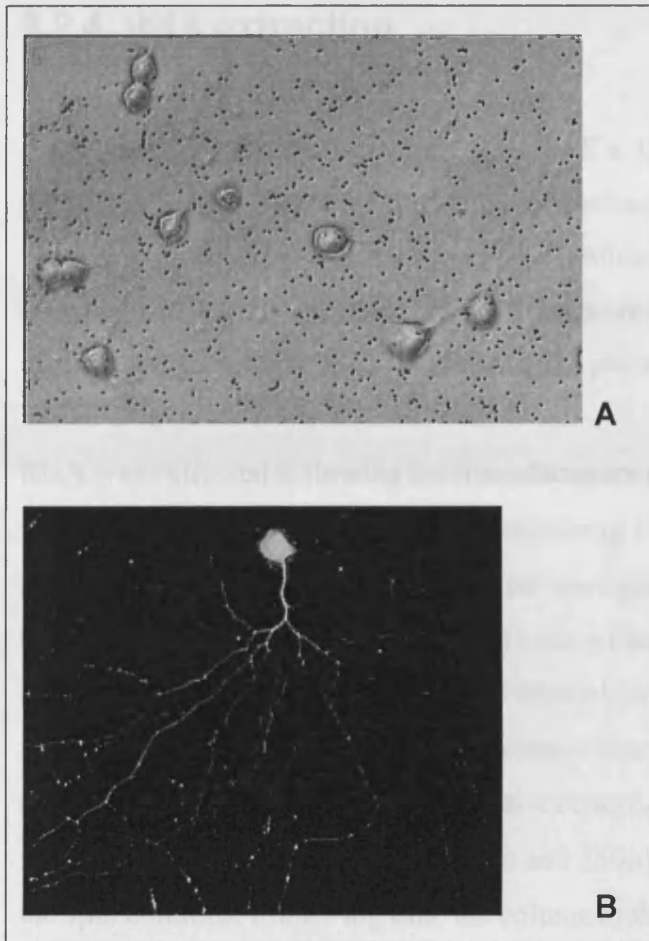


Figure 3.3a: *Representative photomicrographs of cultured sensory neurons.*

A) Nodose neurons cultured for 4 hours. B) A single trigeminal neuron, following 72 hours growth in the presence of NGF. Note the considerable growth of neurites following time in culture.

Survival counts

To ensure that differential survival of neurons in the different culture conditions did not confound the interpretation of gene regulation data, neuronal survival was assessed over the culture period. Initial neuronal counts were done 3 hours after plating (to allow cell attachment to culture dishes), and survival after 24 hours and 48 hours was expressed as a percentage of this 3 hour count.

N.B. Details of all tissue culture reagents, their preparation and storage are given in appendix II.

3.2.4. RNA extraction

A new method of RNA extraction (RNeasy Kit, Qiagen) was chosen for QPCR as preliminary experiments showed that the previous RNA extraction method, used in chapter 2, resulted in RNA that contained inhibitors of the QPCR reaction. In addition, the RNeasy protocol proved to be more reliable, faster to carry out and avoided the use of hazardous chemicals like phenol. (Data not included).

RNA was extracted following the manufacturers protocol. In brief, cells/ganglia were immersed in 350 μ l RLT lysis buffer containing 1% β mercaptoethanol. In the case of whole ganglia, the ganglia in lysis buffer were passed up and down through a 25G needle to break them up and ensure all cells within the ganglion were exposed to the lysis buffer. The same volume of 70% ethanol (analytical reagent grade) was added and samples thoroughly mixed. Each sample was transferred to an RNeasy filter mini-column in a 2ml collecting tube and centrifuged, in a microfuge, at 10,000 rpm for 15s. The supernatant was discarded and 350 μ l RW wash solution was added to the spin columns. Following this, the columns/tubes were centrifuged again, for 15s at 10,000rpm, and the supernatant was discarded. Next, a DNase step was included to remove any contaminating genomic DNA. 80 μ l DNase solution (10 μ l DNase (273U) + 70 μ l RDD buffer, Qiagen) was added directly to the filter membrane and tubes were left at room temperature for 30 minutes. Several more wash steps, using an ethanol based wash solution and centrifugation, were performed after DNase treatment to remove residual DNase and RDD buffer. After the final wash step, the RNA was eluted in nuclease free water. The volume in which RNA was eluted was determined according to the amount of starting tissue and the RNA concentration required for RT-QPCR. In general, RNA was eluted in 100 μ l for whole ganglia and 40 μ l in the case of neuronal RNA. Samples were stored at -80°C until required for RT-QPCR.

3.2.5. Reverse-transcription real-time quantitative PCR

Real Time quantitative PCR (QPCR), after reverse transcription of mRNAs to cDNA, was used to quantify the mRNA levels of the genes of interest in total RNA from both neuronal cultures and from whole ganglia collected from transgenic mice. The MX3000P (Stratagene) was used as outlined in section 2.2.5. Stratascript reverse transcriptase was used for the reverse transcription reaction as previously. Details of reaction preparation and conditions for both RT and PCR are outlined in section 2.2.5. For each gene, the primers were designed on 'Beacon Designer' computer software, to ensure the utmost efficiency and specificity. The annealing temperature for all primer pairs for genes studied in this chapter was 51°C, and 40 cycles of amplification was sufficient to allow accurate quantification of all reverse transcribed mRNAs. The optimal reaction conditions to amplify each cDNA of interest were determined empirically in a series of pilot experiments. This ensured the highest reaction efficiencies and the absence of mispriming and primer dimer artifacts. These optimal conditions are outlined in table 3.4.

In the experimental work that is documented in this chapter, I investigated the roles that the neurotrophic factors NGF and NT-3 play in regulating neonatal sensory neuron expression of all the mRNAs studied in chapter 2 (α CGRP, β CGRP, SP, Nav1.8, Nav1.9 and GAPDH). In addition, I investigated the regulation of three additional mRNAs that encode proteins/peptides that play important roles within the peripheral sensory nervous system, namely Pituitary Adenylate Cyclase Activating Peptide (PACAP), Galanin and Vanilloid Receptor 1 mRNAs. Primers and reaction conditions for amplifying the cDNAs of these additional mRNAs are outlined below:

GENE	PRIMER SEQUENCES	MGCL ₂ CONCENTRATION (MM)	ANNEALING TEMP. (°C)
Pituitary Adenylate Cyclase-Activating Peptide (PACAP)	Forward: 5'-TGG TGT ATG GGA TAA TAA TGC-3' Reverse: 5'-TTC CGT CCT GGT CGT AAG-3'	4	51
Galanin	Forward: 5'-GTT ACA ACT GGA GGT GGA G- 3' Reverse: 5'-TAG GTC TTC TGA GGA GGT G-3'	3	51
Vanilloid Receptor 1 (VR-1)	Forward: 5'-CAA TGT GGG TAT CAT CAA CG- 3' Reverse: 5'-GGT GCT ATG CCT ATC-3'	5	51

Table 3.4. *Primer sequences for additional genes.*

Due to the relatively low abundance of β -CGRP mRNA, some difficulty was encountered when assaying its cDNA by QPCR (recalcitrant primer artifacts and insufficient sensitivity when input total RNA concentrations were low as in the case of total RNA from nodose ganglia). To ameliorate these problems a Molecular Beacon probe was employed to assay β -CGRP mRNA expression in nodose ganglia

from transgenic and wild type. The sequence of the beacon is shown below. It was labelled with the fluorophore Quasar670 at its 5' end , which was visible on the Cy5 channel of the MX3000 QPCR machine, and had blackhole quencher 2 at its 3' end.

β-CGRP Beacon

5' Quasar670 d(CGC GAT AAA TAT GAT GGT GTC TCC CAC TGG ATC
GCG)BHQ-2 3'

Molecular Beacons probes, unlike Sybr green, emit a fluorescent signal only when they bind to a specific DNA sequence and do not detect primer artifacts.

Consequently, melting curve analysis after cycling is not required to validate the identity of the correct PCR products. Molecular Beacon probes emit very little background fluorescence compared to TaqMan probes, and are not as vulnerable to exonuclease attack resulting in a progressive increase in background fluorescence as the QPCR reaction proceeds. SYBR green was not included in QPCR reactions containing the β-CGRP Molecular Beacon. Molecular Beacon probes were not routinely used for QPCR throughout the work contained in this thesis because, despite their advantages in terms of specificity and insensitivity to primer artefacts, they are very expensive and complicate the design of QPCR assays.

3.3. Results

3.3.1. Time course of expression

Initially, the expression of α - and β -CGRP, SP, galanin, PACAP, Nav1.8, Nav1.9 and VR1 mRNAs in each of the sensory ganglia were studied over a developmental time course to determine how the expression of these genes changes throughout development and into adulthood in normal wild-type mice. Once again, as in chapter 2 (See 2.3.1), data was statistically analysed by use of the t-test.

Trigeminal, nodose and dorsal root ganglia were dissected from E16, P0, P5 and adult (approximately P60) CD-1 mice and RNA was extracted using the Qiagen RNeasy kit (see 3.2.4.). Two ganglia were collected per replicate and six replicates were collected for each kind of ganglion at each time point. RNA was reverse transcribed and the expression of α -CGRP, β -CGRP, SP, Nav1.8, Nav1.9, PACAP, VR1 and galanin cDNAs were quantified by QPCR. The expression of GAPDH mRNA was also quantified in all samples and used to normalise all results to account for any differences in the amounts of starting RNA and differences in the efficiency of reverse transcription between samples.

Although they are the products of two separate genes, α -CGRP and β -CGRP mRNAs showed very similar patterns of developmental regulation between E16 and P60 (adult) in all ganglia. In trigeminal, nodose and dorsal root ganglia, both mRNAs showed a steady increase in expression, relative to GAPDH, from E16 to a peak at P60. Both mRNAs were also most highly expressed in DRG and expressed at lowest levels in nodose ganglia (figure 3.4 and 3.5).

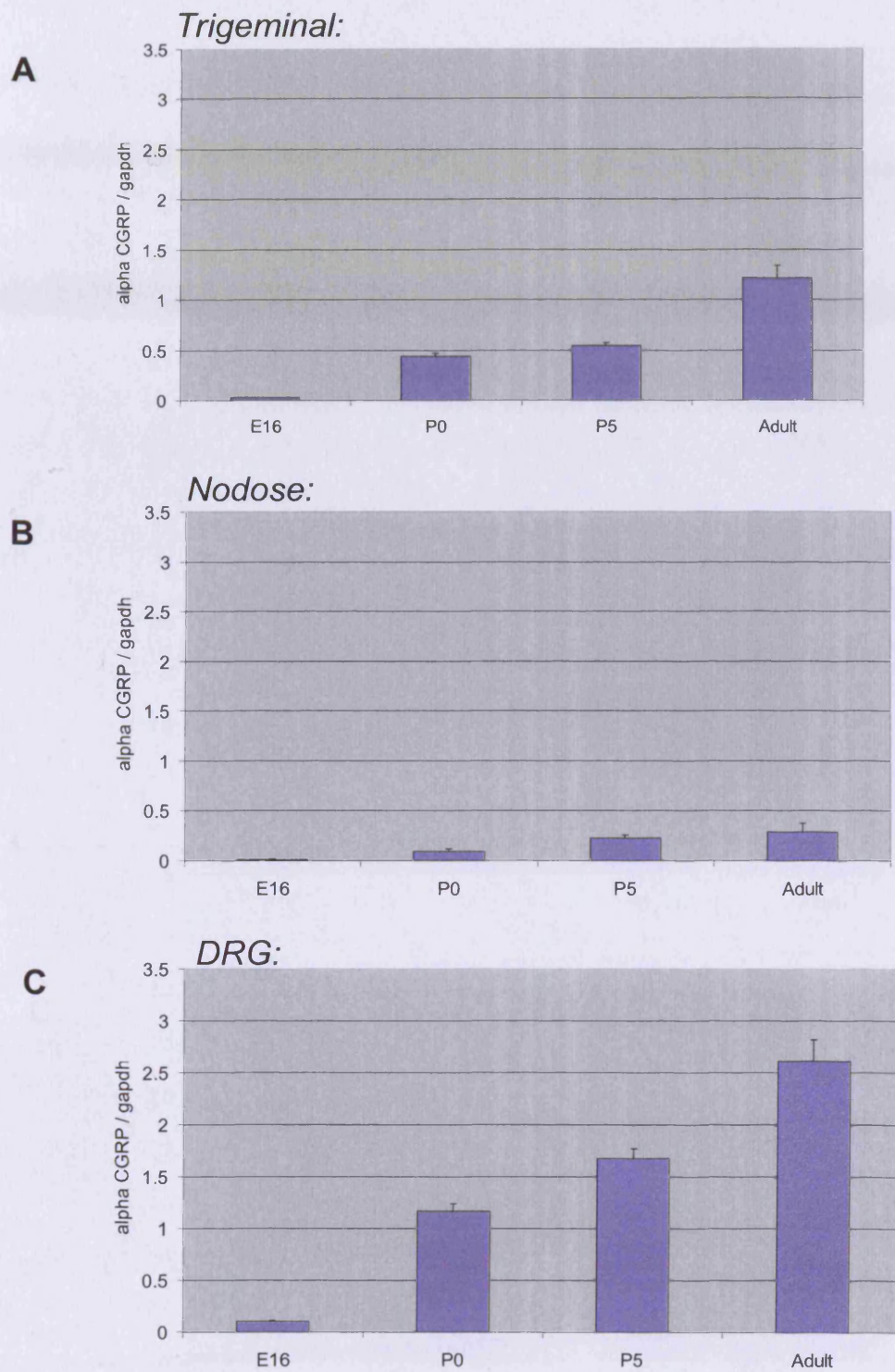


Figure 3.4. Expression of alpha CGRP mRNA in sensory neurons throughout development. Trigeminal ganglia (A), nodose ganglia (B) and DRG (C) were collected from E16, P0, P5 and adult mice and expression of alpha CGRP mRNA was quantified via real-time QPCR. Error bars = +/- standard error, n = 6

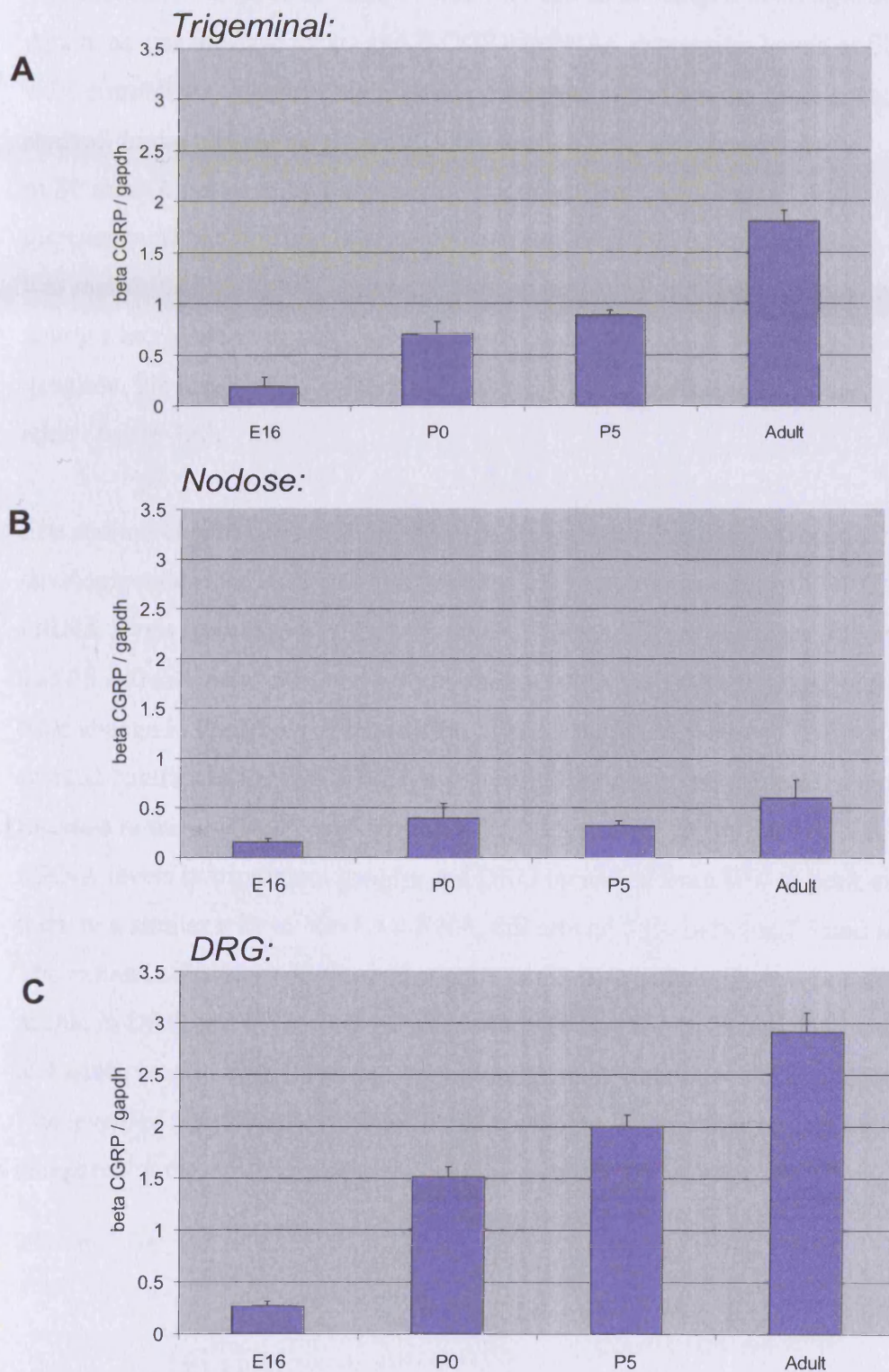


Figure 3.5. Expression of beta CGRP mRNA in sensory neurons throughout development. Trigeminal ganglia (A), nodose ganglia (B) and DRG (C) were collected from E16, P0, P5 and adult mice and expression of beta CGRP mRNA was quantified via real-time QPCR. Error bars = +/- standard error, n = 6

The expression level of SP mRNA was very low in all ganglia at all ages studied. Again, as was the case for α - and β -CGRP mRNAs, expression levels of SP mRNA were consistently lower in the nodose ganglion compared to the other sensory ganglia studied. In the trigeminal ganglion, there was little developmental change in the levels of SP mRNA between E16 and adult. In contrast, there was a small, but significant, increase in SP mRNA levels between E16 and P5 in DRG neurons and the level at P5 was maintained until P60. Similarly, the amount of SP mRNA expressed by nodose neurons increased gradually from E16 to P5, however, in the case of the nodose ganglion, the levels of SP mRNA expressed by neurons dropped between P5 and the adult (figure 3.6).

The sodium channels Nav1.8 and Nav1.9, showed similar, but not identical, developmental mRNA expression patterns. In trigeminal ganglia and DRG, Nav1.8 mRNA levels increased markedly between E16 and P0, remain constant between P0 and P5 and fall about 50% from P5 to adulthood. In the nodose ganglion, there was little change in the levels of expression of Nav1.8 mRNA between E16 and P5 and, in marked contrast to the two other sensory ganglia studied, there was an almost 100% increase in the levels of Nav1.8 mRNA from P5 to P60. In the case of Nav1.9, mRNA levels in trigeminal ganglia and DRG increased from E16 to peak at P5 and then, in a similar way to Nav1.8 mRNA, fall around 50% between P5 and adulthood. The expression pattern of Nav1.9 mRNA in the nodose ganglia was broadly the same as that in DRG and trigeminal ganglia, although the drop in expression between P5 and adult, seen in both DRG and trigeminal ganglia, did not occur in nodose ganglia. The levels of Nav1.9 mRNA were significantly lower in nodose neurons at all ages compared to the other two sensory ganglia (figure 3.7. and 3.8.).

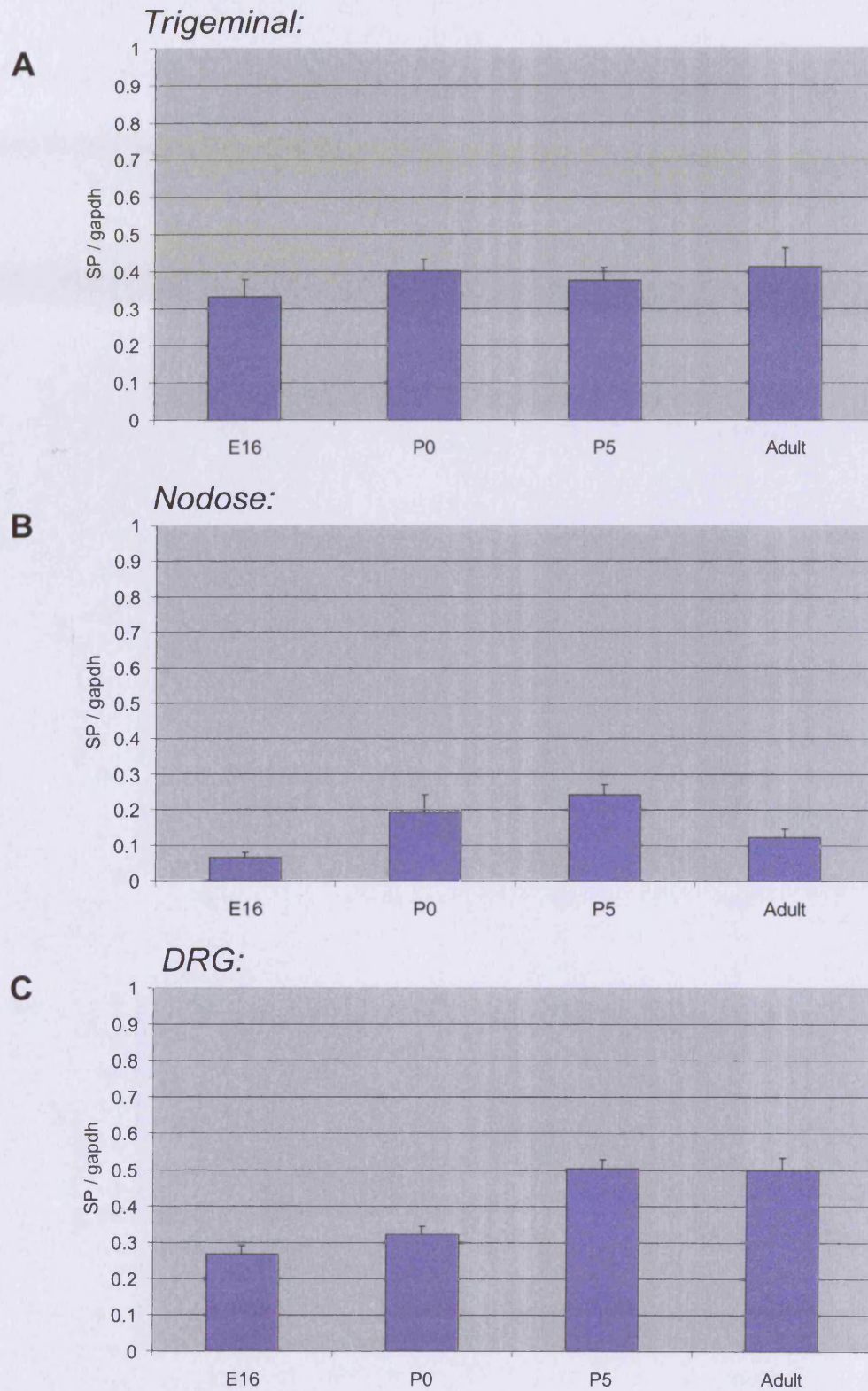


Figure 3.6. Expression of SP mRNA in sensory neurons throughout development
 Trigeminal ganglia (A), nodose ganglia (B) and DRG (C) were collected from E16, P0, P5 and adult mice and expression of SP mRNA was quantified via real-time Q-PCR. Error bars = +/- standard error, n = 6

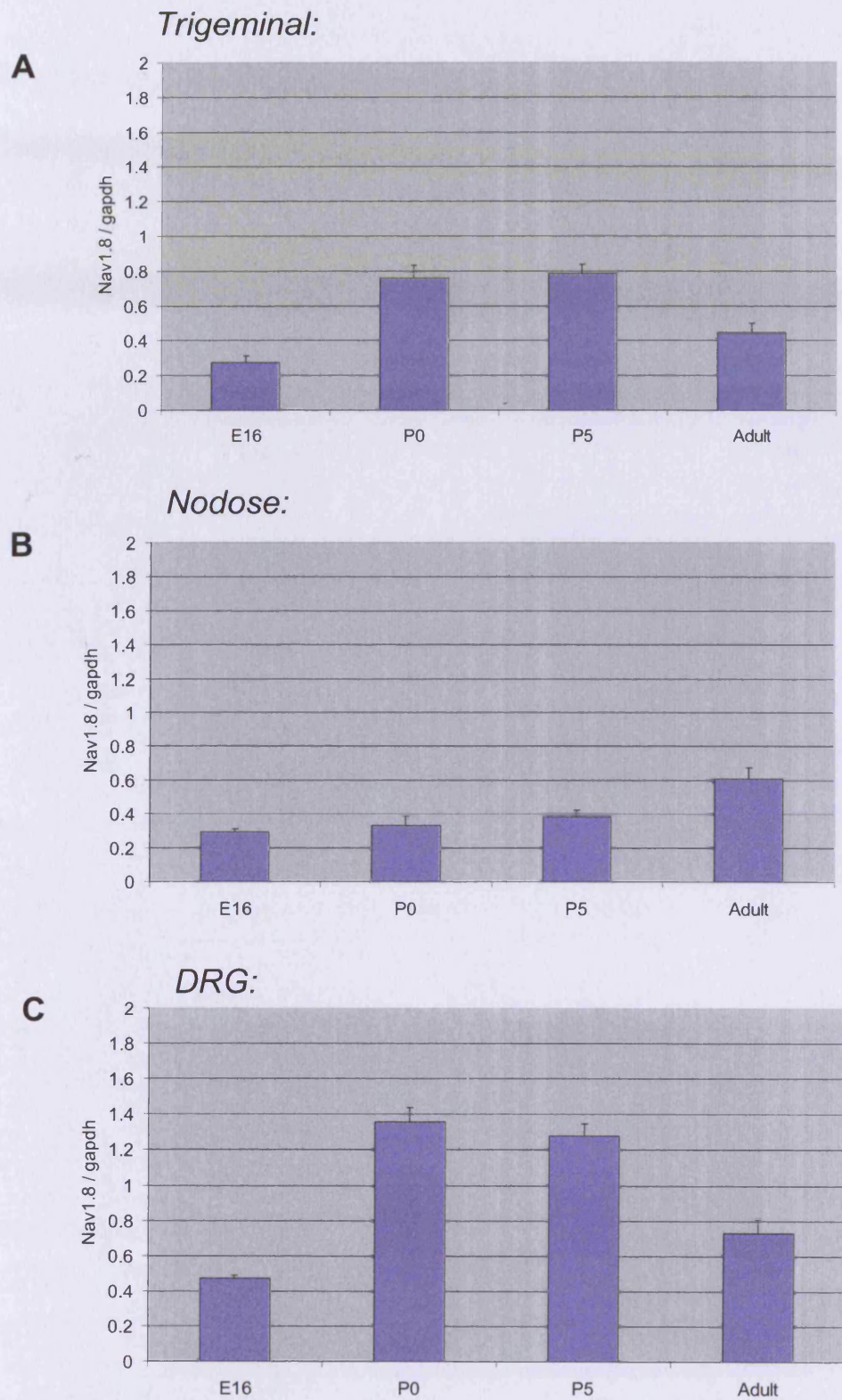


Figure 3.7. Expression of *Nav1.8* mRNA in sensory neurons throughout development. Trigeminal ganglia (A), nodose ganglia (B) and DRG (C) were collected from E16, P0, P5 and adult mice and expression of *Nav1.8* mRNA was quantified via real-time QPCR. Error bars = +/- standard error, n = 6

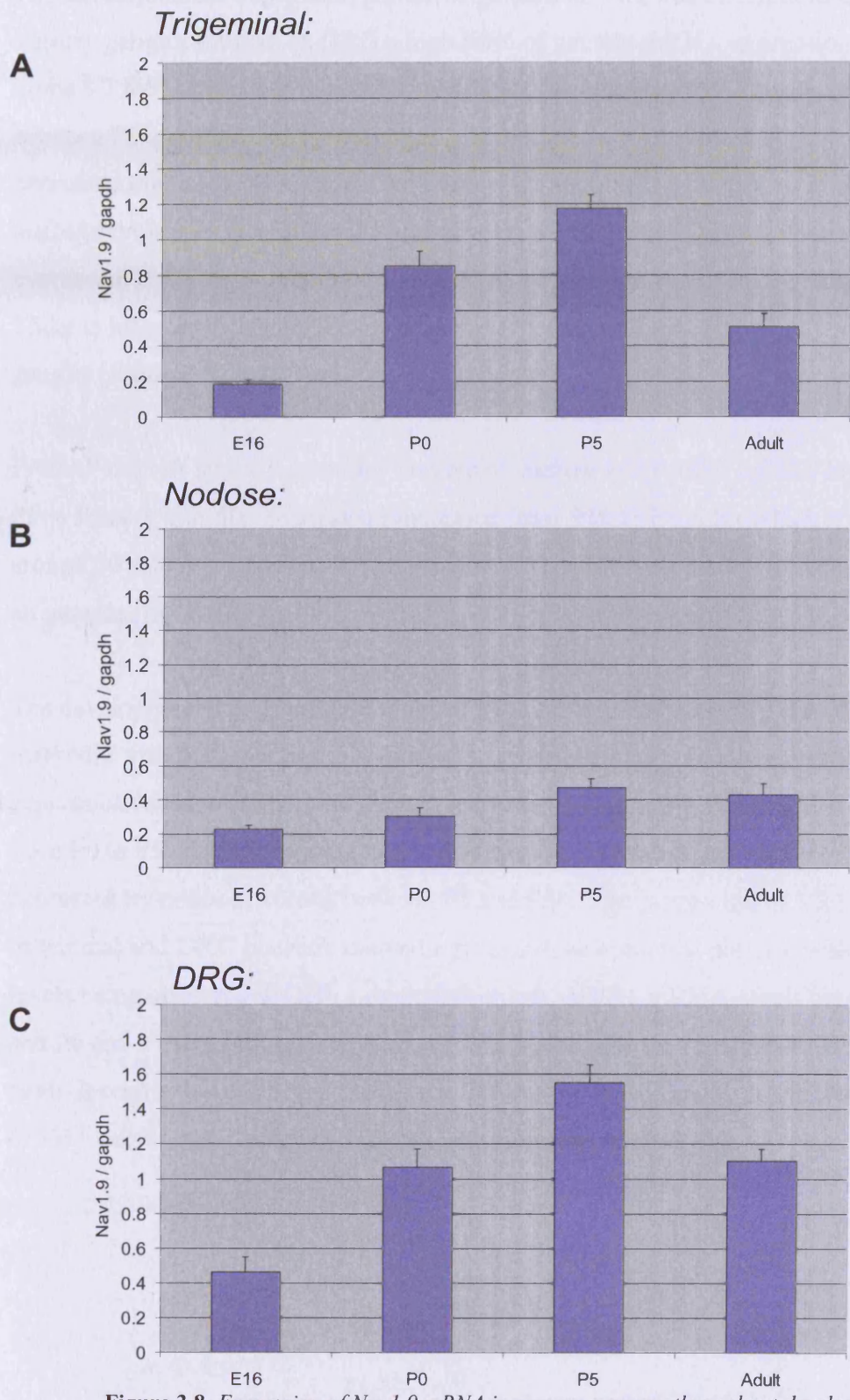


Figure 3.8. Expression of *Nav1.9* mRNA in sensory neurons throughout development
 Trigeminal ganglia (A), nodose ganglia (B) and DRG (C) were collected from E16, P0, P5 and adult mice and expression of *Nav1.9* mRNA was quantified via real-time QPCR. Error bars = +/- standard error, n = 6

The developmental expression pattern of galanin mRNA was different in all three sensory ganglia studied. In DRG a high level of galanin mRNA expression at E16 drops 3.7 fold between E16 and P5, and there was a further 25% drop in expression between P5 and P60. In trigeminal ganglia there were lower levels of galanin mRNA expressed than in DRG neurons, but there was a similar, 2.9-fold drop in galanin mRNA levels between E16 and P5. However, in contrast to DRG, galanin mRNA expression did show a 50% increase between P5 and adulthood in trigeminal ganglia. There is little developmental change in the expression of galanin mRNA in nodose ganglia (figure 3.9).

PACAP mRNA showed a similar expression pattern and similar mRNA levels in all three sensory ganglia. Expression increased from E16 to P0, after which it dropped by around 50% to P5. Between P5 and P60 PACAP mRNA levels fell a further 25% in all ganglia (figure 3.10.).

The developmental expression pattern of VR1 mRNA in the nodose ganglia, contrasts markedly with that seen in the DRG and trigeminal ganglia. In nodose ganglia, expression levels were low at E16, but increased 3-fold to P0 and a further 2-fold from P0 to P5. There was a marked 5-fold drop in the amount of VR1 mRNA expressed by nodose neurons between P5 and P60. The expression of VR1 mRNA in trigeminal and DRG neurons showed a similar developmental pattern, with highest levels being observed at E16, a gradual decrease in VR1 mRNA levels between E16 and P5 and a more pronounced drop, especially in DRG neurons, between P5 and adult. It seems that adult trigeminal neurons express significantly lower average levels of VR1 mRNA than either nodose or DRG neurons (figure 3.11).

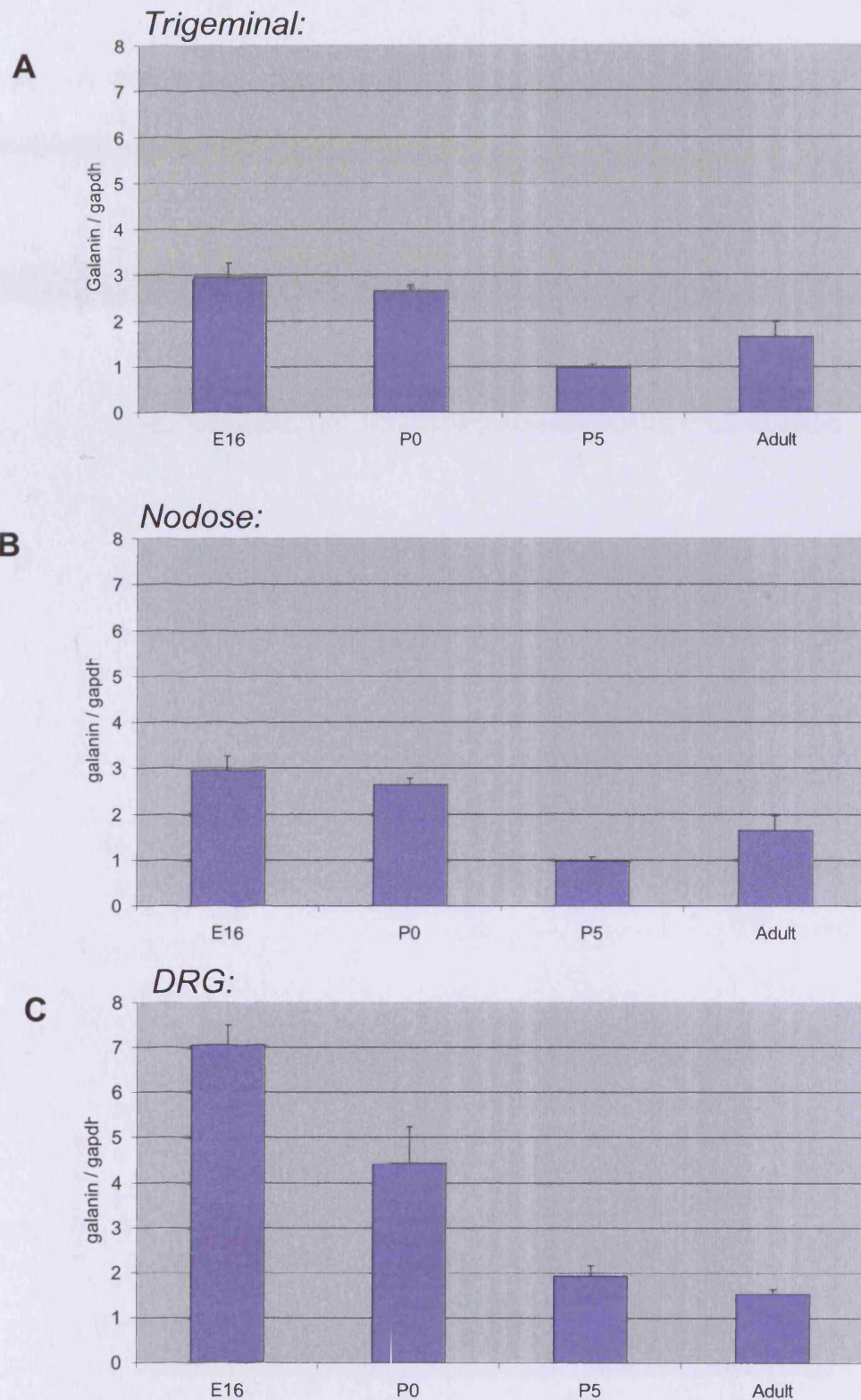


Figure 3.9. Expression of galanin mRNA in sensory neurons throughout development
 Trigeminal ganglia (A), nodose ganglia (B) and DRG (C) were collected from E16, P0, P5 and adult mice and expression of galanin mRNA was quantified via real-time QPCR. Error bars = +/- standard error, n = 6

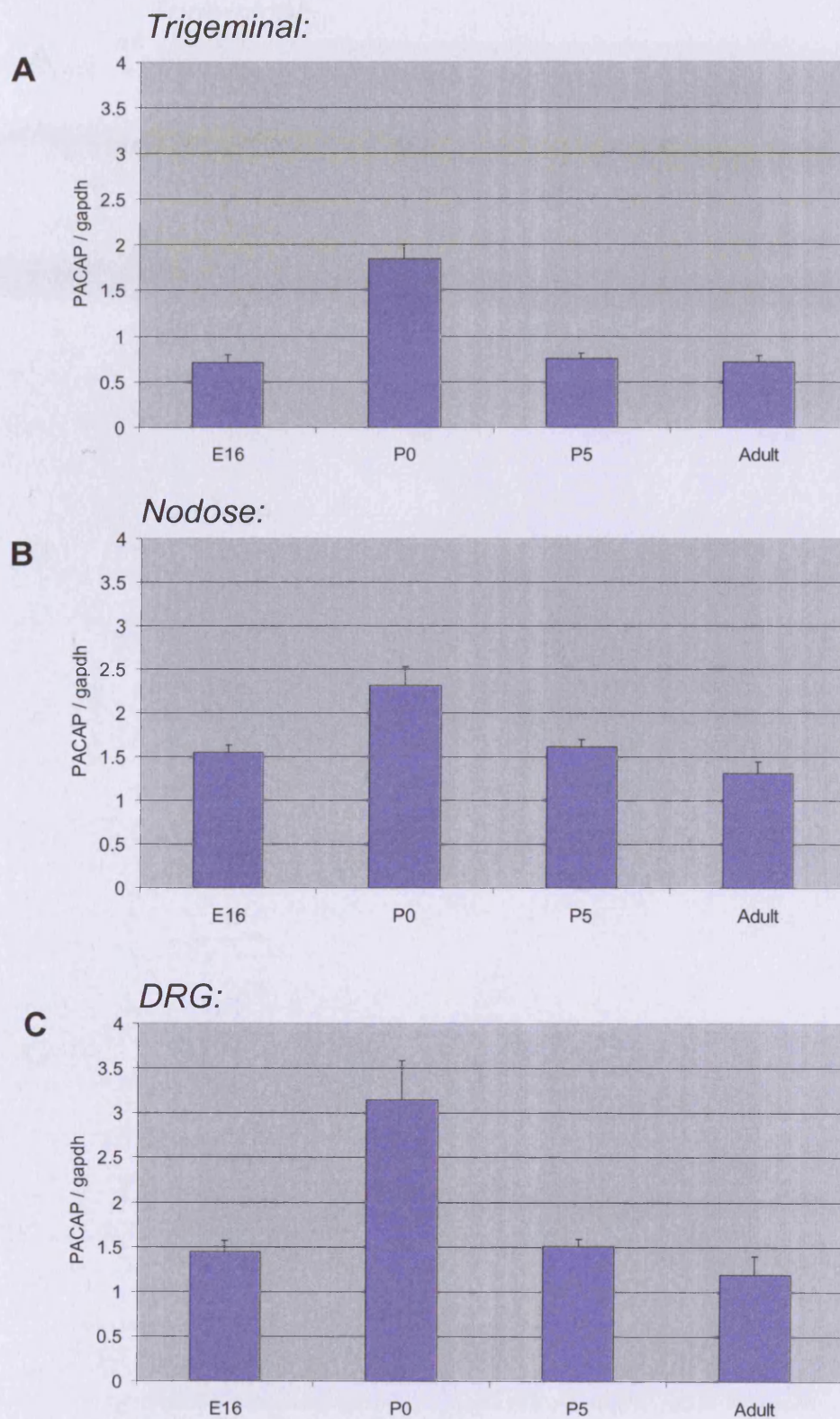


Figure 3.10. Expression of PACAP mRNA in sensory neurons throughout development
 Trigeminal ganglia (A), nodose ganglia (B) and DRG (C) were collected from E16, P0, P5 and adult mice and expression of PACAP mRNA was quantified via real-time QPCR.
 Error bars = +/- standard error, n = 6

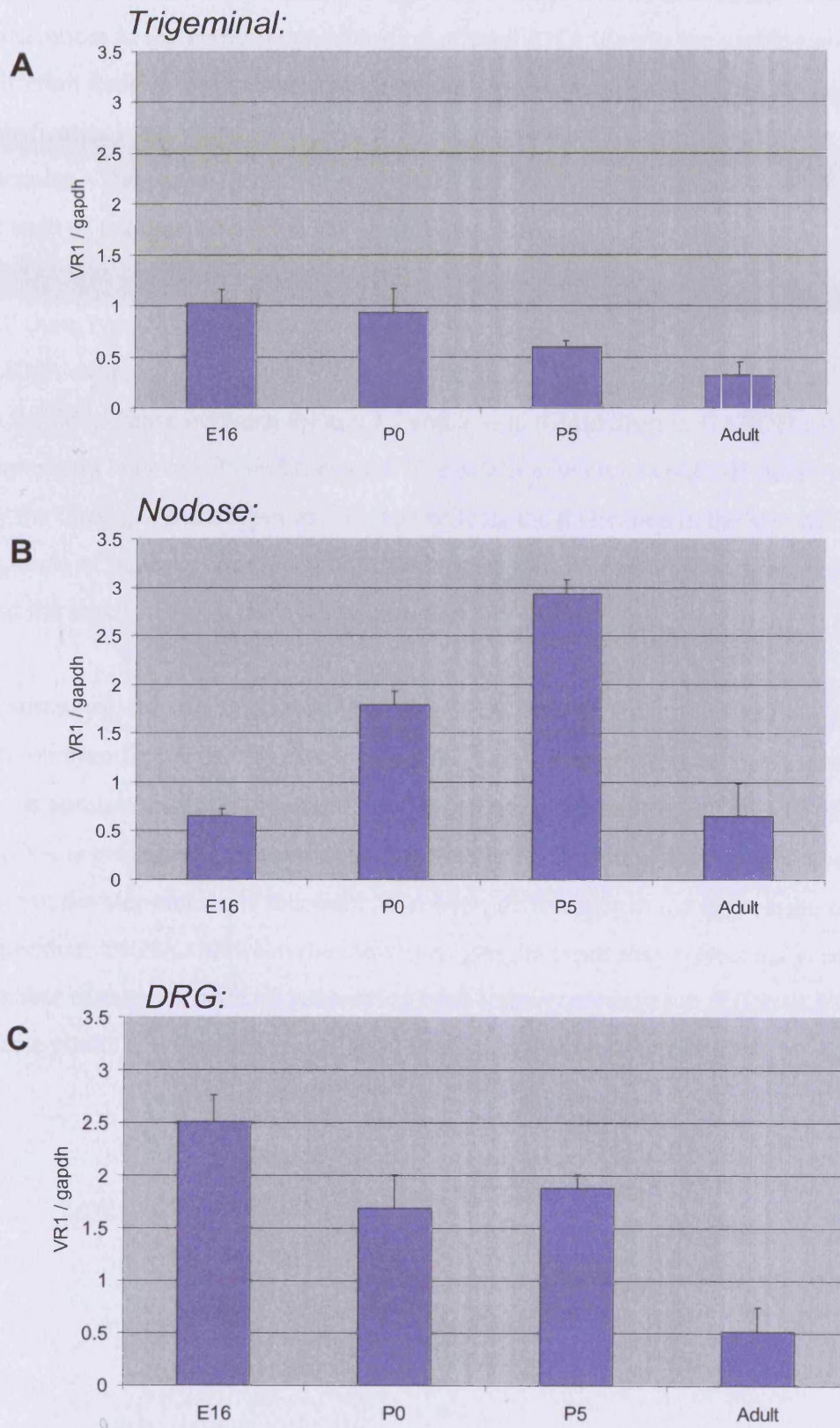


Figure 3.11. Expression of *VR1* mRNA in sensory neurons throughout development
Trigeminal ganglia (A), nodose ganglia (B) and DRG (C) were collected from E16, P0, P5 and adult mice and expression of *VR1* mRNA was quantified via real-time QPCR. Error bars = +/- standard error, n = 6

All mRNA levels were normalised against GAPDH mRNA to account for any differences in the starting concentration of total RNA (due to the varying size of the different ganglia and inconsistencies in the efficiency of RNA extraction and purification) and variations in the efficiency of reverse transcription between different samples. The expression level of GAPDH mRNA/ganglion in each type of ganglion at each of the ages studied is shown in figure 3.12.

All three types of ganglion showed a similar developmental pattern of GAPDH mRNA expression, with a 50-75% drop in GAPDH levels between E16 and P0, a 7- to 8-fold increase between P0 and P5 and a 3- to 4-fold drop in GAPDH mRNA expression between P5 and the adult. The relative levels of GAPDH mRNA expressed by the three ganglion types at each age reflects the difference in the size of the ganglia in terms of neuronal numbers, with the largest ganglion being the trigeminal ganglion and the smallest being the nodose ganglion.

In summary, the expression patterns of mRNA from all these predominantly sensory neuron specific genes, varied considerably. Some showed highest levels of expression in the adult, whilst others peaked at younger ages. The expression pattern of each mRNA is probably indicative of the discrete roles of each of these genes in sensory neuron development and function. Moreover, differences in the expression of individual mRNAs between the individual ganglia types may reflect differences in the number of sensory neurons subserving each sensory modality in different ganglia. These points are discussed more fully in the conclusion to this chapter.

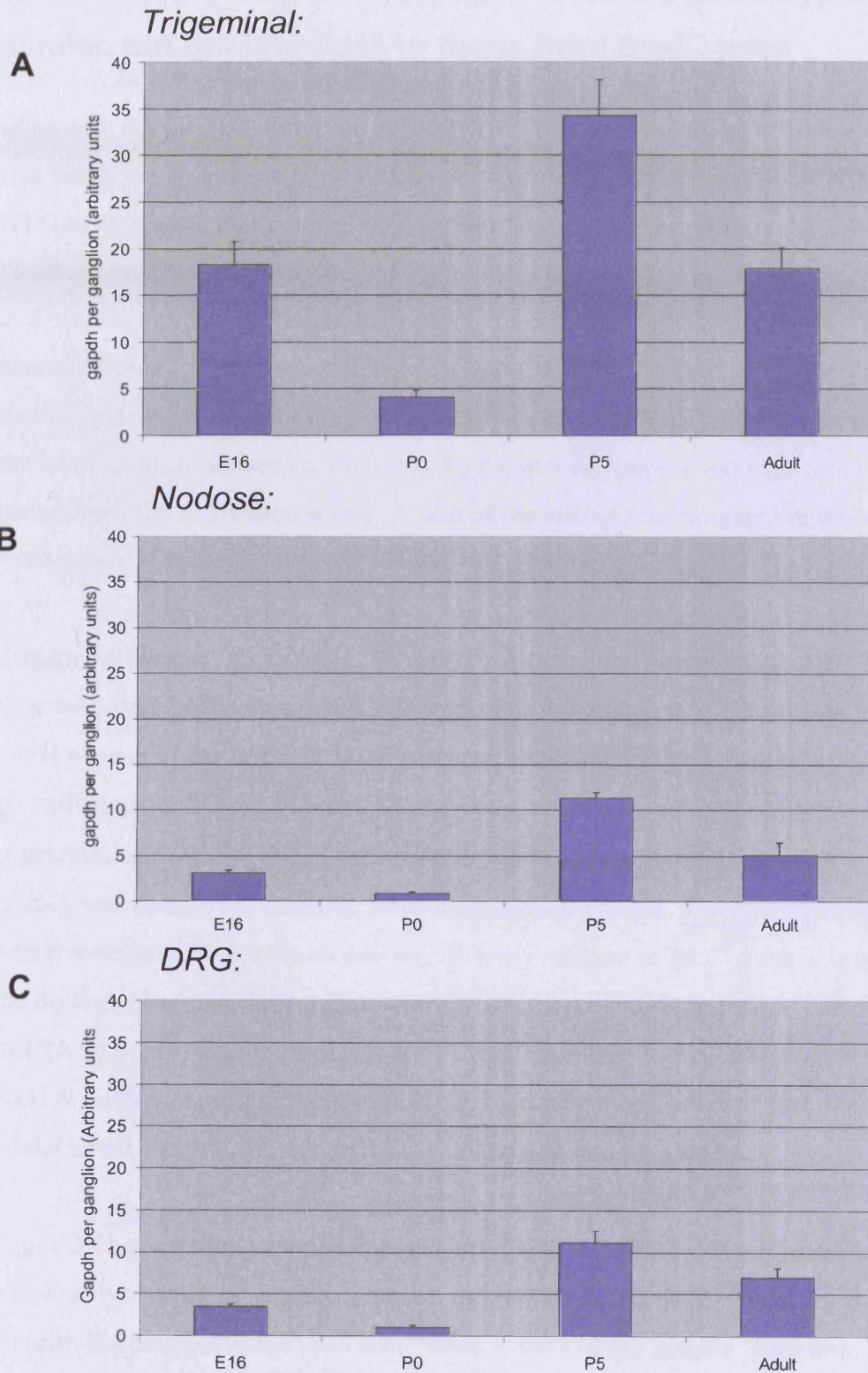


Figure 3.12. Expression of GAPDH mRNA per ganglion in sensory neurons throughout development

Trigeminal ganglia (A), nodose ganglia (B) and DRG (C) were collected from E16, P0, P5 and adult mice and expression of GAPDH mRNA was quantified via real-time QPCR and expression per ganglion calculated.

Error bars = +/- standard error, n = 6

3.3.2. Sensory neurons from $Bax^{-/-}$ mice are phenotypically similar, but not identical, to those from $Bax^{+/+}$ mice

Mice with double null mutations in either the TrkA or NT-3 locus, together with the Bax locus, were used in this chapter to obtain data reflecting the roles of NGF and NT-3 in regulating a number of sensory neuron mRNAs. As described above, by knocking out the gene encoding the proapoptotic protein Bax, the apoptotic cell death that normally occurs in the single TrkA or NT-3 null mutants is prevented and sensory neurons that are normally lost in the single null mutants survive. To ensure these surviving neurons are phenotypically similar to neurons from wild-type animals, and the relative ratios between phenotypically distinct sub-population of neurons are maintained, the expression levels of each of the mRNAs investigated in this chapter were assayed in ganglia from $Bax^{-/-}$ and wild-type mice

Trigeminal ganglia, nodose ganglia and DRG were dissected from P0 $Bax^{-/-}$ mice and their wild-type littermates. RNA was extracted, reverse transcribed and the expression levels of each of the mRNAs to be examined in the double null mutants was quantified using QPCR. Figure 3.13 shows the expression of α -CGRP mRNAs in trigeminal, nodose and dorsal root ganglia from $Bax^{-/-}$ and wild-type mice. α -CGRP mRNA was detected at a similar level in trigeminal ganglia, however in nodose and dorsal root ganglia expression was significantly reduced in $Bax^{-/-}$ mice. Expression of the Sodium channels Nav1.8 and Nav1.9 mRNAs are shown in figure 3.14. Nav1.8 mRNA was expressed at similar levels in $Bax^{-/-}$ and $Bax^{+/+}$ in all ganglia, however Nav1.9 mRNA expression was significantly reduced in the DRG of $Bax^{-/-}$ mice, whilst levels were unaffected in the trigeminal and nodose ganglia.

Figure 3.15 shows the expression of galanin (A) and PACAP (B) mRNAs in $Bax^{-/-}$ and wild-type ganglia. For each mRNA, expression levels were, on the whole, comparable between $Bax^{+/+}$ and $Bax^{-/-}$ mice in each of the ganglia. However, in DRG of $Bax^{-/-}$ mice, galanin mRNA expression was reduced by approximately 30%.

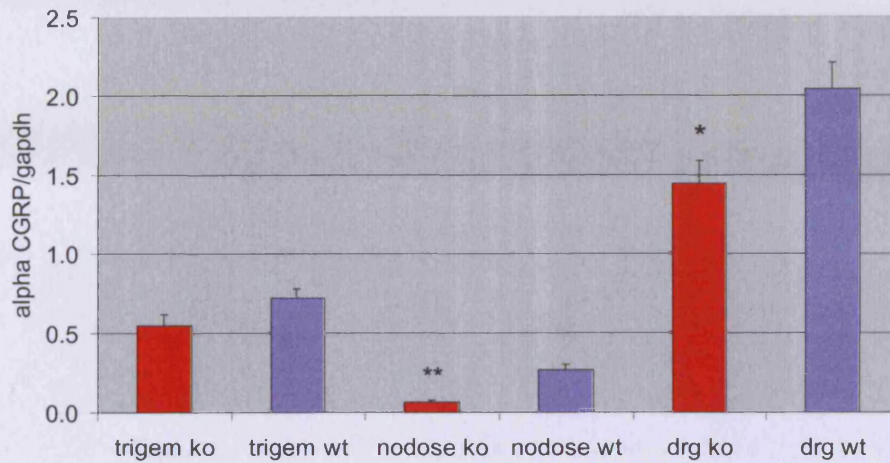


Figure 3.13. Expression of alpha CGRP mRNA in sensory neurons of *Bax* single knockout mice at P0

Trigeminal ganglia, nodose ganglia and DRG were dissected from P0 *Bax*^{-/-} mice and wild-type litter mates and expression of alpha CGRP mRNA was quantified using real-time QPCR. Expression was similar in trigeminal ganglia of both wild-type and *Bax*^{-/-} mice, however in the nodose and dorsal root ganglia mRNA was significantly lower in the double knockout.

Error bars = +/- standard error, n = 6. * = p = <0.05, ** = p = <0.01 as determined by two tailed unpaired t-test.

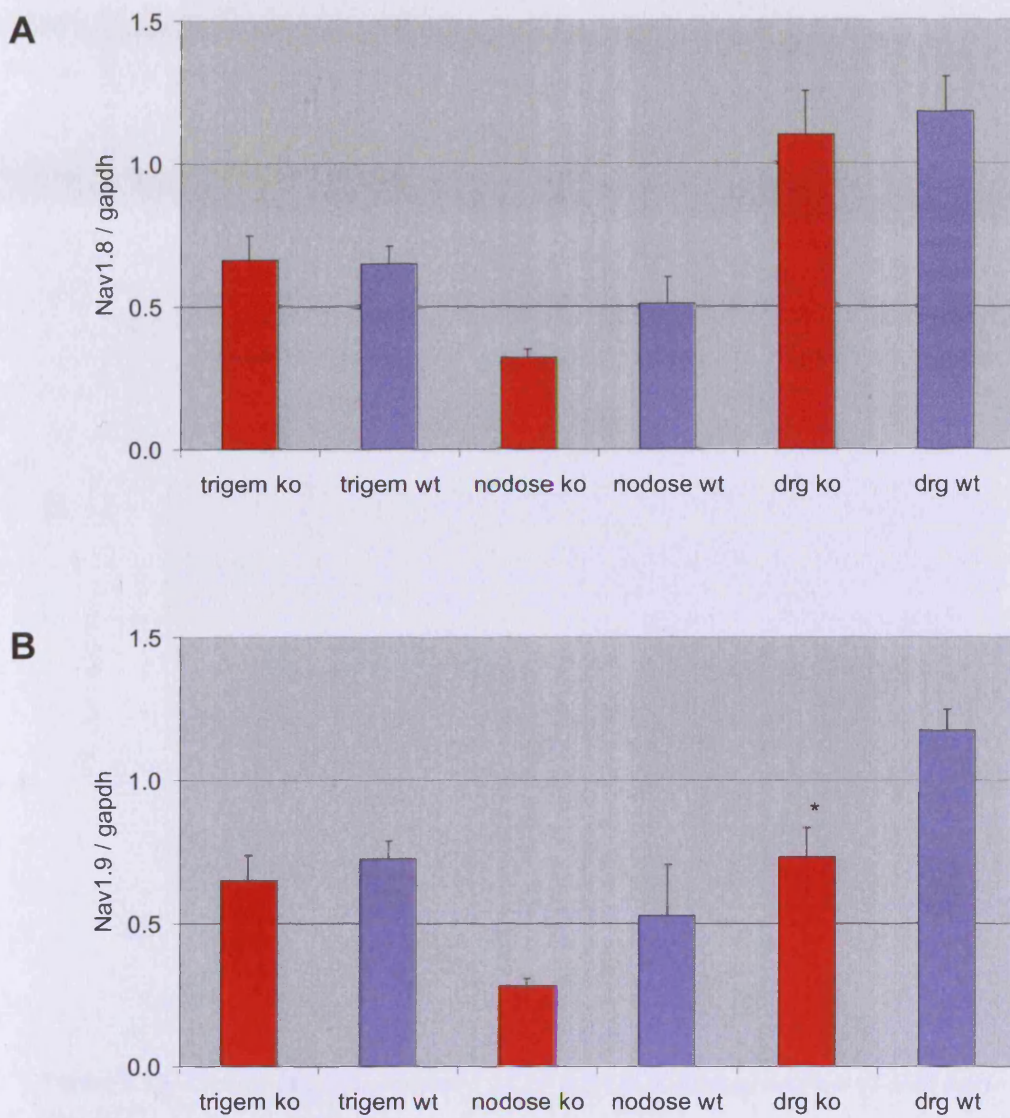


Figure 3.14. Expression of *Nav1.8* and *Nav1.9* mRNAs in sensory neurons of *Bax* single knockout mice at P0

Trigeminal ganglia, nodose ganglia and DRG were dissected from P0 *Bax*^{-/-} mice and wild-type litter mates and expression of *Nav1.8* (A) and *Nav1.9* (B) mRNAs were quantified using real-time QPCR. For the majority, expression was similar in both wild-type and *Bax*^{-/-} mice, however in the DRG, expression of *Nav1.9* mRNA was significantly lower in *Bax*^{-/-} mice.

Error bars = +/- standard error, n = 6. * = p < 0.05, ** = p < 0.01 as determined by two tailed unpaired t-test.

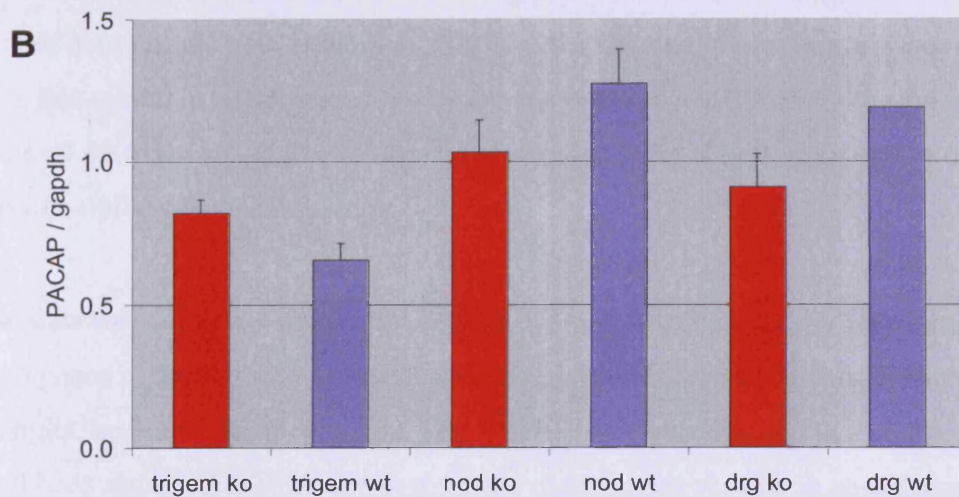
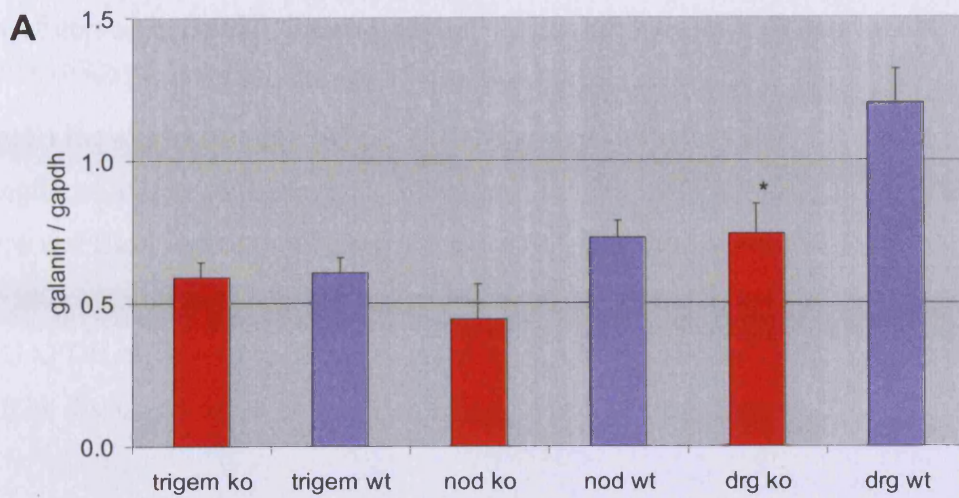


Figure 3.15. Expression of galanin and PACAP mRNAs in sensory neurons of *Bax* single knockout mice at P0

Trigeminal ganglia, nodose ganglia and DRG were dissected from P0 *Bax*^{-/-} mice and wild-type litter mates and expression of galanin (A) and PACAP (B) mRNAs were quantified using real-time QPCR.

Expression is similar in both wild-type and *Bax*^{-/-} in most cases, although in the DRG of *Bax*^{-/-} mice expression of galanin mRNA was significantly lower.

Error bars = +/- standard error, n = 6. * = p = <0.05, ** = p = <0.01 as determined by two tailed unpaired t-test.

VR1 mRNA was unaffected in trigeminal and dorsal root ganglia of $Bax^{-/-}$, however in the placode derived nodose ganglia, expression is reduced to approximately 50% of that observed in $Bax^{+/+}$ (figure 3.16).

Finally the expression of GAPDH mRNA was quantified in $Bax^{-/-}$ and wild-type ganglia, as shown in figure 3.17. Levels of GAPDH mRNA were similar in $Bax^{-/-}$ mice and $Bax^{+/+}$ mice in all ganglia, suggesting that little or no cell death occurred in the knockout mouse. Because of the reduction of cell death in $Bax^{-/-}$ mice, an increase in GAPDH mRNA in these mice might be expected. The lack of an apparent increase will be discussed below in the concluding section of this chapter

Although, on the whole, levels of mRNA expression were similar for the genes studied in $Bax^{-/-}$ and wild-type ganglia, significant differences in mRNA expression levels between the two genotypes do occur in some ganglia for some genes. For this reason, ganglia from $Bax^{-/-}$ mice and not true wild-type mice were used as a control when investigating the effects of neurotrophins on gene regulation using double-null mutant mice.

No data was collected for SP and β -CGRP. There was difficulty in detecting these two genes in these initial experiments, due to the low levels of expression in these ganglia, however due to cost and time limitations it was decided that sufficient data had been obtained to conclude that further experiments should go ahead using Bax single knockout litter-mates mice as a control comparison rather than wild-type mice.

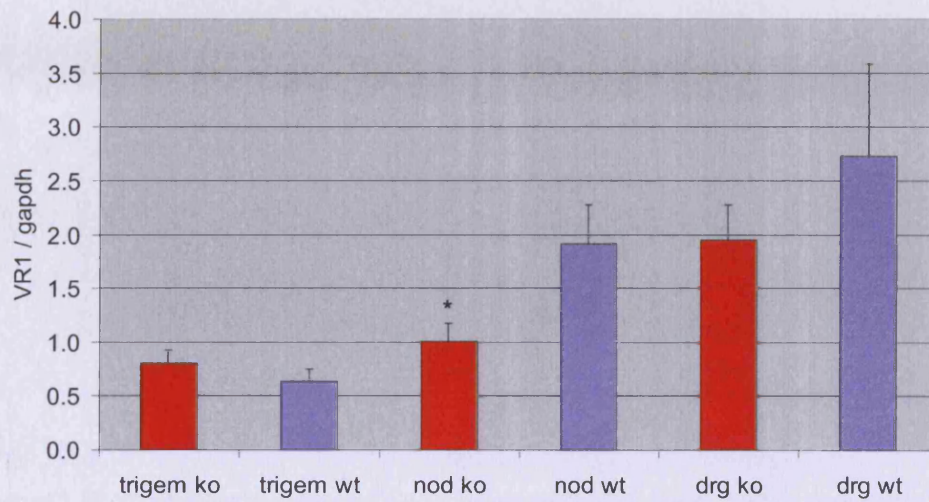


Figure 3.16. Expression of VR1 mRNA in sensory neurons of *Bax* single knockout mice at P0

Trigeminal ganglia, nodose ganglia and DRG were dissected from P0 *Bax*^{-/-} mice and wild-type litter mates and expression of VR1 mRNA was quantified using real-time QPCR.

Expression is similar in both wild-type and *Bax*^{-/-} in trigeminal and dorsal root ganglia, although in the nodose of *Bax*^{-/-} mice expression of VR1 mRNA is significantly lower in comparison to *Bax*^{+/+}.

Error bars = +/- standard error, n = 6. * = p = <0.05, ** = p = <0.01 as determined by two tailed unpaired t-test.

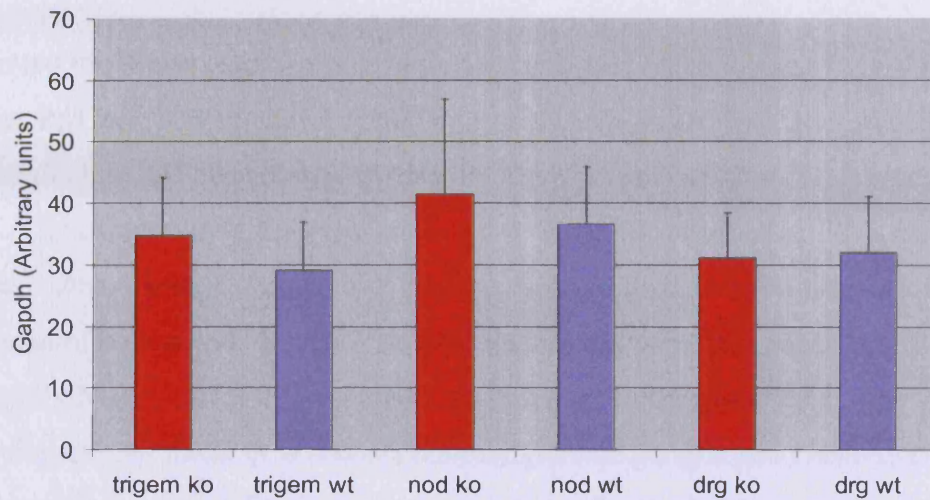


Figure 3.17. *Expression of gapdh mRNA in Trigeminal, DRG and nodose of Bax single knockout mice at P0*

Trigeminal ganglia, nodose ganglia and DRG were dissected from P0 $Bax^{-/-}$ and wild-type litter mates and expression of gapdh mRNA was quantified using real-time QPCR. No significant difference between wild-type and $Bax^{-/-}$ mice is obvious.

Error bars = \pm standard error, $n = 6$. * = $p = <0.05$, ** = $p = <0.01$ as determined by two tailed unpaired t-test.

3.3.3. The use of TrkA^{-/-}/Bax^{-/-} null mutant mice to study the role of TrkA signalling in gene expression in early postnatal sensory neurons.

Real time-QPCR was used to quantify the expression levels of α - and β -CGRP, SP, Galanin, PACAP, Nav1.8, Nav1.9 and VR1 mRNAs in trigeminal, nodose and dorsal root ganglia that had been dissected from P0 TrkA^{-/-}/Bax^{-/-} and Bax^{-/-} mice using RT-QPCR. The expression of both isoforms of CGRP was determined in P0 TrkA^{-/-}/Bax^{-/-} and Bax^{-/-} mice (figure 3.18). By P0, the data clearly shows that the normal levels of expression of both α - and β -CGRP mRNA are reliant upon TrkA signalling (and presumably NGF induced TrkA signalling), as a significant reduction in the expression of both mRNAs was observed in trigeminal ganglia and DRG of TrkA^{-/-}/Bax^{-/-} neonates compared to Bax^{-/-} neonates (figure 3.18). Nodose ganglia also showed a significant reduction in α -CGRP mRNA expression in the double null mutant compared to the single Bax knockout. β -CGRP mRNA levels were also reduced in nodose ganglia of TrkA^{-/-}/Bax^{-/-} mice compared to the single Bax null mutant, although this drop was not quite statistically significant. The reduction of β -CGRP mRNA levels, as a result of the loss of TrkA signalling, is less dramatic than the reduction in α CGRP mRNA levels in all three sensory ganglia.

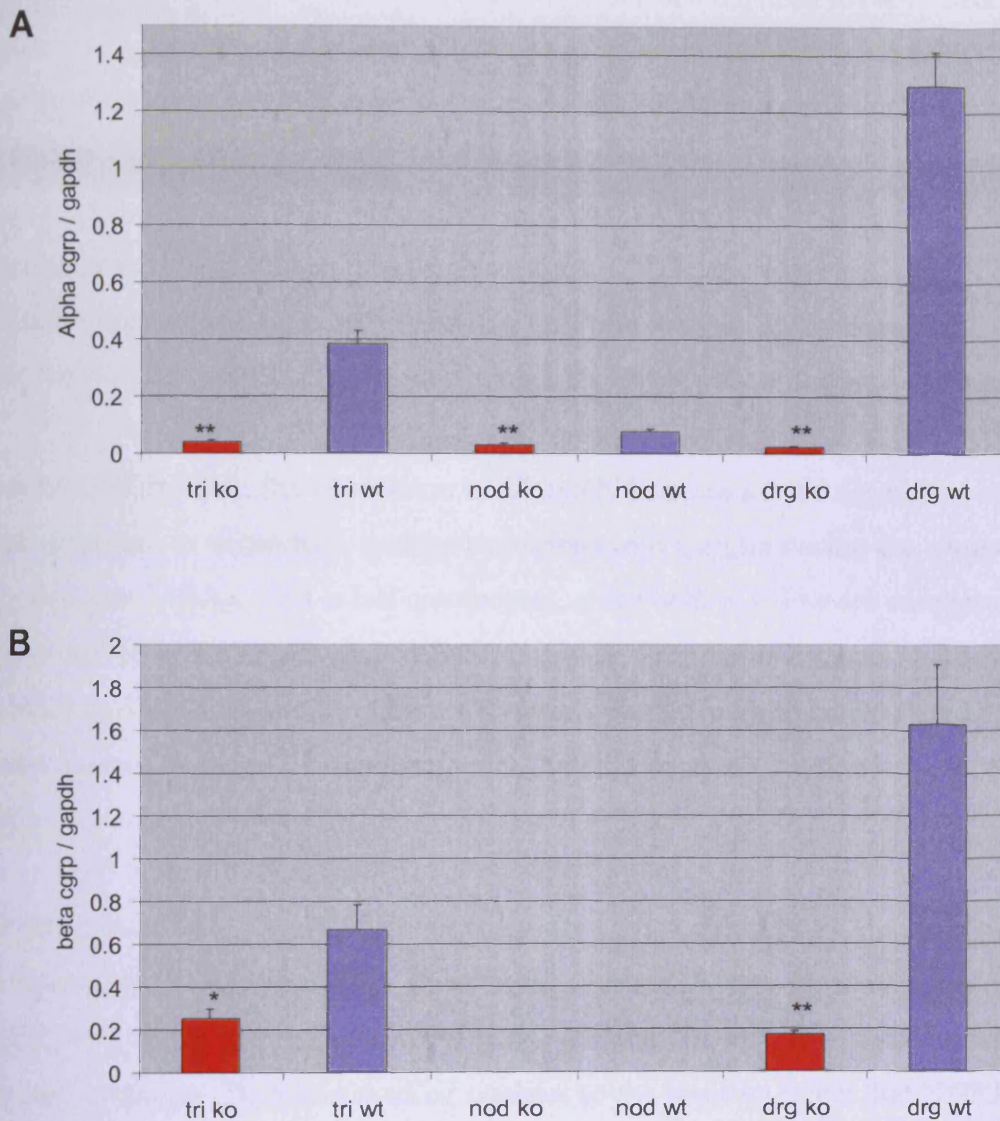


Figure 3.18. Expression of alpha CGRP and beta CGRP mRNAs in sensory neurons of P0 *TrkA^{-/-}/Bax^{-/-}* mice

Trigeminal ganglia, nodose ganglia and DRG were dissected from P0 *Bax^{-/-}/TrkA^{-/-}* and also *Bax^{-/-}* mice, and expression of alpha CGRP (A) and beta CGRP(B) mRNAs were quantified by real-time QPCR.

Expression of both alpha and beta CGRP mRNA were significantly reduced in sensory ganglia of the *TrkA^{-/-}/Bax^{-/-}* mice.

N.B. where wt actually represents data from *Bax* single knockout mice.

Error bars +/- standard error, n = 6 - 8. * = p = <0.05, ** = p = <0.01 as determined by two-tailed unpaired t-test.

Figure 3.19 shows the expression of Nav1.8 and Nav1.9 mRNAs in P0 sensory ganglia from both genotypes. In accordance with the embryonic data presented in chapter 2, the normal levels of expression of both mRNAs was dependent upon TrkA signalling as illustrated by the significant reduction in mRNA levels observed in both trigeminal ganglia and DRG of TrkA^{-/-}/Bax^{-/-} neonates compared to Bax^{-/-} neonates (figure 3.19.). Interestingly, although nodose ganglia contain a sub-population of NGF responsive neurons, no difference in the levels of sodium channel mRNAs was apparent between nodose ganglia from the two genotypes. This would suggest that either the expression of both these mRNAs is not confined to the subset of TrkA expressing, NGF responsive, nodose neurons, or, if it is, the expression of TTX resistant sodium channels is regulated in a different manner in these neurons compared to TrkA expressing neurons within the trigeminal and dorsal root ganglia.

Like α -CGRP mRNA, the expression of SP mRNA appears to be dependent upon TrkA signalling in trigeminal, nodose and dorsal root ganglia during the neonatal period (figure 3.20A). This is not unexpected, since both mRNAs are co-expressed predominantly in the same sub-population of NGF responsive sensory neurons during postnatal development and both have previously been shown to be regulated in a similar manner in the adult (Lundberg et al., 1985; Lee et al., 1985; Skofitsch et al., 1985)

The data presented in figure 3.20 B suggests that TrkA signalling does not regulate the expression of PACAP mRNA in neonatal trigeminal, nodose, or dorsal root ganglia, with levels of PACAP mRNA being comparable in TrkA^{-/-}/Bax^{-/-} neonates and Bax^{-/-} neonates. This is in marked contrast to the positive effect that NGF has on PACAP mRNA expression in adult rat nociceptive neurons (Jongsma Wallin et al., 2001, 2003).

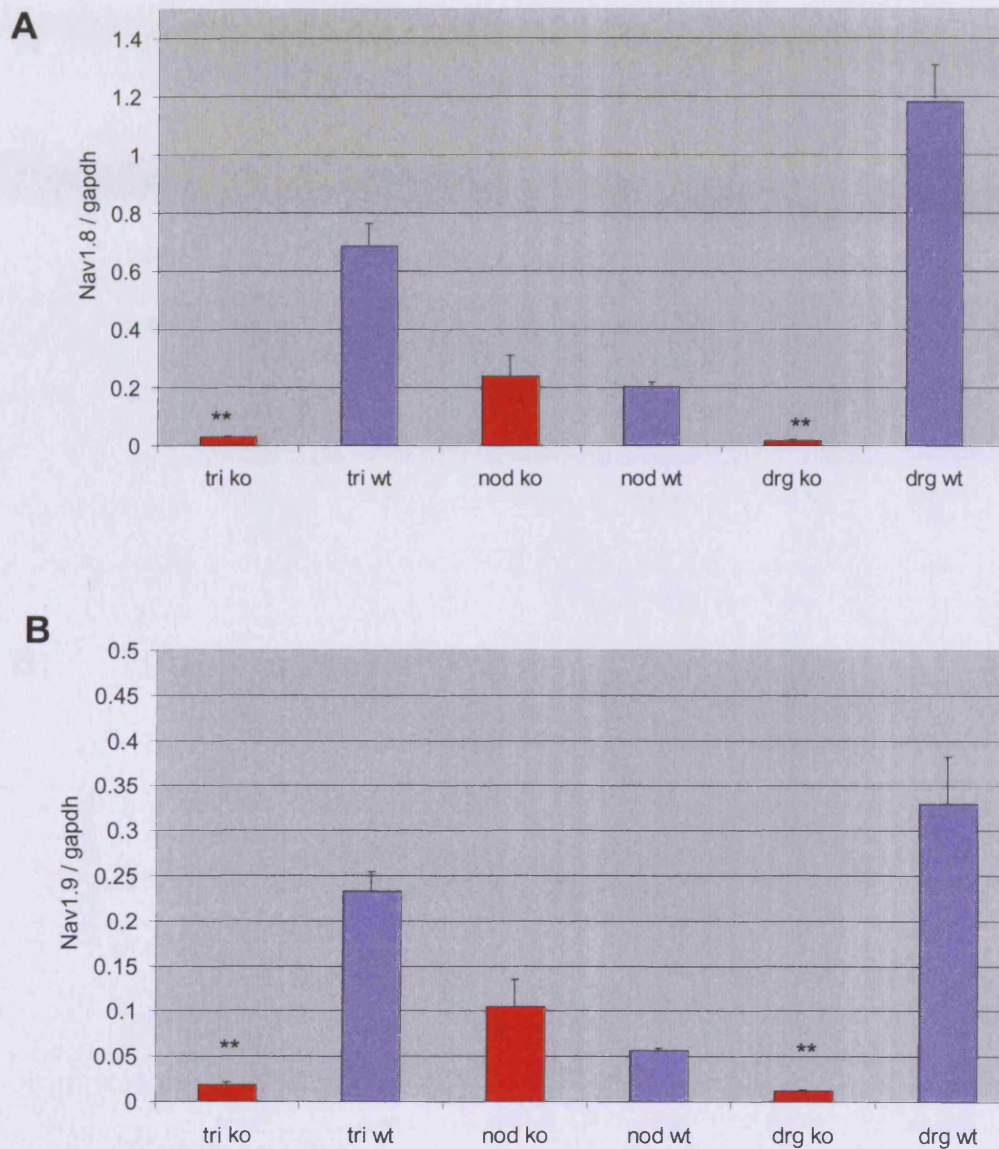


Figure 3.19. Expression of *Nav1.8* and *Nav1.9* mRNAs in sensory neurons of P0 *TrkA^{-/-}/Bax^{-/-}* mice

Trigeminal ganglia, nodose ganglia and DRG were dissected from P0 *Bax^{-/-}/TrkA^{-/-}* and also *Bax^{-/-}* mice, and expression of *Nav1.8* (A) and *Nav1.9* (B) mRNAs quantified by real-time QPCR. Expression of both *Nav1.8* and *Nav1.9* mRNA was dramatically down-regulated in trigeminal ganglia and DRG of the *TrkA^{-/-}/Bax^{-/-}* mice. No alteration of expression was observed in the nodose ganglia.

N.B. where wt actually represents data from *Bax* single knockout mice.

Error bars +/- standard error, n = 6 - 8. * = p = <0.05, ** = p = <0.01 as determined by two-tailed unpaired t-test

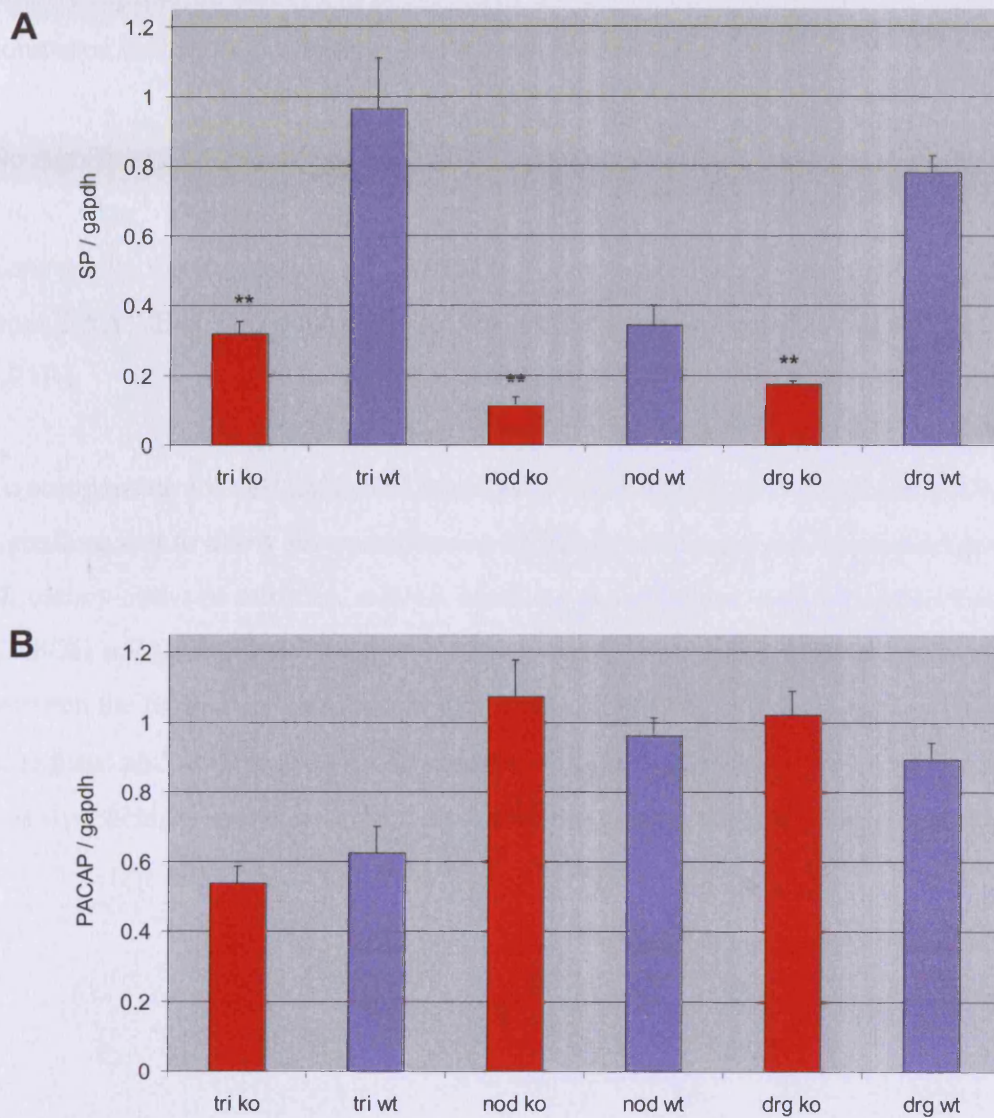


Figure 3.20 Expression of SP and PACAP mRNA in sensory neurons of *TrkA*^{-/-}/*Bax*^{-/-} mice

Trigeminal ganglia, nodose ganglia and DRG were dissected from P0 *TrkA*^{-/-}/*Bax*^{-/-} and *Bax*^{-/-} mice, and expression of SP (A) and PACAP (B) mRNAs were quantified by real time QPCR.

N.B. Where wt actually represents data from *Bax* single knockout mice.

Error bars +/- standard error, n = 6 - 8. * = p = <0.05, ** = p = <0.01 as determined by two-tailed unpaired t-test.

The expression levels of galanin and VR1 mRNAs in P0 sensory ganglia from the two genotypes was also explored (figure 3.21). Galanin mRNA expression levels were the same in the trigeminal and nodose ganglia of double null neonatal mice as they were in $Bax^{-/-}$ neonates (figure 3.21A). However, a small but significant decrease in galanin mRNA expression levels was observed in the DRG of double mutant neonates compared to the DRG of single Bax mutant neonates.

No significant differences in VR1 mRNA expression levels were apparent between $TrkA^{-/-}/Bax^{-/-}$ and $Bax^{-/-}$ neonates in both trigeminal ganglia and DRG (figure 3.21B). Conversely, the expression of VR1 mRNA was markedly higher in nodose ganglia from $TrkA^{-/-}/Bax^{-/-}$ P0 pups compared to nodose ganglia from $Bax^{-/-}$ pups (figure 3.21B).

To compensate for cell death that may occur in the absence of functional $TrkA$ signalling and to allow for variations in RNA extraction and reverse transcription efficiency between samples, mRNA levels were normalised against expression of GAPDH mRNA (shown in figure 3.22). No significant difference was observed between the different genotypes in the levels of GAPDH mRNA expressed in P0 trigeminal and nodose ganglia. In contrast, the expression of GAPDH mRNA in DRG was significantly lower in double-knockout neonates compared to $Bax^{-/-}$ neonates.

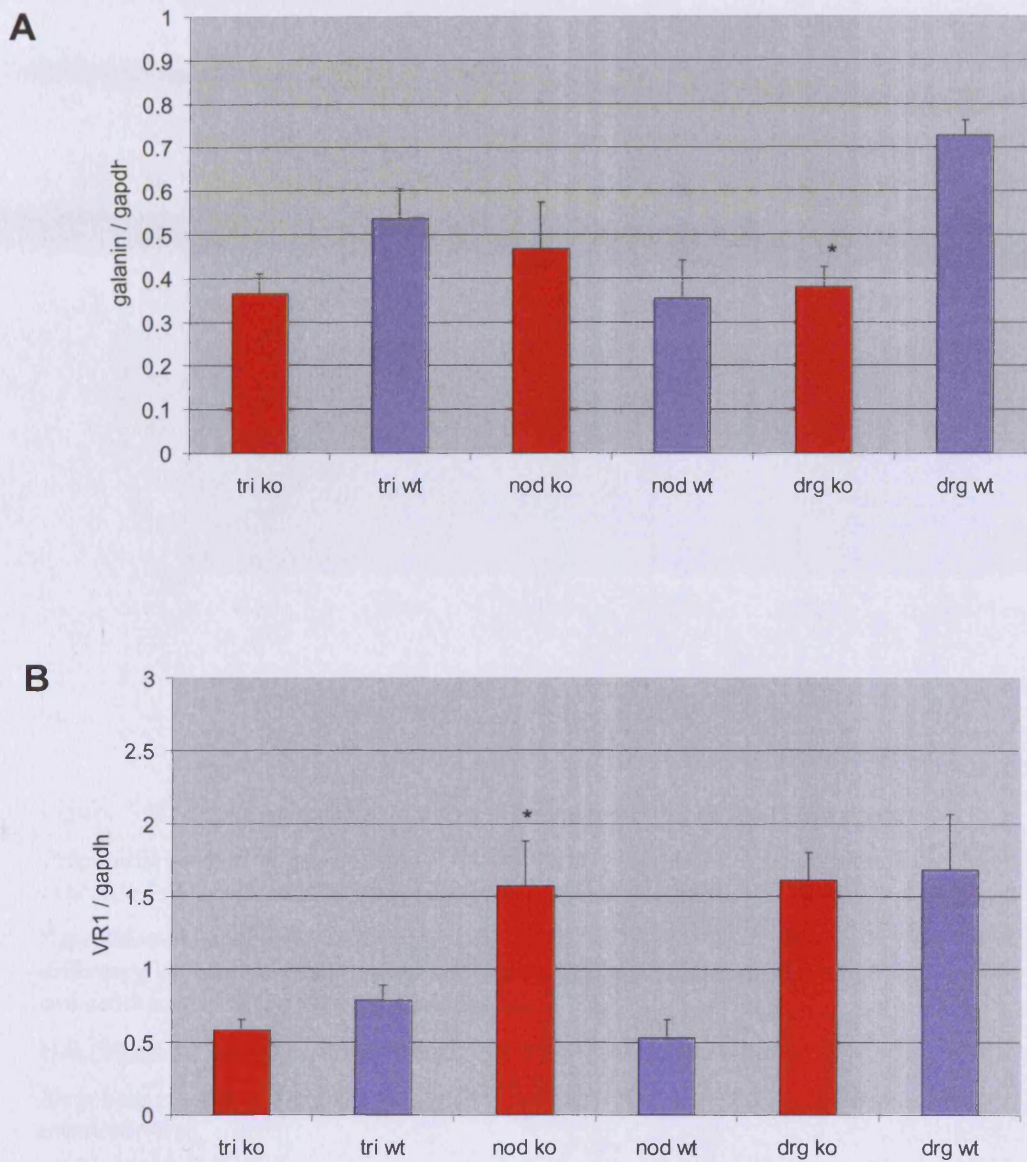


Figure 3.21. Expression of galanin and VR1 mRNA in sensory neurons of *TrkA*^{-/-}/*Bax*^{-/-} mice

Trigeminal ganglia, nodose ganglia and DRG were dissected from P0 *TrkA*^{-/-}/*Bax*^{-/-} and *Bax*^{-/-} mice, and expression of galanin (A) and VR1(B) mRNAs were quantified by real time QPCR.

Expression of galanin mRNA was significantly decreased in DRG of *TrkA*^{-/-}/*Bax*^{-/-} mice, it is however still expressed at a relatively high level in the null mutant. No difference was observed in expression in trigeminal or nodose ganglia. Expression of VR1 mRNA was upregulated in nodose ganglia of *TrkA*^{-/-}/*Bax*^{-/-} mice, no difference in mRNA expression was observed in trigeminal ganglia or DRG.

N.B. Where wt actually represents data from *Bax* single knockout.

Error bars +/- standard error, n = 6 - 8. * = p = <0.05, ** = p = <0.01 as determined by two-tailed unpaired t-test.

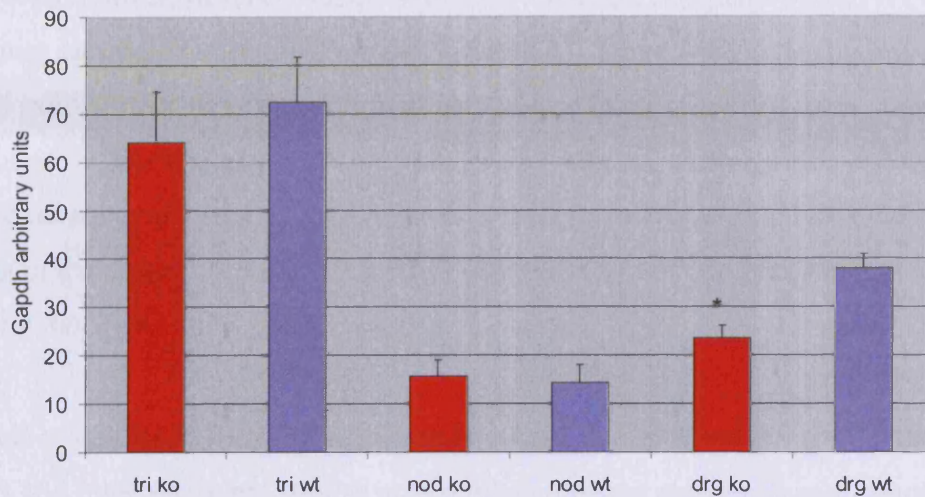


Figure 3.22. Expression of *gapdh* mRNA in sensory neurons of *TrkA^{-/-}/Bax^{-/-}* mice

Trigeminal ganglia, nodose ganglia and DRG were dissected from *TrkA^{-/-}/Bax^{-/-}* and *Bax^{-/-}* mice and expression of *Gapdh* mRNA was quantified by real time QPCR.

Expression of *gapdh* was significantly less in DRG of *TrkA^{-/-}/Bax^{-/-}* than single *Bax^{-/-}* mice, but no difference is observed in trigeminal or nodose ganglia. The decreased level of *gapdh* mRNA, suggests cell death in the DRG of double knockout mice.

N.B. Where wt actually represents data from *Bax* single knockout mice.

Error bars +/- standard error, n = 6 – 8. * = p = <0.05 ** = p = <0.01 as determined by two-tailed unpaired t-test.

3.3.4. The use of NT-3^{-/-}/Bax^{-/-} null mutant mice to study the role of NT-3 in gene expression in early postnatal sensory neurons

The expression levels of GAPDH mRNA in the three sensory ganglia from NT-3^{-/-}/Bax^{-/-} mice and Bax^{-/-} mice are shown in figure 3.23. There was markedly less GAPDH mRNA expressed by trigeminal ganglia and DRG dissected from double knockout neonates compared to those from Bax^{-/-} neonates, although this difference only reaches statistical significance in the case of trigeminal ganglia. No difference was seen in the amount of GAPDH mRNA expressed by nodose ganglia from the two different genotypes

The levels of α -CGRP, β -CGRP (figure 3.24), Nav1.8, Nav1.9 (figure 3.25) and SP (figure 3.26A) mRNAs were similar in trigeminal, nodose and dorsal root ganglia of NT-3^{-/-}/Bax^{-/-} and Bax^{-/-} neonates, suggesting that the expression of these mRNAs is not regulated by NT-3 at P0.

Although PACAP mRNA (figure 3.26B) levels were similar in nodose ganglia from single and double transgenic P0 pups, there was a marked increase in the expression of PACAP mRNA in trigeminal and dorsal root ganglia in the absence of NT-3. In the case of DRG, this increase did not quite reach statistical significance ($p = 0.061$). Similarly, the expression levels of VR1 mRNA were also greater in both DRG and trigeminal ganglia dissected from the double knockout neonatal mice compared to those from Bax^{-/-} neonates (figure 3.27A). There was no difference in the expression of VR1 mRNA between the nodose ganglia of the two genotypes.

In the case of galanin mRNA, significantly higher levels of expression were found in trigeminal ganglia from NT-3^{-/-}/Bax^{-/-} neonatal mice than trigeminal ganglia from Bax^{-/-} neonates (figure 3.27B). In contrast, there was no significant difference between the two mouse strains in the expression of galanin mRNA in DRG and nodose ganglia (figure 3.27B).

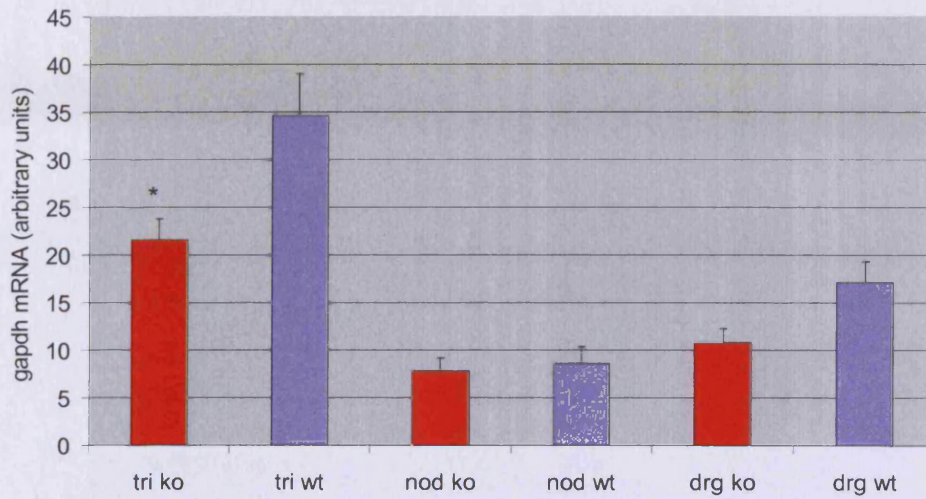


Figure 3.23. Expression of GAPDH mRNA in sensory neurons of *NT-3^{-/-}/Bax^{-/-}* mice

Trigeminal ganglia, nodose ganglia and DRG were dissected from P0 *NT-3^{-/-}/Bax^{-/-}* and *Bax^{-/-}* mice and expression of gapdh mRNA quantified by real time PCR.

N.B. Where wt actually represents data from *bax* single knockout mice.

Error bars = +/- standard error, n = 5 – 8. * = p < 0.05, ** = p < 0.01

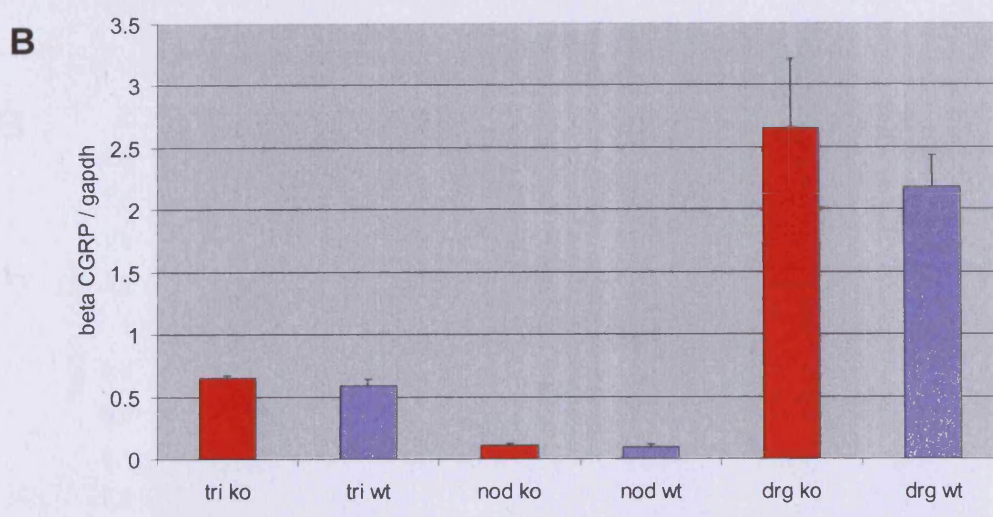
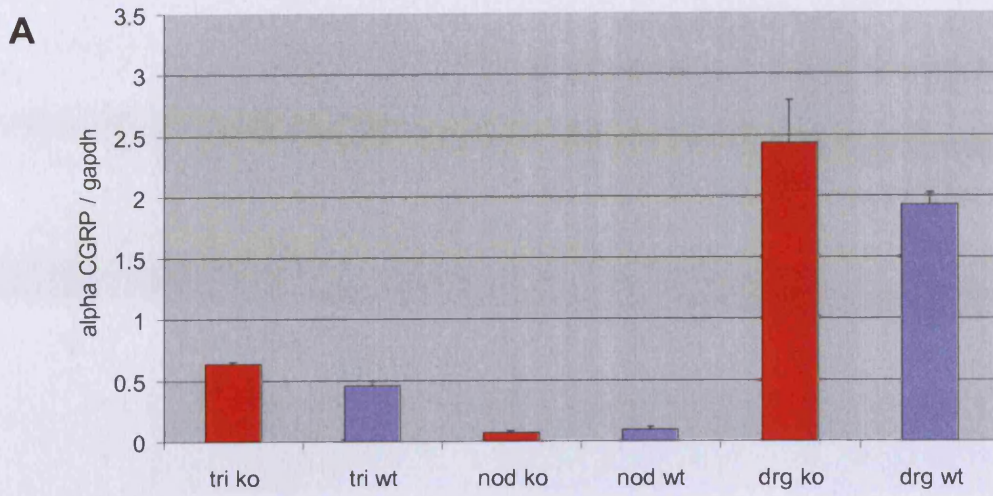


Figure 3.24. Expression of alpha CGRP and beta CGRP mRNAs in sensory neurons of *NT-3^{-/-}/Bax^{-/-}* mice

Trigeminal ganglia, nodose ganglia and DRG were dissected from P0 *NT-3^{-/-}/Bax^{-/-}* and *Bax^{-/-}* mice and expression of alpha CGRP (A) and beta CGRP (B) mRNAs were quantified by real time QPCR.

N.B. Where wt actually represents data from *bax* single knockout mice.

Error bars +/- standard error, n = 5 - 8. * = p = <0.05, ** = p = <0.01 as determined by two-tailed unpaired t-test.

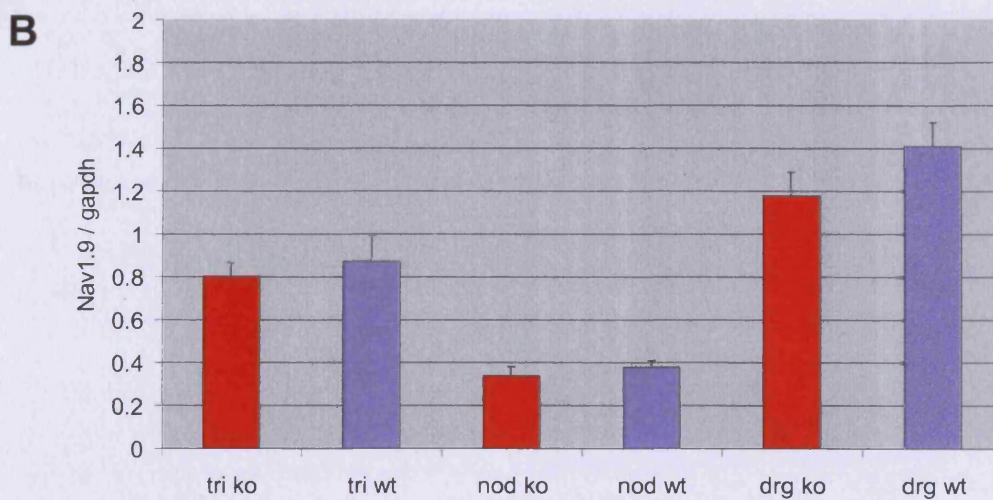
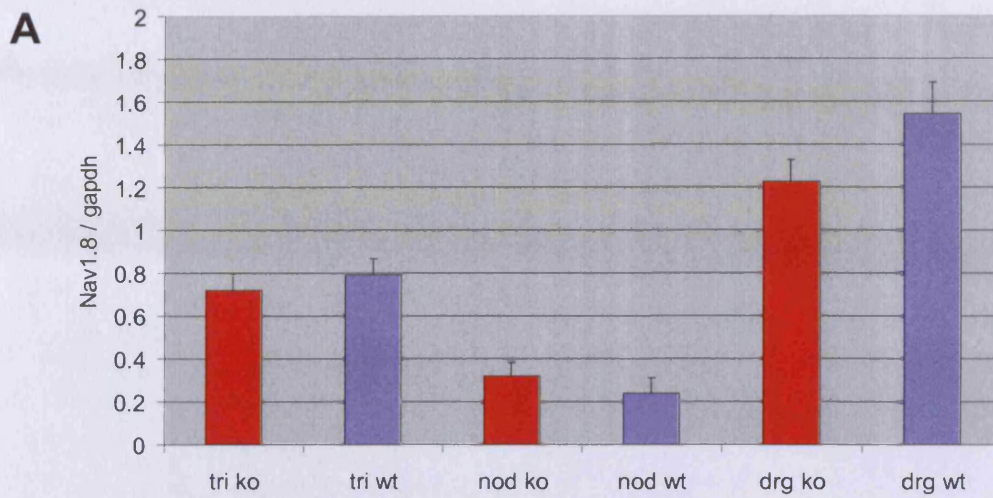


Figure 3.25. Expression of *Nav1.8* and *Nav1.9* in sensory neurons of *NT-3^{-/-}/Bax^{-/-}* mice

Trigeminal ganglia, nodose ganglia and DRG were dissected from P0 *NT-3^{-/-}/Bax^{-/-}* and *Bax^{-/-}* mice and expression of alpha *Nav1.8* (A) and *Nav1.9* (B) mRNAs quantified by real time QPCR.

N.B. Where wt actually represents data from *bax* single knockout mice.

Error bars +/- standard error, n = 5 - 8 . * = p = <0.05, ** = p = < 0.01 as determined by two-tailed unpaired t-test.

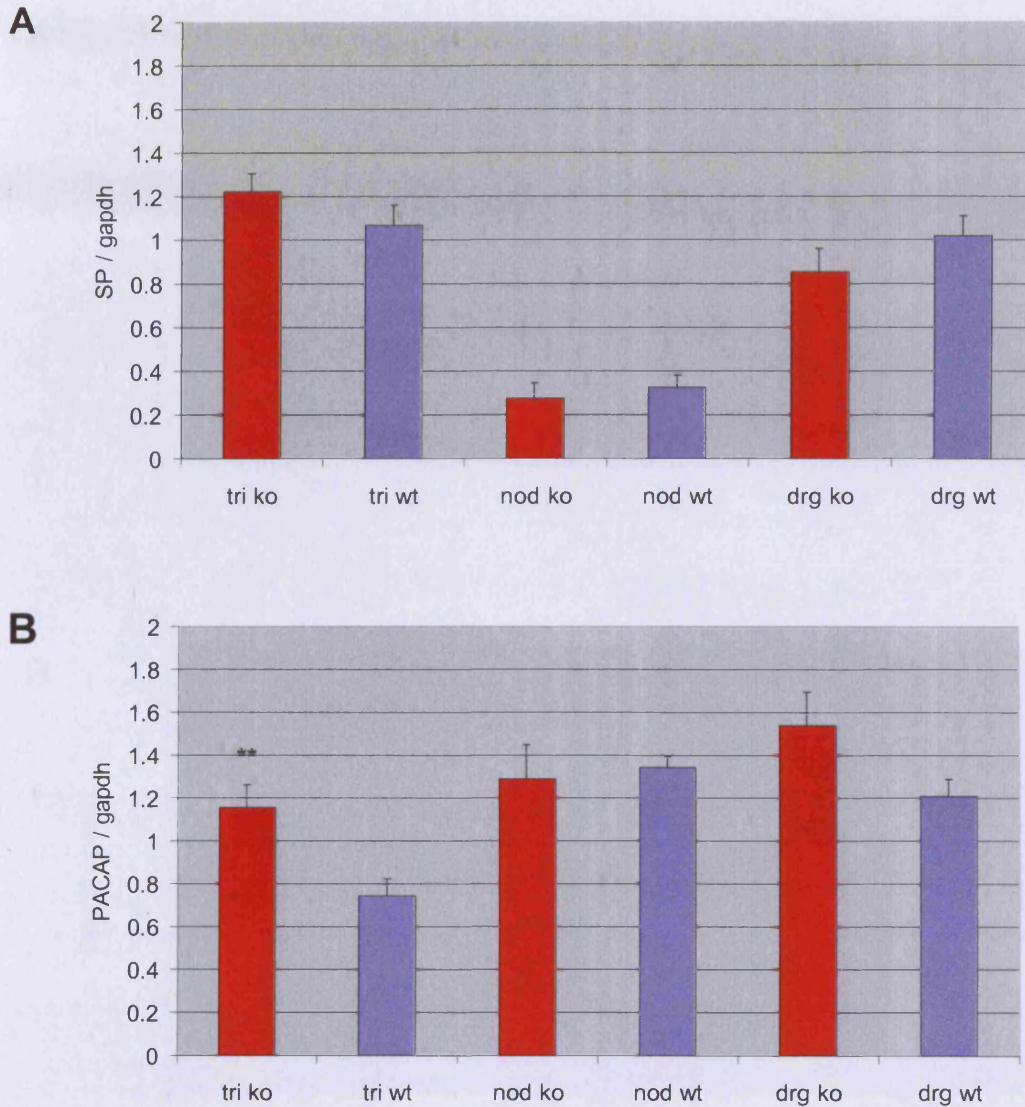


Figure 3.26. Expression of SP and PACAP mRNAs in sensory neurons of NT-3^{-/-}/Bax^{-/-} mice

Trigeminal ganglia, nodose ganglia and DRG were dissected from P0 NT-3^{-/-}/Bax^{-/-} and Bax^{-/-} mice and expression of SP (A) and PACAP (B) mRNAs were quantified by real time QPCR.

N.B. Where wt actually represents data from bax single knockout mice.

Error bars +/- standard error, n = 5-8. * = p = <0.05, ** = p = < 0.01 as determined by two-tailed unpaired t-test.

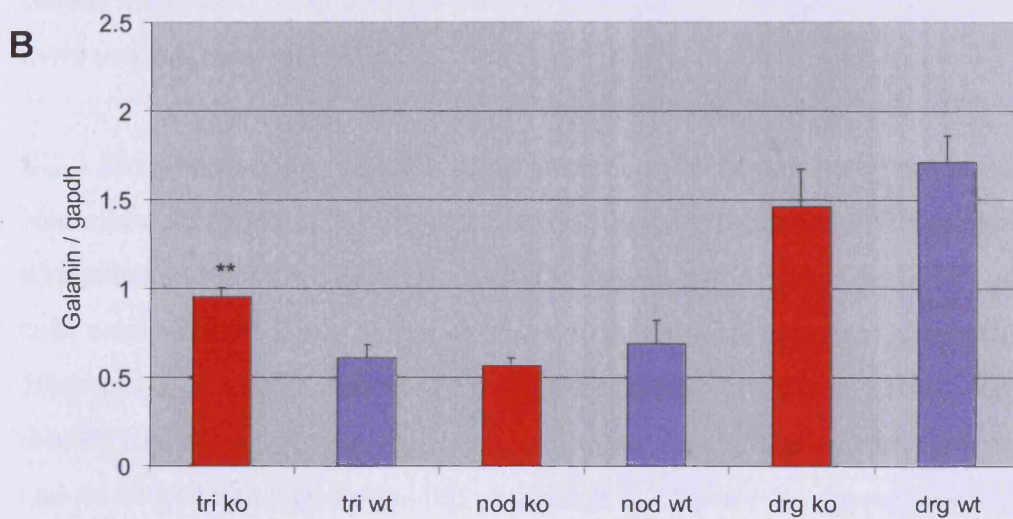
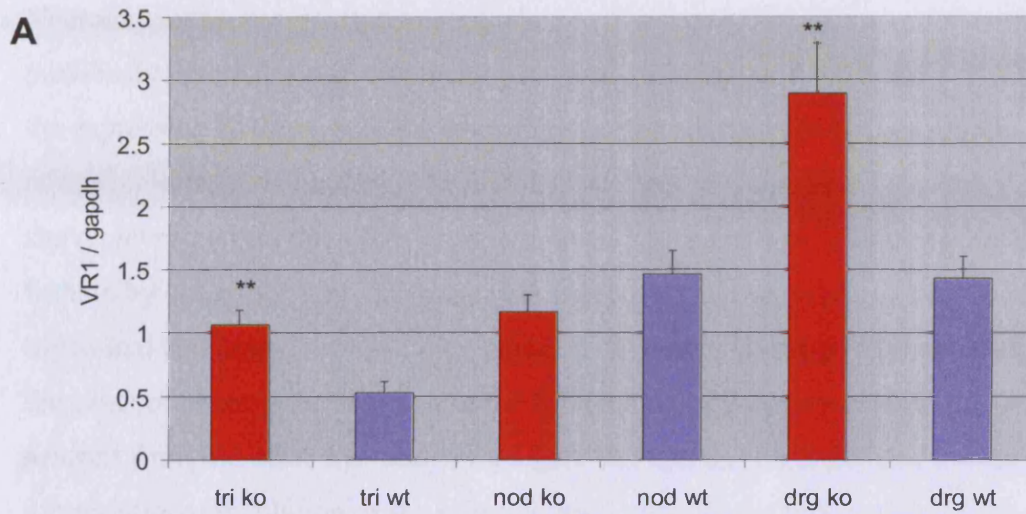


Figure 3.27. Expression of *VR1* and *galanin* mRNAs in sensory neurons of *NT-3^{-/-}/Bax^{-/-}* mice

Trigeminal ganglia, nodose ganglia and DRG were dissected from P0 *NT-3^{-/-}/Bax^{-/-}* and *Bax^{-/-}* mice and expression of *VR1* (A) and *galanin* (B) mRNAs were quantified by real time QPCR.

N.B. Where wt actually represents data from *bax* single knockout mice.

Error bars +/- standard error, n = 5 – 8. * = p = <0.05, ** = p = < 0.01 as determined by two-tailed unpaired t-test.

3.3.5. The use of primary neuronal cultures to explore the role of NGF and NT-3 in gene expression in early postnatal sensory neurons

Neuronal cultures were used to study the *in-vitro* expression of the mRNAs that were previously investigated in transgenic neonates to ascertain if NGF and NT-3 regulate the expression of these mRNAs when the neurons are cultured independently of their normal cellular environment. The previous analysis of transgenic neonates, detailed above, revealed that the mRNA levels of any given gene were modulated in a similar fashion by either NT-3 or TrkA dependent neurotrophic factor signalling in both trigeminal and dorsal root ganglion neurons. In contrast, transcriptional regulation of the panel of genes was often markedly different in nodose neurons compared to neurons from the other two sensory ganglia. For this reason, I decided to compare the transcriptional regulation of the selected panel of genes by neurotrophic factors only in cultured trigeminal and nodose ganglia neurons (trigeminal ganglia neurons were chosen rather than DRG neurons because P0 trigeminal ganglia contain significantly more neurons than P0 DRG).

CD-1 litters were collected at birth and cultures were set up as outlined in 3.2.3. Neurons were plated onto poly-ornithine/ laminin pre-treated dishes and placed into an incubator (5% CO₂, 37°C). In the case of trigeminal ganglion neuronal cultures, cells were cultured for 0, 24 and 48 hours, in either the presence or absence of 10ng/ml NT-3 or NGF. Because P0 nodose ganglia contain significantly less neurons than P0 trigeminal ganglia, only two time points, 0 and 24 hours, were examined for nodose neuron cultures. Following incubation at 37°C for the appropriate time period, the neuronal cultures were lysed by the addition of a chaotropic RNA extraction buffer, RNA was purified with the RNeasy kit and mRNA expression was quantified using RT- QPCR.

3.3.5.1. Survival of cultured P0 trigeminal and nodose neurons

Caspase Inhibitors were included in all culture dishes to prevent cell death in the absence of neurotrophic support confounding the interpretation of mRNA expression

data. To ensure that neuronal survival was similar in all the culture conditions, the number of healthy neurons in each culture was counted at 24 hours and/or 48 hours (depending on the neuron type) and neuronal survival was expressed as a percentage of the number of neurons in each culture at 3 hours (3 hours was chosen as the time point for the initial neuronal count as this period allowed time for neurons to adhere to the culture dishes and therefore be easily identified by their round phase bright appearance).

The survival of both trigeminal and nodose ganglia neurons, in the presence and absence of NGF, is shown in figure 3.28. Approximately all trigeminal neurons survive for 48 hours when cultured in the presence of caspase inhibitors and NGF, as would be anticipated. However, the number of surviving neurons falls to 80% of the initial starting number by 24 hours and 60% by 48 hours in the presence of caspase inhibitors alone. A similar decrease in the number of surviving neurons at 24 hrs (to 60% of initial numbers) was seen for all conditions in the case of nodose ganglia neurons, which are largely dependent upon BDNF for survival at this age. Although there was significant death of nodose and trigeminal neurons in culture in the presence of caspase inhibitors alone (due to a lack of appropriate neurotrophic factor support), survival is much improved from the cell death that was seen in the absence of caspase inhibitor support. In the case of trigeminal neurons, approximately 30% trigeminal neurons survived after 24 hours with no factors, and by 48 hours this was down to 5%. In the case of nodose neurons, approximately only 25% of neurons survived in the absence of neurotrophic factors and caspase inhibitors following 24 hours in culture.

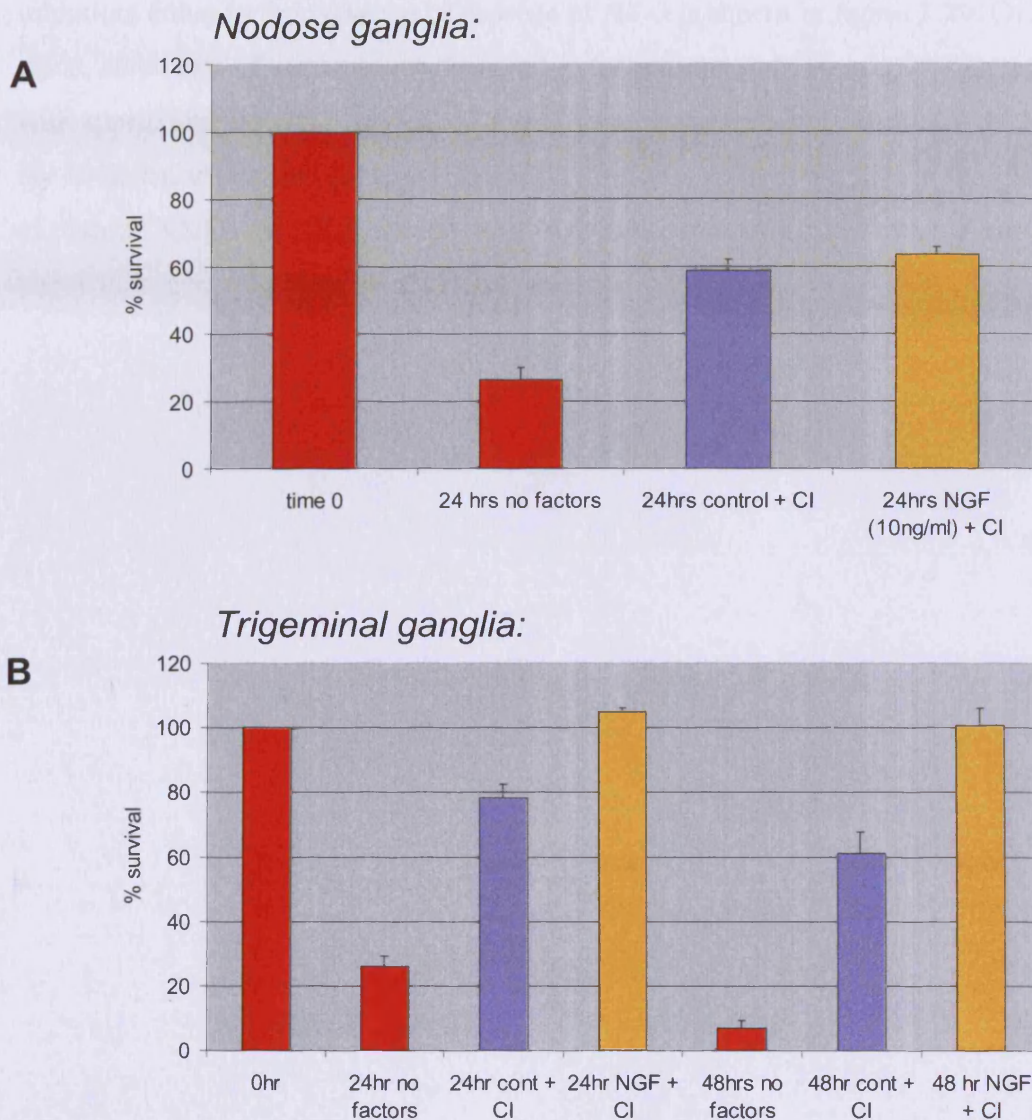


Figure 3.28. Survival of P0 nodose ganglia (A) and trigeminal ganglia (B) neurons cultured in the presence or absence of NGF and caspase inhibitors

Trigeminal and nodose were dissected from P0 CD-1 mice and cultured *in-vitro* for 24 (nodose) or 48 (trigeminal) hours in the presence or absence of NGF (10ng/ml). Caspase inhibitors (50 μ M) were added to the growing medium to allow survival of the neurons at this young age in the absence of trophic support.

60- 80% of cells survive after 24hours in culture and 60% survival is observed after 48hours in the absence of factors. Presence of NGF does not have an effect on survival of nodose neurons (A), however an increased survival is observed with trigeminal neurons (B) which are largely dependent upon NGF for survival.

Any gene expression analysed will be normalised against GAPDH mRNA to account for the small loss in cell number observed here.

N.B. Survival is calculated as a percentage of starting cell number in a 1cm grid at time 0 (counted 4 hours after plating)

Error bars = +/- standard error, n = 4

The survival of trigeminal and nodose neurons in cultures supplemented with caspase inhibitors either in the presence or absence of NT-3 is shown in figure 3.29. Once again, some loss of neurons was observed in the presence of caspase inhibitors alone, with approximately 80% survival of both nodose and trigeminal neurons at 24 hours. By 48 hours, trigeminal ganglia neurons survival was reduced to 60% in the presence of caspase inhibitors. NT-3 was not able to promote the survival of either neuron type above that seen with caspase inhibitors alone.

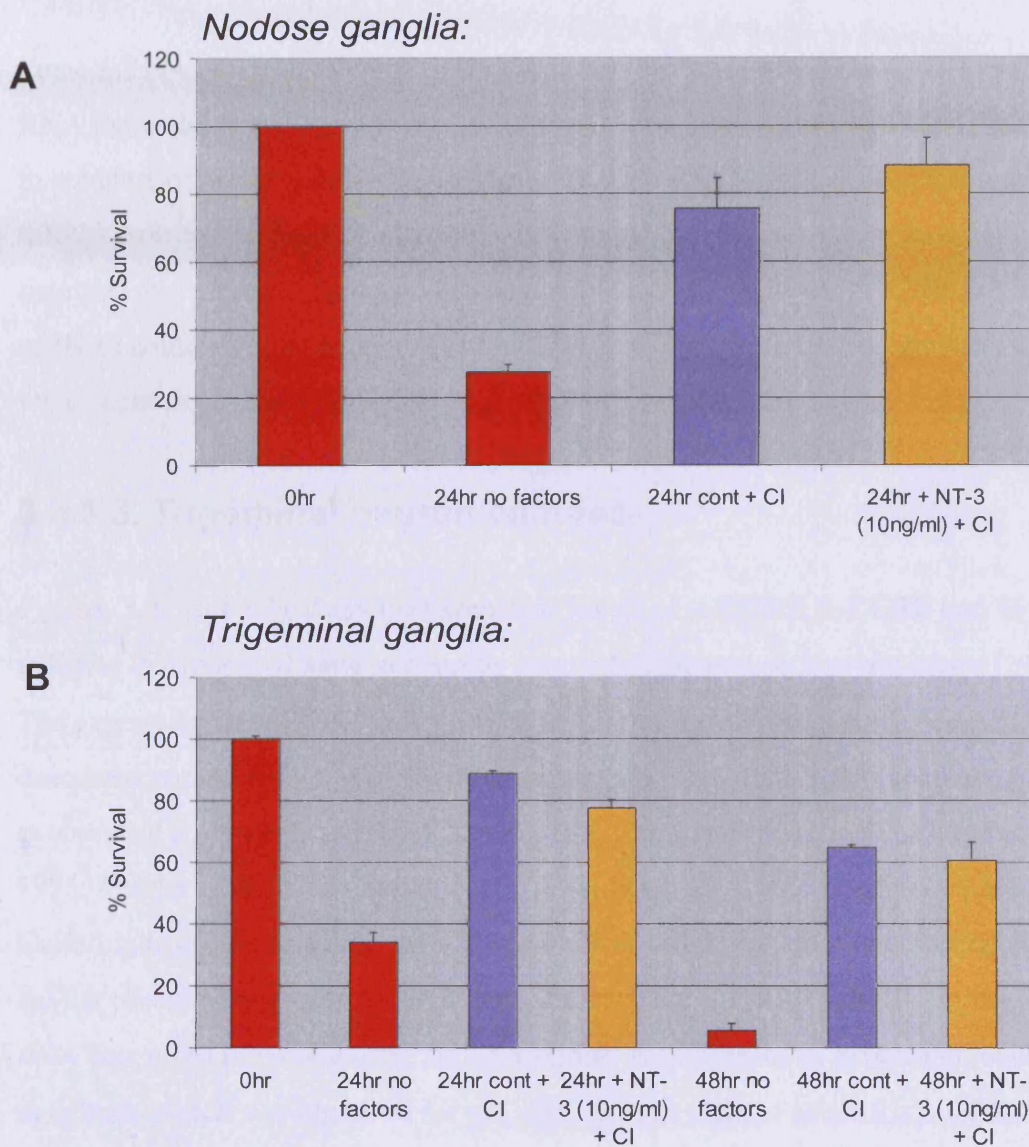


Figure 3.29. Survival of P0 nodose ganglia (A) and trigeminal ganglia neurons (B) cultured in the presence or absence of NT-3 and caspase inhibitors

Trigeminal and nodose were dissected from P0 CD-1 mice and cultured *in-vitro* for 24 (nodose) or 48 (trigeminal) hours in the presence or absence of NT-3 (10ng/ml). Caspase inhibitors, 50 μ M were added to the growing medium to allow survival of the neurons at this young age in the absence of trophic support.

Approximately 80% of cells survive after culture for 24 hours for both nodose and trigeminal ganglia. Approximately 60% survival of trigeminal ganglia neurons are observed after 48hours. No difference is observed with or without factors.

Any gene expression analysed will be normalised against GAPDH mRNA to account for the small loss in cell number observed here.

N.B. Survival is calculated as a percentage of starting cell number in a 1cm grid at time 0 (counted 4 hours after plating)

Error bars = +/- standard error, n = 4

3.3.5.2. The regulation of mRNA expression in cultured P0 sensory neurons by NT-3 and NGF

Some mRNAs were difficult to accurately quantify using real-time-QPCR analysis of RNA extracted from sensory neuron cultures, since these cultures yielded little RNA in comparison to the whole ganglia that were used for previous RT-QPCR analysis of mRNA expression. For this reason no data for galanin mRNA expression could be obtained for cultured P0 nodose and trigeminal ganglia neurons. β CGRP and VR1 mRNAs could also not be quantified in RNA extracted from nodose neuron cultures, which contained fewer cells than trigeminal neuron cultures.

3.3.5.3. Trigeminal neuron cultures

Figures 3.30 and 3.31 show the expression levels of α -CGRP, β -CGRP and SP mRNAs in trigeminal ganglia neurons cultured in the presence or absence of NGF. The expression of α -CGRP mRNA (figure 3.30A) and SP mRNA (figure 3.31) both decreased around 5-fold over 48 hrs in culture, an effect that is also seen in axotomised adult neurons (Nielsch et al., 1987; Noguchi et al., 1990; Zhang et al., 1995; Mulder et al., 1997; Sterne et al., 1998; Shi et al., 2001; Shadiack et al., 2001). Culturing trigeminal neurons with 10ng/ml NGF effectively prevents this decrease. A similar positive regulation by NGF was observed for β -CGRP mRNA (figure 3.30B) at 24 hours, but not at 48hours, and in addition the decrease in expression over time in culture, which was observed for α -CGRP mRNA did not occur for β -CGRP mRNA. This suggests that the two isoforms respond to the same environment in different ways at this developmental stage. This provides another example of the distinct regulation of the two transcripts, despite their high sequence homology.

Figure 3.32 shows the expression levels of Nav1.8 and Nav1.9 mRNAs in trigeminal ganglia neurons cultured in the presence or absence of NGF. Both Nav1.8 and Nav1.9, mRNAs all showed a greater than 10-fold decrease in expression over 48hrs in culture, again an effect which has been observed in adult DRG following axotomy (Okuse et al., 1997; Cummins et al., 1997; Tate et al., 1998; Dib-hajj et al., 1998;

Novakovic et al., 1998 Sleeper et al., 2000; Decosterd et al., 2002). The presence of 10 ng/ml NGF totally prevented the time dependent decrease in Nav1.8 mRNA expression, but only partially prevented the decrease in Nav1.9 mRNA levels.

In a similar fashion to the TTX sodium channels, expression of VR1 was reduced approximately 10 fold over time in culture to levels that cannot be detected accurately by PCR (figure 3.33A). Such a decrease has been observed in axotomised sensory neurons previously (Michael and Priestly, 1999; Michael and Priestly 2002; Fukuoka T et al., 2002). The presence of NGF inhibited this decrease, with levels returned close to those at time 0 by 48 hours (figure 3.33A).

In contrast to the mRNAs for CGRP, SP and TTX-resistant sodium channels, PACAP mRNA expression levels increased 5-fold during the first 24 hours in culture (figure 3.33B). Over the next 24 hrs, the markedly elevated levels of PACAP mRNA decreased some 2.5-fold to remain at twice the initial level at 48 hrs. An increase in PACAP mRNA expression has also been observed in axotomised DRG neurons (Jongsma Wallin et al., 2001). No regulation of PACAP mRNA expression by NGF was observed in culture.

The expression of GAPDH mRNA in cultured trigeminal neurons is shown in figure 3.34. As would be expected, the levels of GAPDH mRNA were higher in cultures containing NGF compared to those with caspase inhibitors alone (approximately a 60% reduction compared to time 0 in cultures supplemented with only caspase inhibitors). This was due to a combination of decreased survival in cultures containing caspase inhibitors alone (40% reduction- see figure 3.28), along with the fact that cells cultured in caspase inhibitors alone are atrophic and are probably less healthy than those supplemented with NGF. This result re-affirms the need to normalise results against GAPDH mRNA to provide a true representation of mRNA expression that is not just a reflection of decreased cell vigour and cell death.

Data representing the effects of exogenous NT-3 on the expression of α -CGRP, β -CGRP and SP mRNAs in cultured trigeminal neurons is shown in figures 3.35 and 3.36. As in previous trigeminal ganglion neuronal cultures, a decrease in the expression of α -CGRP (figure 3.35A) and SP (figure 3.36) mRNAs was observed

over time in culture, but β -CGRP mRNA expression was slightly increased after 24 hrs in culture and returned to initial levels at 48 hrs (figure 3.35B). The expression of α - and β -CGRP and SP mRNAs were not regulated by NT-3.

The expression levels of Nav1.8 and Nav1.9 in trigeminal neurons from control cultures, containing caspase inhibitors alone, and cultures supplemented with 10ng/ml NT-3 are illustrated in figure 3.37. Both Nav1.8 and Nav1.9 mRNAs respond to the culture environment in a similar way to the previous NGF cultures (figure 3.32.), with marked decreases in the expression of both over time in culture. In contrast to NGF, culturing trigeminal neurons in the presence of 10ng/ml NT-3 did not appear to ameliorate the decrease in Nav1.8 and Nav1.9 mRNA levels. Expression of VR1 mRNA also showed similar effects to that seen in NGF trigeminal cultures (figure 3.33A), with a substantial decrease observed over time in culture. Presence of NT-3 did not affect this culture-induced reduction. In contrast to other genes, an increase in the expression of PACAP mRNA over the same time period was observed (figure 3.33B). Additionally NT-3 did appear to increase the levels of PACAP mRNA expressed by trigeminal neurons after 24 and 48 hours in culture, although this increase only reached significance at 48 hours.

The expression of GAPDH mRNA in trigeminal neurons cultured in caspase inhibitors alone or with caspase inhibitors supplemented with 10ng/ml NT-3 is shown in figure 3.39. In cultures containing caspase inhibitors alone, there was approximately a 60% reduction in the expression of GAPDH mRNA by 48 hrs, probably reflecting cell death in the cultures (40% see figure 3.28) and the compromised health of remaining neurons (discussed below). This is in agreement with the control trigeminal neuron cultures set up for the NGF experiments described above. However, in contrast to the effects of NGF, NT-3 was not able to prevent the decrease in GAPDH mRNA expression that occurs over time in culture, reflecting the inability of NT-3 to prevent the death of trigeminal neurons in culture or improve the vitality of those that avoid apoptosis.

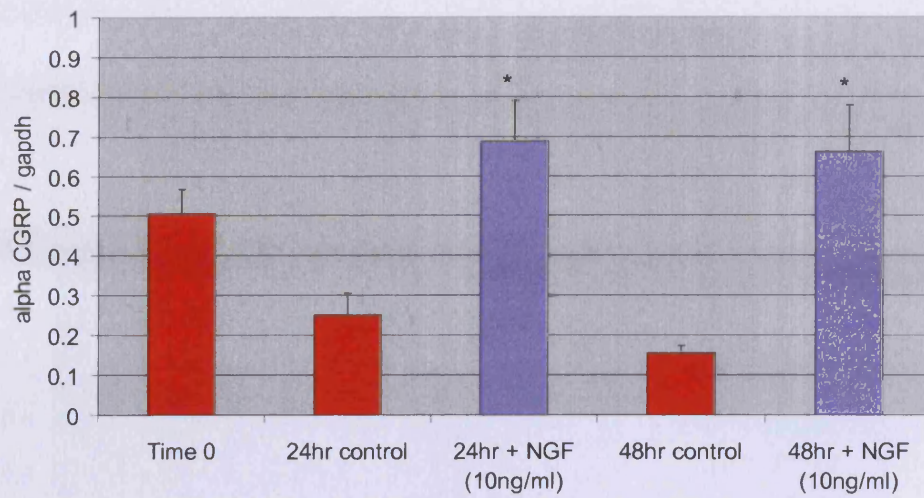
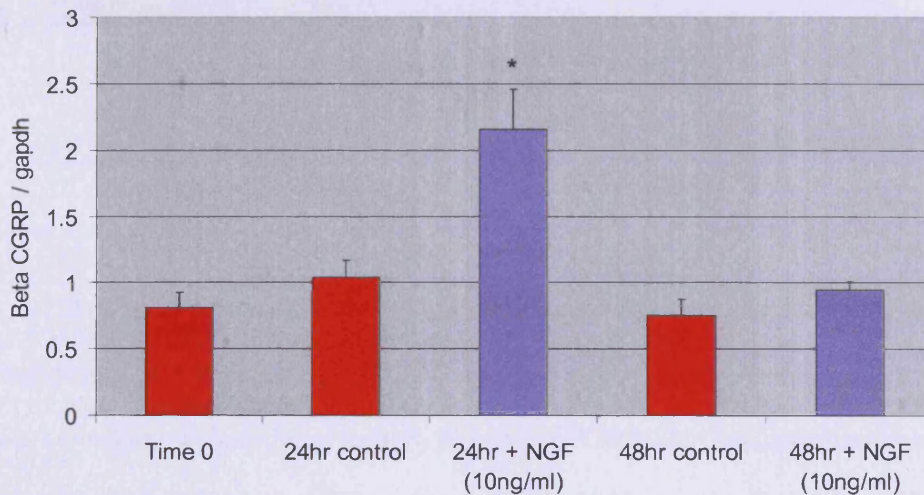
A**B**

Figure 3.30. Expression of alpha- and beta-CGRP mRNAs in P0 trigeminal ganglia neurons cultured in the presence or absence of NGF (10ng/ml) and caspase inhibitors.

Trigeminal ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NGF (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of alpha CGRP (A) and beta CGRP (B) were quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = p < 0.05 and ** = p < 0.01 as determined by two-tailed unpaired t test.

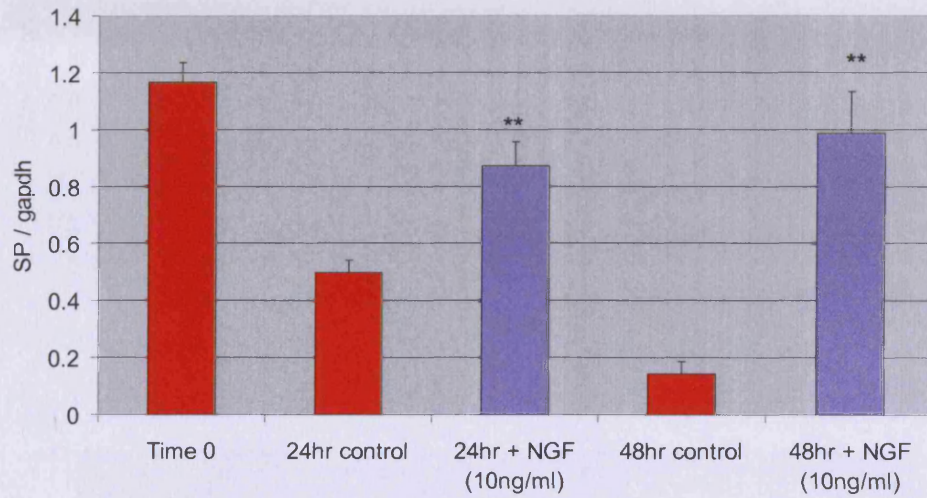


Figure 3.31. Expression of SP mRNA in P0 trigeminal ganglia neurons cultured in the presence or absence of NGF (10ng/ml) and caspase inhibitors.

Trigeminal ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NGF (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of SP mRNA was quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = $p < 0.05$ and ** = $p < 0.01$ as determined by two-tailed unpaired t test.

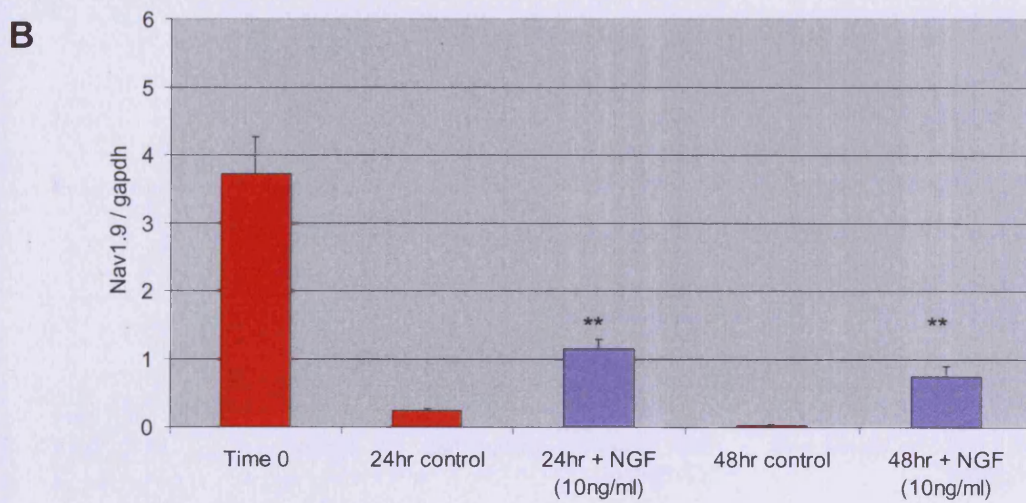
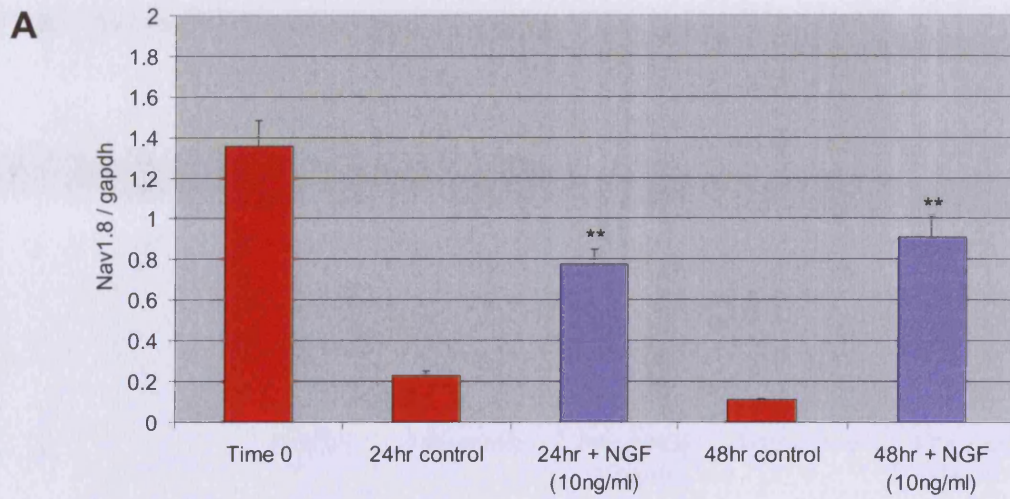


Figure 3.32. Expression of *Nav1.8* and *Nav1.9* mRNAs in trigeminal ganglia neurons cultured in the presence or absence of NGF (10ng/ml) and caspase inhibitors.

Trigeminal ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NGF (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of *Nav1.8* (A) and *Nav1.9* (B) were quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = $p < 0.05$ and ** = $p < 0.01$ as determined by two-tailed unpaired t test.

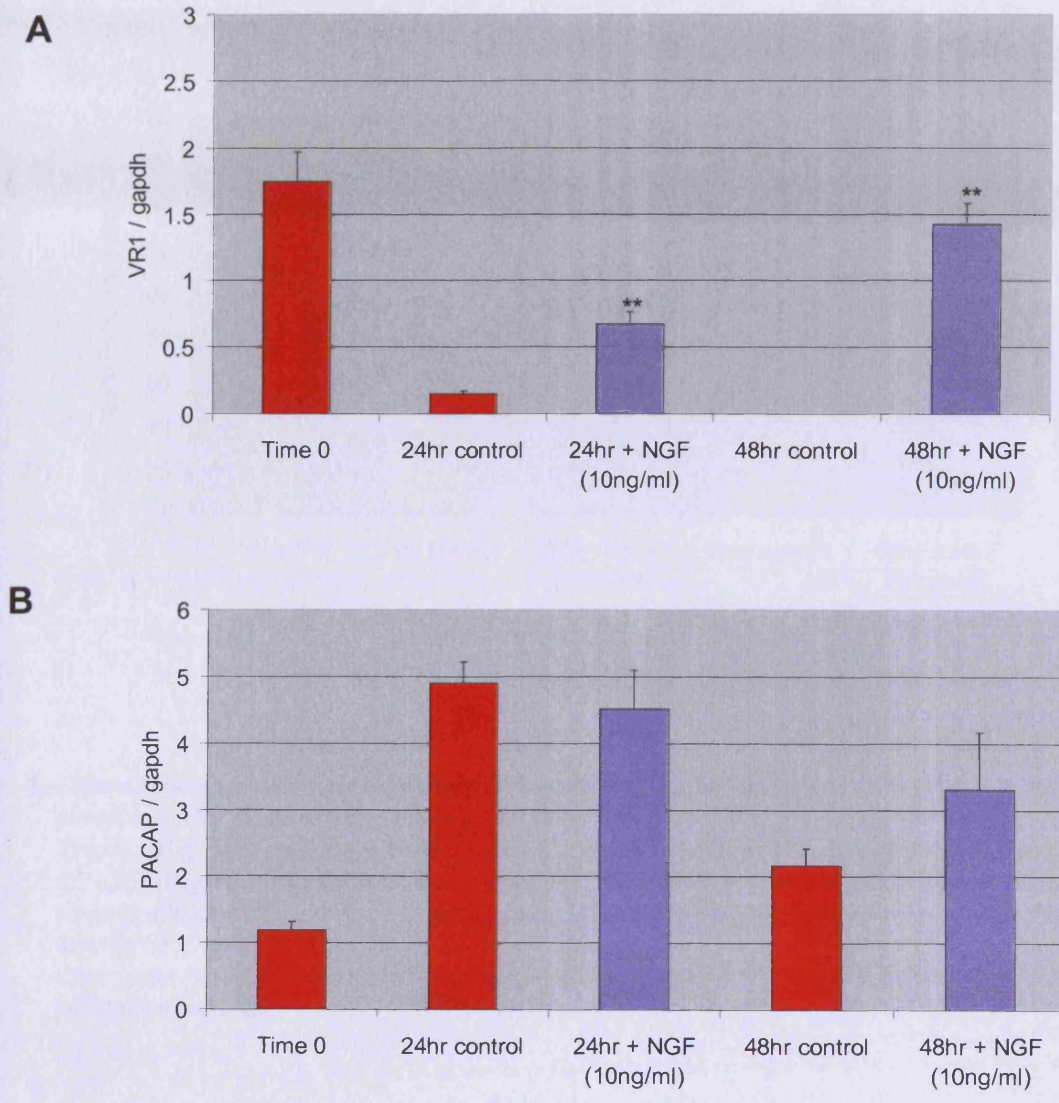


Figure 3.33. Expression of *VR1* and *PACAP* mRNAs in trigeminal ganglia neurons cultured in the presence or absence of NGF (10ng/ml) and caspase inhibitors.

Trigeminal ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NGF (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of *VR1* (A) and *PACAP* (B) mRNAs were quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = p = <0.05 and ** = p = < 0.01 as determined by two-tailed unpaired t test.

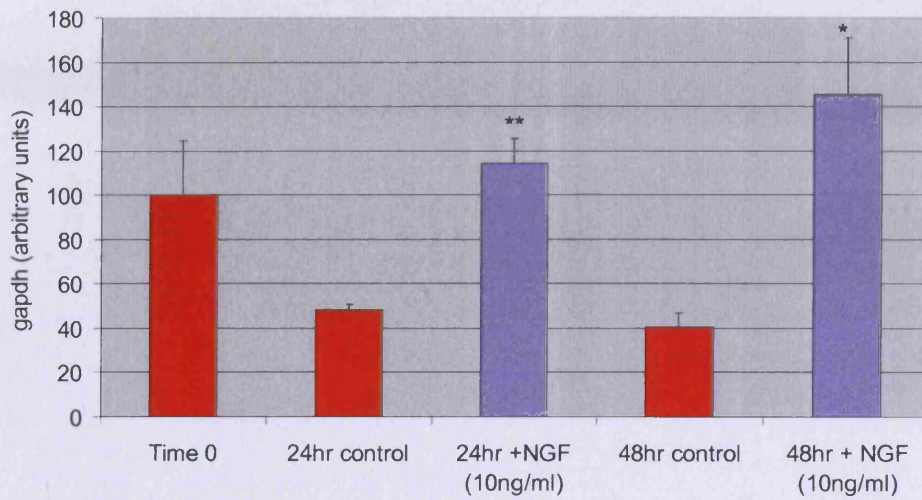


Figure 3.34. Expression of GAPDH mRNA in trigeminal ganglia neurons cultured in the presence or absence of NGF (10ng/ml) and caspase inhibitors.

Trigeminal ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NGF (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of GAPDH mRNA was quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = p = <0.05 and ** = p = < 0.01 as determined by two-tailed unpaired t test.

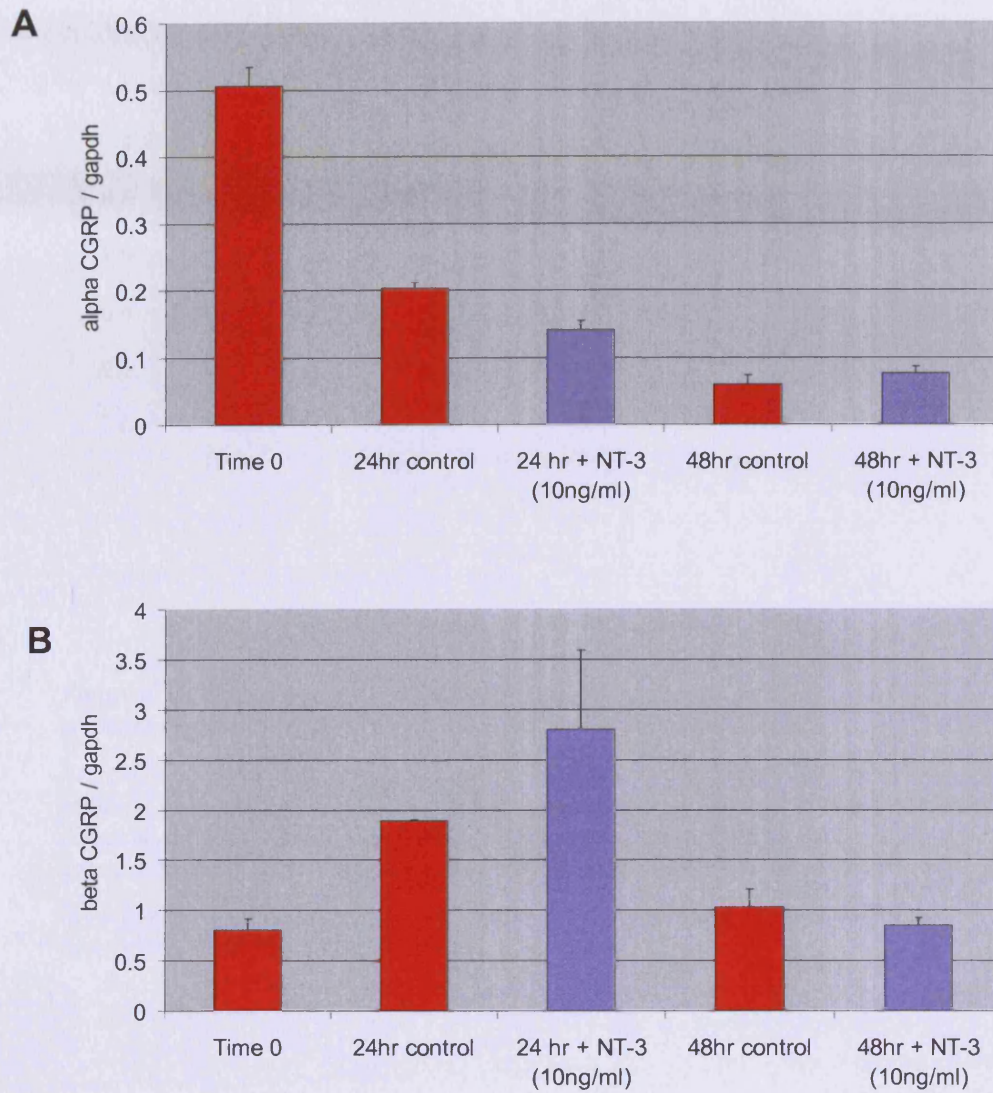


Figure 3.35. Expression of alpha- and beta-CGRP mRNAs in P0 trigeminal ganglia neurons cultured in the presence or absence of NT-3 (10ng/ml) and caspase inhibitors.

Trigeminal ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NT-3 (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of alpha-CGRP (A) and beta-CGRP (B) mRNAs were quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = p < 0.05 and ** = p < 0.01 as determined by two-tailed unpaired t test.

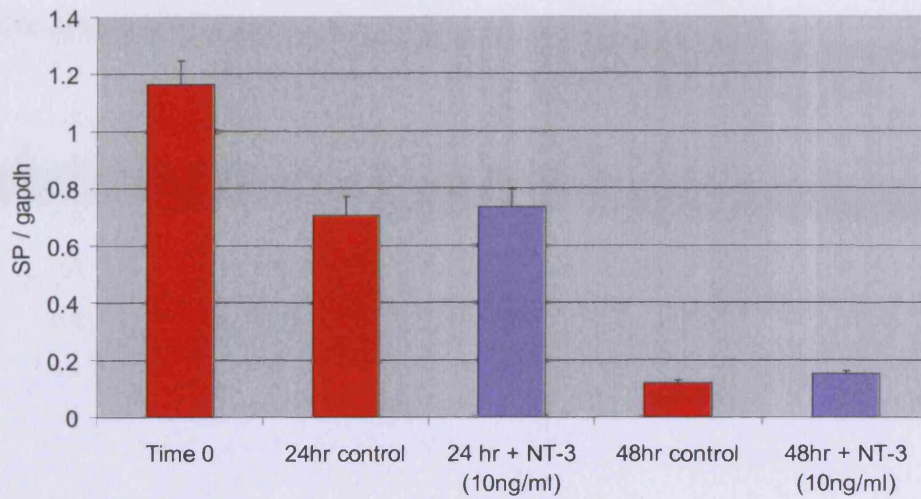


Figure 3.36. Expression of SP mRNA in P0 trigeminal ganglia neurons cultured in the presence or absence of NT-3 (10ng/ml) and caspase inhibitors.

Trigeminal ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NT-3 (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of SP mRNA was quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = p = <0.05 and ** = p = < 0.01 as determined by two-tailed unpaired t test.

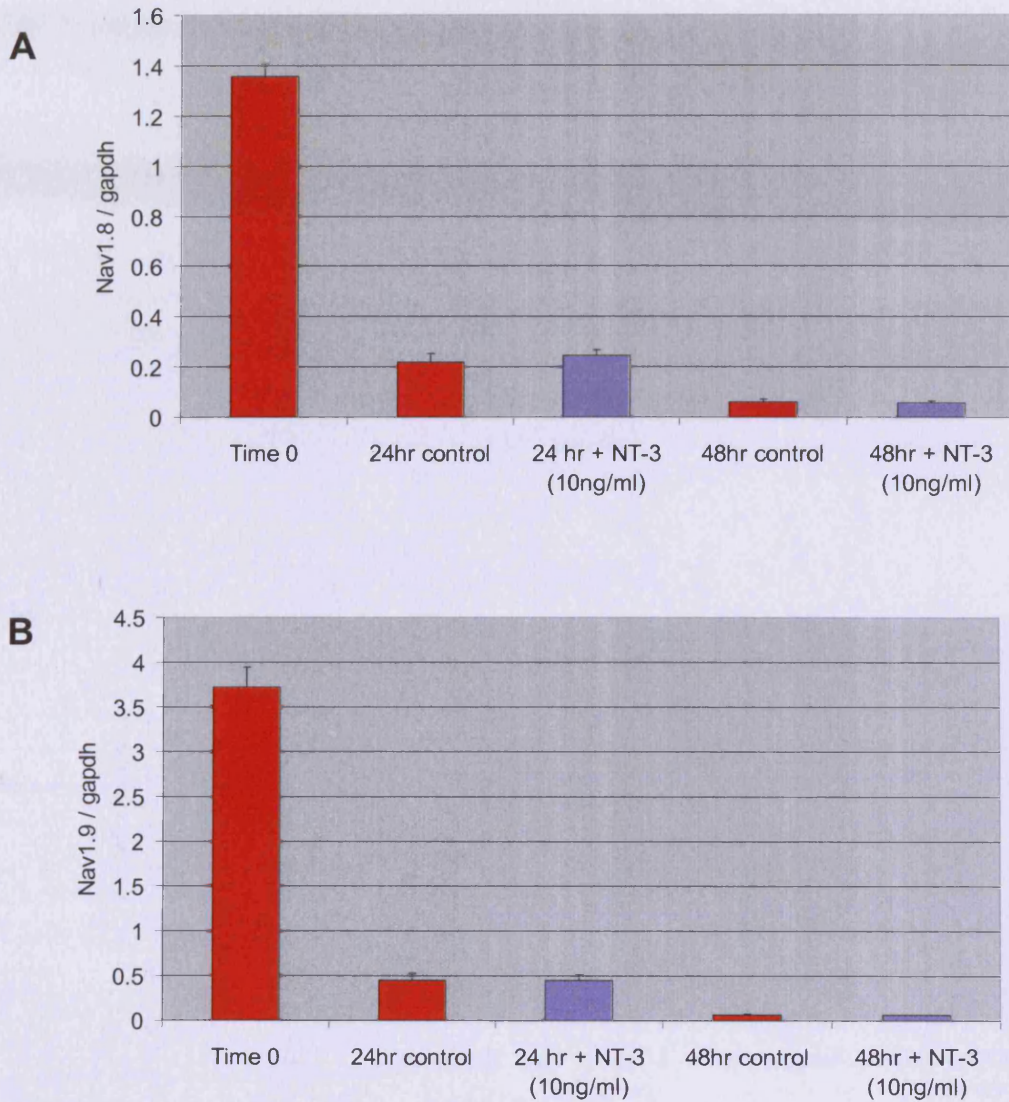


Figure 3.37. Expression of *Nav1.8* and *Nav1.9* mRNAs in P0 trigeminal ganglia neurons cultured in the presence or absence of NT-3 (10ng/ml) and caspase inhibitors.

Trigeminal ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NT-3 (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of *Nav1.8* (A) and *Nav1.9* (B) mRNAs were quantified by real-time QPCR.

Error bars = \pm standard error, n = 4. Where * = $p < 0.05$ and ** = $p < 0.01$ as determined by two-tailed unpaired t test.

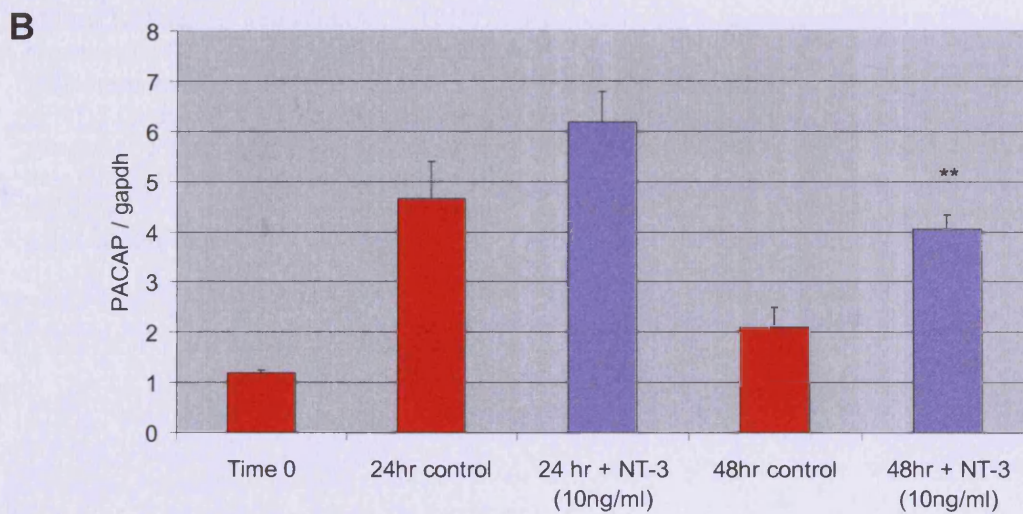
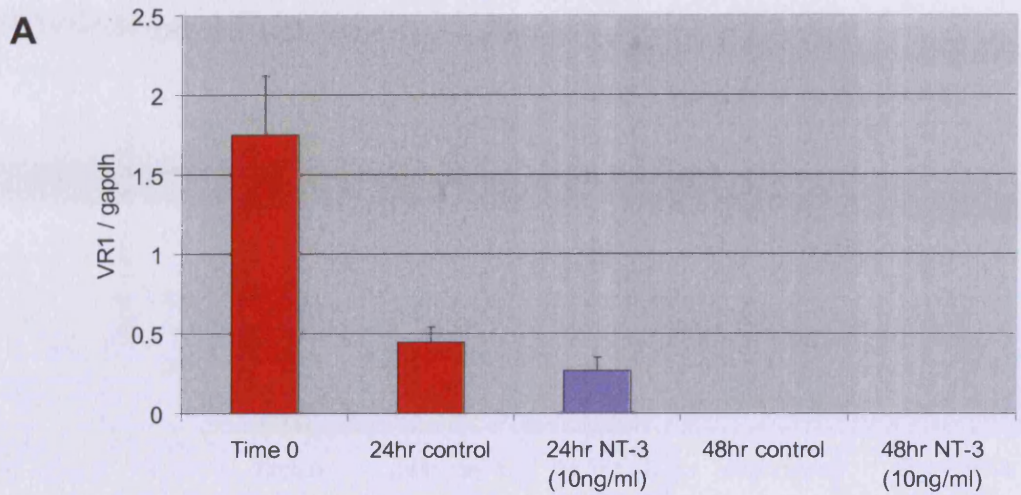


Figure 3.38. Expression of *VR1* and *PACAP* mRNAs in P0 trigeminal ganglia neurons cultured in the presence or absence of NT-3 (10ng/ml) and caspase inhibitors.

Trigeminal ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NT-3 (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of *VR1* (A) and *PACAP* (B) mRNAs were quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = $p < 0.05$ and ** = $p < 0.01$ as determined by two-tailed unpaired t test.

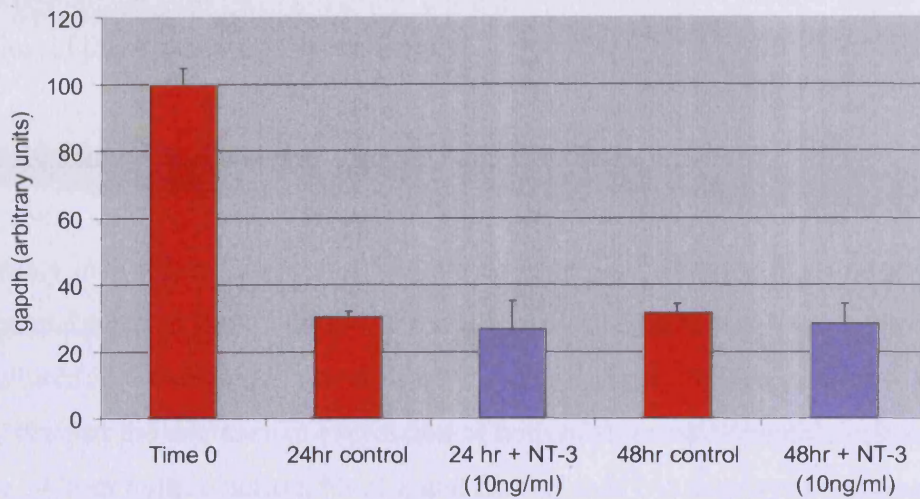


Figure 3.39. Expression of mRNA of GAPDH trigeminal ganglia neurons cultured in the presence or absence of NT-3 (10ng/ml) and caspase inhibitors.

Trigeminal ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NT-3 (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of GAPDH mRNA was quantified by real-time QPCR and used to normalise data for other genes.

Error bars = +/- standard error, n = 4. Where * = $p < 0.05$ and ** = $p < 0.01$ as determined by two-tailed unpaired t test.

3.3.5.4 Nodose neuron cultures

β -CGRP and VR1 mRNAs could not be accurately quantified in total RNA extracted from nodose neurons. This was due to a combination of low neuronal number in these cultures (due to the small size of nodose ganglia) combined with the low levels of expression of these genes in nodose ganglia.

Figure 3.40 shows the effect that 10ng/ml NGF has on the expression of α -CGRP and SP mRNAs in cultured nodose neurons. The expression levels of both mRNAs were considerably lower than those found in cultured trigeminal neurons. As was observed for trigeminal neurons, both mRNAs showed decreased expression levels over time when cultured in the absence of neurotrophic factor support. NGF was able to partially prevent the decrease in expression of both of these neuropeptide mRNAs. Over the 24 hour culture period, Nav1.8 and Nav1.9 mRNAs displayed a dramatic, greater than 10-fold, decrease in their levels of expression in nodose neurons cultured without neurotrophic factor support (figure 3.41). NGF was able to increase the levels of both mRNAs by around 2-fold compared to neurons cultured in caspase inhibitors alone, a rise that was statistically significant. As in the case of trigeminal neurons, the expression of PACAP mRNA in nodose neurons increased some 5-fold over a 24 hour period in culture (figure 3.42). The addition of 10ng/ml to the culture medium was able to reduce the up-regulation of PACAP mRNA expression slightly, although the effect did not reach statistical significance ($p = 0.0625$) (figure 3.42).

GAPDH mRNA expression levels decreased by around 40% in nodose neurons, cultured in caspase inhibitors for 24 hours (figure 3.43). NGF was able to partially reduce the decrease in GAPDH mRNA expression levels, although the rescue of GAPDH mRNA expression was not statistically significant.

Unlike NGF, NT-3 cannot prevent any of the decrease in the expression of α -CGRP and SP mRNAs that occurs when nodose neurons are cultured for 24 hours without neurotrophic support (figure 3.44). Similarly, NT-3 was not able to enhance the expression of Nav1.8 and Nav1.9 mRNAs and thereby prevent the marked drop in expression of these two mRNAs that occurs in nodose neurons cultured for 24 hours in caspase inhibitors (figure 3.45).

The expression of PACAP mRNA in cultured nodose neurons is shown in figure 3.46. In accordance with the NGF series of experiments, the amount of PACAP mRNA expressed by nodose neurons increased markedly (over 5-fold) over a 24 hour culture period when the neurons were cultured with caspase inhibitors alone. NT-3, like NGF, appeared to be able to partially prevent the increase in PACAP mRNA expression, although, as in the case of NGF, this did not reach statistical significance ($p = 0.123$), possibly due to error bars. This data is in contrast to the positive regulatory effect that NT-3 appears to have on PACAP mRNA expression in cultured trigeminal neurons (figure 3.46), but is in agreement with the data for nodose ganglia from $Bax^{-/-}$ / NT-3⁻ transgenic neonates (figure 3.26).

As for NGF cultures (figure 3.43), expression of GAPDH was reduced in nodose neurons following 24 hours in culture in the absence of neurotrophic factor support and presence of caspase inhibitors (figure 3.47). NT-3 could not prevent this decrease, illustrating the inability of this factor to support survival of such sensory neuronal populations.

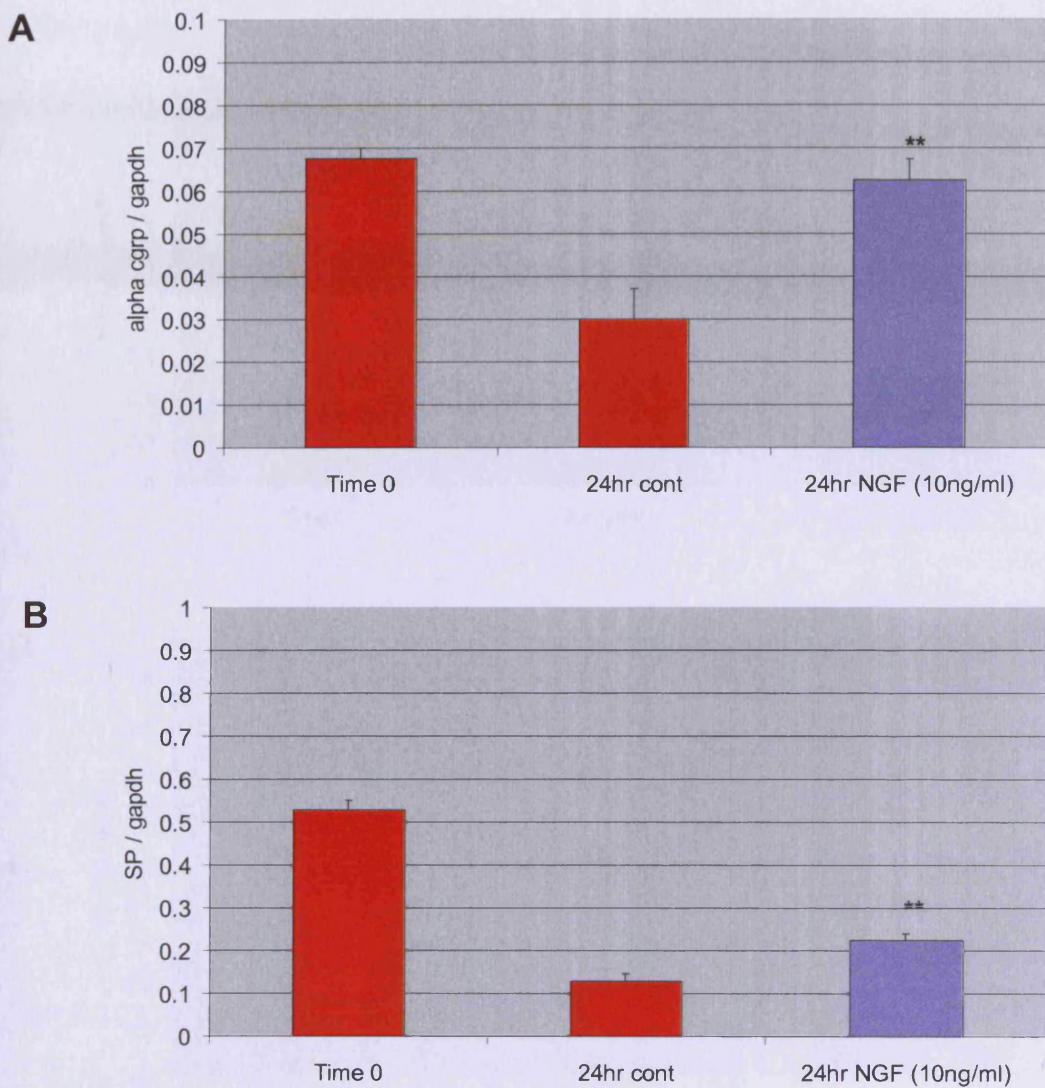


Figure 3.40. Expression of alpha CGRP and SP in P0 nodose neurons ganglia cultured in the presence or absence of NGF (10ng/ml) and caspase inhibitors.

Nodose ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NGF (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of alpha CGRP (A) and SP (B) mRNAs were quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = p = <0.05 and ** = p = < 0.01 as determined by two-tailed unpaired t test.

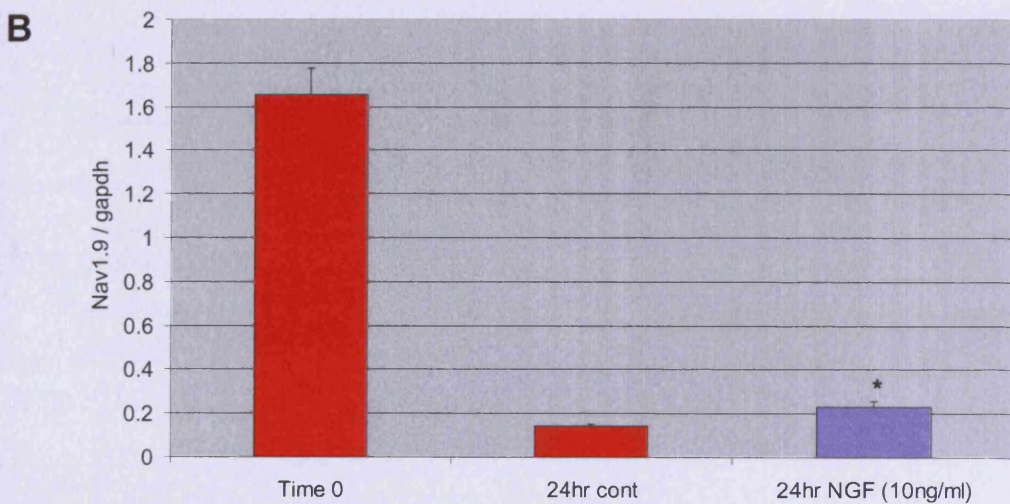
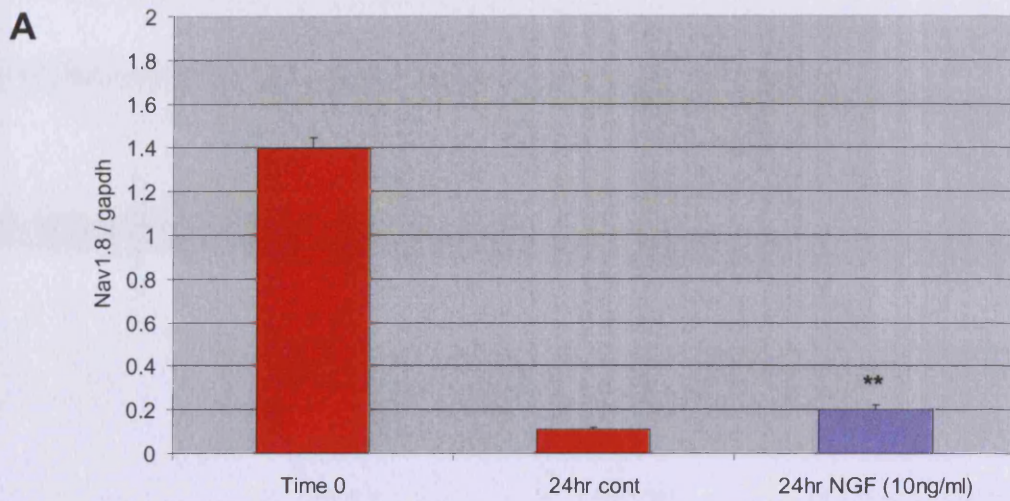


Figure 3.41. Expression of mRNAs of Nav1.8 and Nav1.9 in P0 nodose ganglia neurons cultured in the presence or absence of NGF (10ng/ml) and caspase inhibitors.

Nodose ganglia were dissected from P0, CD-1 mice and cultured *in-vitro* in the presence or absence of NGF (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of Nav1.8 (A) and Nav1.9 (B) mRNAs were quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = p = <0.05 and ** = p = < 0.01 as determined by two-tailed unpaired t test.

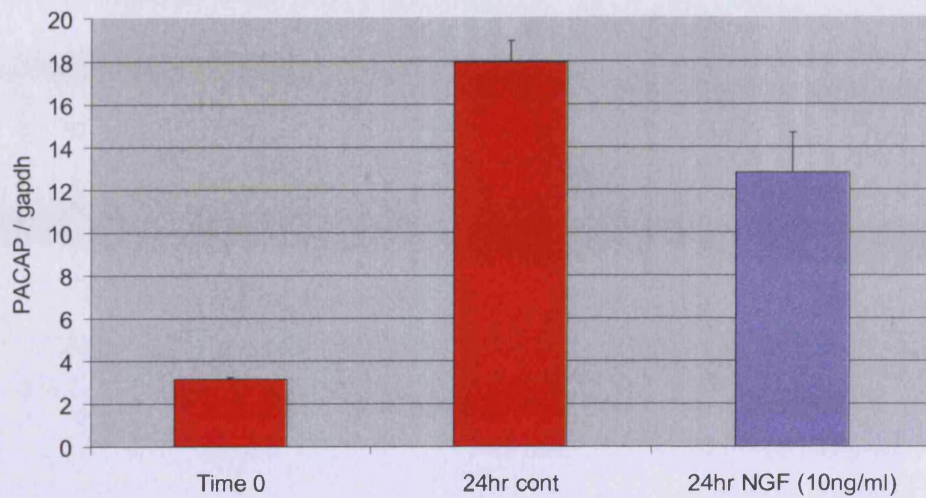


Figure 3.42. Expression of PACAP mRNA in P0 nodose ganglia neurons cultured in the presence or absence of NGF (10ng/ml) and caspase inhibitors.

Nodose ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NGF (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of PACAP mRNA was quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = p = <0.05 and ** = p = < 0.01 as determined by two-tailed unpaired t test.

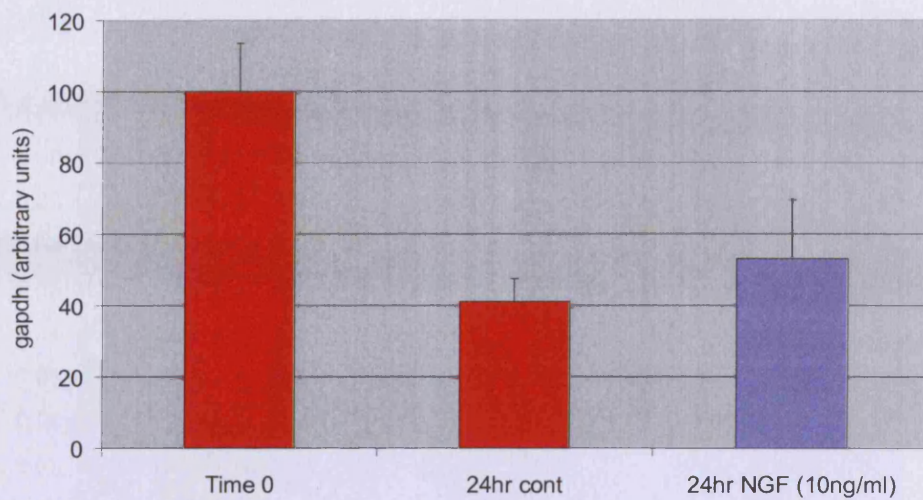


Figure 3.43. Expression of GAPDH mRNA in P0 nodose ganglia neurons cultured in the presence or absence of NGF (10ng/ml) and caspase inhibitors.

Nodose ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NGF (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of GAPDH mRNA was quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = p = <0.05 and ** = p = < 0.01 as determined by two-tailed unpaired t test.

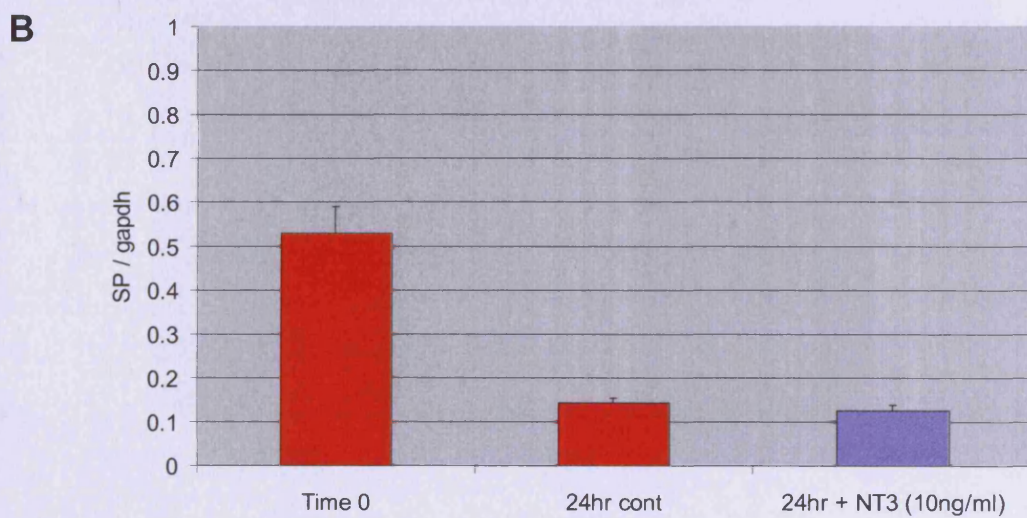
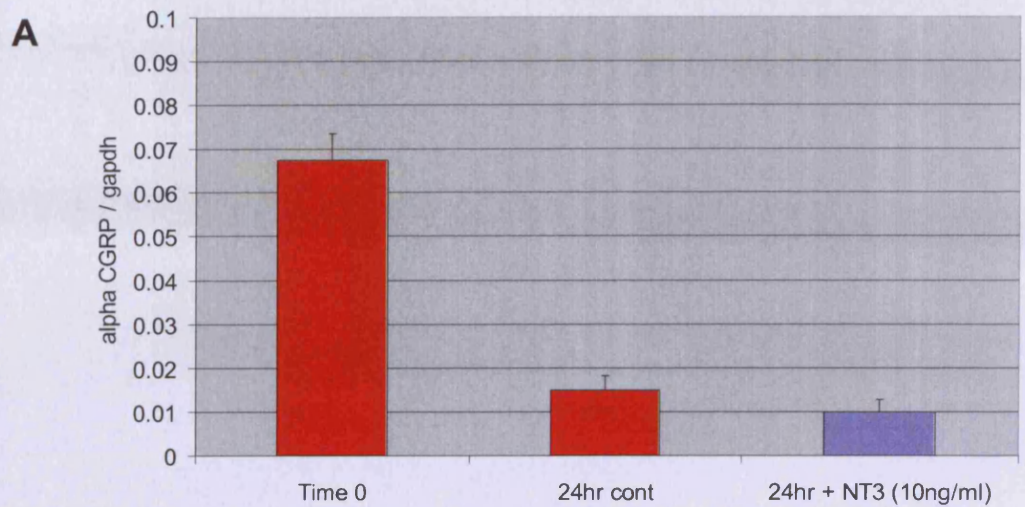


Figure 3.44. Expression of alpha CGRP and SP mRNAs in P0 nodose ganglia neurons cultured in the presence or absence of NT-3 (10ng/ml) and caspase inhibitors.

Nodose ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NT-3 (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of alpha CGRP (A) and SP (B) mRNAs were quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = p < 0.05 and ** = p < 0.01 as determined by two-tailed unpaired t test.

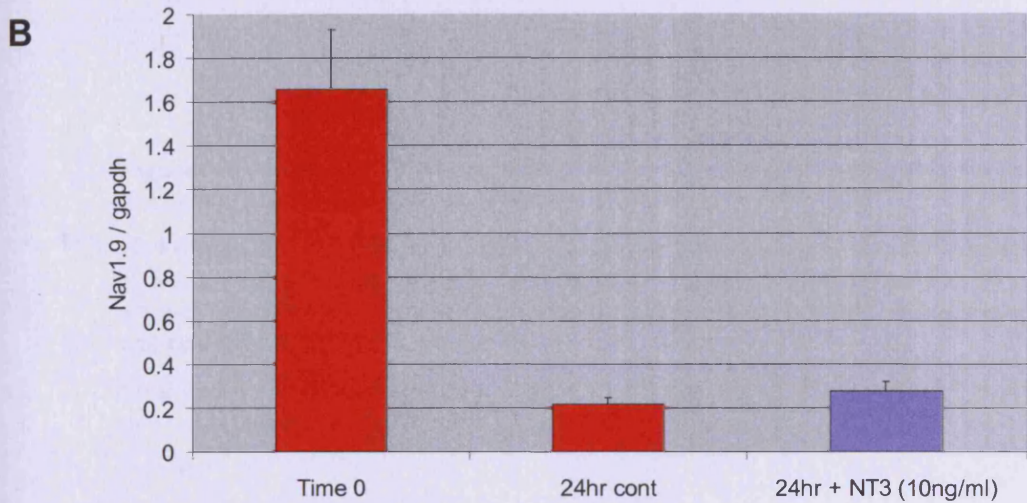
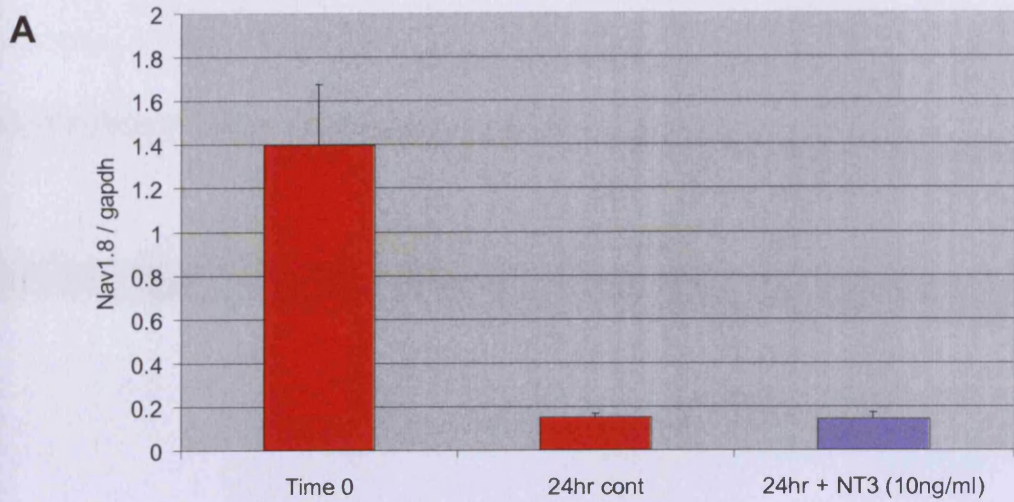


Figure 3.45. Expression of *Nav1.8* and *Nav1.9* mRNAs in P0 nodose ganglia neurons cultured in the presence or absence of NT-3 (10ng/ml) and caspase inhibitors.

Nodose ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NT-3 (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of *Nav1.8* (A) and *Nav1.9* (B) mRNAs were quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = p < 0.05 and ** = p < 0.01 as determined by two-tailed unpaired t test.

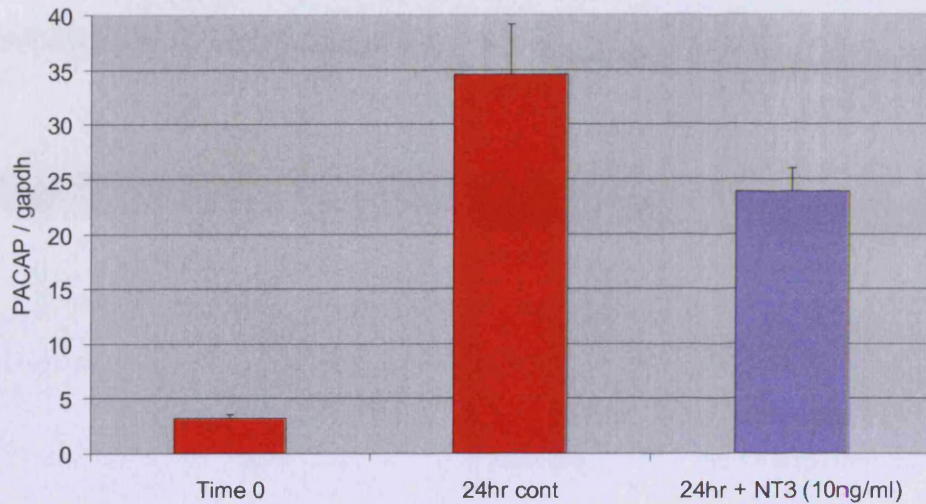


Figure 3.46. Expression of PACAP mRNA in P0 nodose ganglia neurons cultured in the presence or absence of NT-3 (10ng/ml) and caspase inhibitors.

Nodose ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NT-3 (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of PACAP mRNA was quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = $p < 0.05$ and ** = $p < 0.01$ as determined by two-tailed unpaired t test.

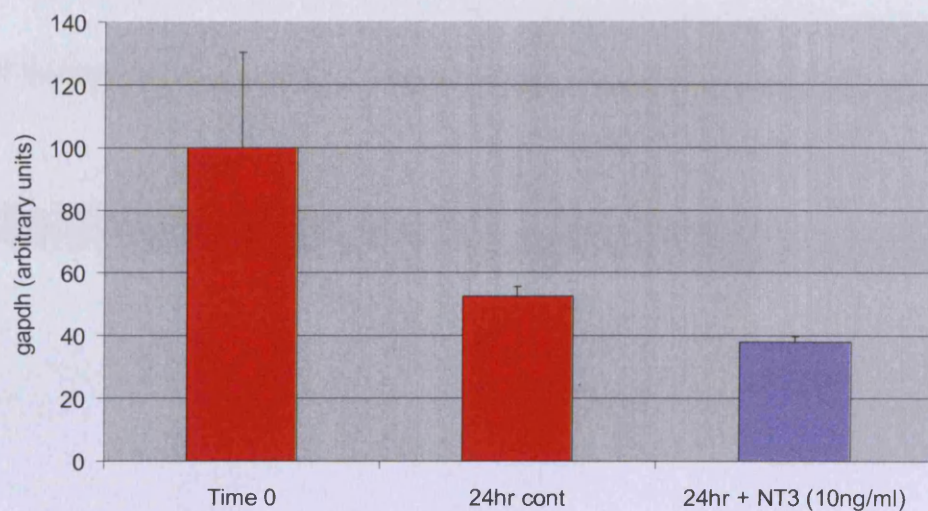


Figure 3.47. Expression of GAPDH mRNA in P0 nodose ganglia neurons cultured in the presence or absence of NT-3 (10ng/ml) and caspase inhibitors.

Nodose ganglia were dissected from P0 CD-1 mice and cultured *in-vitro* in the presence or absence of NT-3 (10ng/ml). Caspase inhibitors (50 μ M) were included in all dishes to allow survival in the absence of correct neurotrophic factor support. Expression of GAPDH mRNA was quantified by real-time QPCR.

Error bars = +/- standard error, n = 4. Where * = p = <0.05 and ** = p = < 0.01 as determined by two-tailed unpaired t test.

3.4. Discussion

In this chapter, I used RT-QPCR to determine the roles that NT-3 and NGF (via TrkA dependent signalling) play in regulating the mRNA expression of several functionally important sensory neuron genes in neonatal trigeminal, nodose and dorsal root ganglion neurons. The roles of these neurotrophic factors in regulating neonatal sensory neuron mRNA expression was studied *in vivo* in transgenic mice and *in vitro* in primary neuronal cultures.

One important caveat should be born in mind when interpreting the data presented in this chapter. Previously published data suggest that all the genes investigated in this chapter are predominantly or exclusively expressed by neurons within peripheral sensory ganglia of “normal” adult rodents, *in-vivo*. Whilst this is probably the case in both neonatal and transgenic animals as well as in neuronal cultures, the possibility exists that under these circumstances that non-neuronal cells within sensory ganglia also begin to express the mRNAs being investigated. The only way to rule out this, albeit remote, possibility is to carry out a comprehensive in-situ hybridisation, immuno-histochemistry and immnuo-cytochemistry study for each of the genes under investigation. Unfortunately, limited time precluded such a study. Therefore, the formal possibility exists that some of the data presented in this chapter reflects unexpected mRNA expression by glial cells within whole sensory ganglia and non-neuronal cells in dissociated cultures.

I will discuss the *in-vitro* and *in-vivo* regulation of each mRNA, in the various ganglia, in turn.

3.4.1. GAPDH

Non-neuronal cells within sensory ganglia contain extremely small relative levels (approximately 1000-fold to 5000 fold-less depending on neuron size) of GAPDH mRNA compared to the much larger neurons (S Wyatt unpublished observation), and as such only make up a small-percentage of the total amount of GAPDH mRNA expressed by neonatal sensory ganglia. The potential effects that TrkA and NT-3

deletion have on non-neuronal cells and how this relates to alterations in the expression of GAPDH mRNA by non-neuronal cells can, therefore, be discounted.

In general the amount of GAPDH expressed by cells directly reflects the size of the cells (S Wyatt unpublished data). The smaller a cell is, the less energy it requires for maintenance and function. A reduced energy requirement would be expected to be reflected by a lowered rate of glycolysis and hence a reduced requirement for GAPDH (although obviously this is over simplistic and cell energy requirements are also dictated by cell function). The loss of TrkA did not significantly alter the levels of GAPDH mRNA expressed by neonatal trigeminal and nodose ganglia (figure 3.22). In some respects the data for trigeminal ganglia is surprising, since the lack of NGF/TrkA signalling would be expected to make NGF-responsive neurons atrophic and would also be expected to limit their ability to innervate peripheral targets and decrease their degree of terminal arborisation (e.g. Patel et al., 1990; Lentz et al., 1999; Genc et al., 2004). Moreover, cultured P0 trigeminal neurons display a significant reduction in neurite outgrowth, cell soma size and expression of GAPDH mRNA in the absence of NGF (data not shown and figure 3.30), making the in-vivo data even more curious. In nodose ganglia, it is not surprising that the loss of TrkA had no discernable effects on GAPDH mRNA levels, since nodose ganglia contain such a small number of NGF-responsive neurons (Forgie et al., 1999). In contrast to the other two sensory ganglia studied, neonatal DRG from TrkA/Bax double-null mutants do show significantly lower levels of GAPDH mRNA compared to Bax single-null mutants, probably reflecting the atrophic nature of NGF-responsive neurons in the absence of NGF/TrkA signalling (figure 3.22).

Both neural crest-derived sensory ganglia showed a loss of GAPDH mRNA expression in NT-3/Bax double-null mutant neonates compared to Bax^{-/-} neonates (figure 3.23). Although the TrkC-positive sub-population of proprioceptive neurons is only a small percentage of all DRG sensory neurons (predominantly being found in DRG innervating the limbs), proprioceptive neurons are large and as a consequence probably express a relatively large proportion of total DRG GAPDH mRNA (see above). NT-3 and Bax deficient DRG are likely to display a significant reduction in proprioceptive neuron cell size and a reduction in the complexity of terminal arborisation of these neurons, with a concomitant reduction in their expression of

GAPDH mRNA. This phenomenon may account for some of the reduction in GAPDH mRNA levels seen in the DRG of NT-3/Bax double-null mutant neonates. In addition, it has recently been shown that NT-3/Bax double-null mutant neonatal DRG contain fewer TrkC-positive neurons and more TrkA-positive neurons than WT animals (Genc et al., 2004). In DRG, TrkA-positive cells are predominantly small nociceptive neurons, expressing relatively low amounts of GAPDH mRNA. In contrast, TrkC positive DRG neurons are predominantly large proprioceptors expressing large amounts of GAPDH mRNA. Therefore, the change in the ratio of TrkA-positive to TrkC-positive neurons is also likely to reduce GAPDH mRNA levels in the absence of NT-3.

The trigeminal ganglion does not have a proprioceptive neuron component (these reside in the trigeminal mesencephalic nucleus) and neonatal trigeminal ganglia contain few TrkC positive neurons (Ernfors et al., 1992). Therefore, the reduction in the levels of GAPDH mRNA expressed by trigeminal ganglia in the absence of NT-3 signalling is less likely to be as a consequence of a reduction in neuron size and/or extent of terminal arborisation of TrkC-positive neurons. It is also unlikely to be the consequence of a change in the ratio of TrkA-positive to TrkC-positive neurons. The reduction in GAPDH mRNA expression in the absence of NT-3, is also unlikely to be as a result of the loss of NT-3/TrkA signalling, since as observed in figure 3.22, TrkA deletion does not reduce GAPDH mRNA levels in trigeminal ganglia. It is of course possible that NT-3 normally signals through its other non-preferred receptor TrkB or through the common neurotrophin receptor, p75, to exert its trophic effects on trigeminal ganglion neurons. Since these receptors (especially p75) are widely expressed in the neonatal trigeminal ganglion, it is possible that a loss of NT-3 will cause a reduction in size and a reduction in target field innervation/terminal arborisation of a large number of neurons, hence leading to a reduction in GAPDH mRNA levels. Analysis of NT-3 null mutant mice has come up with a number of hypotheses to account for the loss of neurons in neural crest-derived sensory ganglia. These range from direct apoptosis of post-mitotic neurons (Wilkinson et al., 1996), to apoptosis of proliferating neuronal precursors (ElShamy and Ernfors 1996 a and b) and finally to premature differentiation of neuronal precursors resulting in a reduction in the precursor pool during the peak period of neurogenesis (Farinas et al., 1996). At

present, it is not clear which of these three scenarios is the correct one. The deletion of Bax will prevent the first two events taking place in the absence of NT-3 signalling, allowing the maintenance of neuronal numbers. However, the premature differentiation of neuronal precursors into neurons will not be prevented by Bax deletion. It is therefore possible that NT-3/Bax double-null mutant neonates contain fewer trigeminal neurons than Bax^{-/-} neonates, and this could account for the drop in GAPDH mRNA levels in trigeminal ganglia of NT-3^{-/-}/Bax^{-/-} neonates. Of course, this hypothesis could also explain some or all of the reduction in GAPDH mRNA levels found in double-null mutant DRG. Careful histology-based analysis of neuron numbers in sensory ganglia from NT-3^{-/-}/Bax^{-/-} mice and Bax^{-/-} mice would determine whether this hypothesis is true.

The deletion of NT-3 did not significantly alter the levels of GAPDH mRNA expressed by neonatal nodose neurons in-vivo (figure 3.23), suggesting that NT-3 does not exert trophic effects on significant number of neonatal nodose neurons. This data is in agreement with a visual inspection of neonatal nodose cultures in the presence or absence of NT-3. NT-3 did not markedly increase neuron cell body size or increase neurite outgrowth compared to nodose neurons grown without trophic factor support (data not shown).

GAPDH mRNA levels were significantly less, at both 24 and 48 hr time points, in P0 trigeminal neurons cultured in the absence of NGF compared to those cultured in 10 ng/ml NGF (figure 3.34). This reduction in GAPDH mRNA levels was not due to a reduction in neuronal survival, since neurons were grown in the presence of caspase inhibitors that block apoptosis. However, neurons grown in the absence of NGF were clearly small and atrophic and displayed very little neurite outgrowth compared to those cultured with NGF. Since cell size generally correlates directly with GAPDH mRNA expression levels (see above), this data is not surprising. NT-3, in contrast to NGF, did not markedly increase the size of neuronal soma in P0 trigeminal cultures, nor did it enhance neurite outgrowth significantly (data not shown). In accordance with this observation, NT-3 did not prevent the decrease in GAPDH mRNA expression that occurs when trigeminal neurons are placed in culture (figure 3.39).

NGF was only able to marginally prevent the drop in GAPDH mRNA expression that occurred when P0 nodose neurons were cultured without neurotrophic factors for 24 hrs (figure 3.43). This is not surprising in light of the small percentage of NGF-responsive nodose neurons at P0 (Forgie et al., 1999). NT-3 did not alter GAPDH mRNA levels in neonatal nodose neuron cultures (figure 3.47)

The GAPDH mRNA data presented in this chapter clearly demonstrates that the levels of GAPDH mRNA expressed by sensory neurons, both *in-vivo* and *in-vitro*, can be modulated by exposure to the neurotrophins NGF and NT-3. This observation is important for interpreting expression data for other transcripts normalised to GAPDH mRNA.

3.4.2. α - and β - CGRP

To date, few studies have addressed the regulation of the expression of α - and β -CGRP separately, either at the mRNA or peptide level. Most mRNA expression studies have either used methods that are unable to distinguish between the two CGRP transcripts (e.g. Patel et al., 2000), or have just studied the expression of the α -CGRP transcript because this has been reported to be the more highly expressed transcript in the adult. Throughout development, however, β -CGRP is the predominant transcript expressed in many tissue types and, despite high sequence homology, α - and β -CGRP are transcribed from separate genes. This would, therefore, suggest functionally distinct roles for the peptide products of each transcript in development.

In chapter 2, the differential regulation of α - and β -CGRP mRNAs was demonstrated in sensory neurons of the embryonic mouse. This research has been extended in this chapter to explore α - and β -CGRP mRNA expression patterns and the regulation of their expression at postnatal ages. An initial investigation into the expression of both mRNAs in sensory neurons from wild-type late embryonic, neonatal and adult mice showed very similar expression patterns for both transcripts. Both mRNAs showed an increase in expression from embryonic ages into the adult in all three sensory ganglia studied. Such relatively high levels of β -CGRP mRNA in the adult were unexpected,

since previous publications have suggested that β -CGRP is poorly expressed in adult tissues (Amara et al., 1985, Gibson et al., 1988). It should be born in mind that the RT-QPCR data, although normalised to GAPDH mRNA expression, does not reflect the absolute levels of mRNA transcripts relative to the amount of GAPDH mRNA. To achieve a measure of this, a standard curve would need to be constructed, prior to reverse transcription of RNA, from known amounts of the cRNAs of GAPDH and the other assayed genes. In fact, the CT (threshold-crossing values) of RT-QPCR amplified β -CGRP transcripts from late embryonic, neonatal and adult sensory ganglia, were higher than those of α -CGRP. Since the efficiency of both QPCR reactions was very similar (data not shown), this demonstrates that α -CGRP is expressed at higher levels than β -CGRP. Nevertheless, it was surprising to find β -CGRP transcripts expressed at higher levels in RNA extracted from adult sensory ganglia compared to RNA from neonatal sensory ganglia given the previously published data (this can be stated with certainty, since the mRNA levels of β -CGRP from the different ganglia at different ages were all assayed in the same QPCR plates using the same standard curve).

During the embryonic period, the presence of NGF was not essential for the initial induction of either α - or β -CGRP mRNAs in trigeminal ganglia or β -CGRP in DRG neurons (see chapter 2). However, NGF/TrkA signalling seems to be important for the expression of both isoforms by birth, since TrkA^{-/-}/Bax^{-/-} neonates showed a significant loss of α - and β -CGRP mRNAs in both trigeminal ganglia and DRG compared to Bax^{-/-} neonates (figure 3.18). NGF/TrkA signalling also appeared to be required for full expression of both α - and β -CGRP mRNAs by nodose neurons (figure 3.18). Interestingly, it would appear that the reduction in β -CGRP mRNA expression in all three ganglia in the absence of NGF/TrkA signalling was less pronounced than the reduction in α -CGRP mRNA expression. This suggests that either other factors co-operate with NGF in regulating the expression of β -CGRP mRNA in NGF responsive neurons, or that β -CGRP mRNA expression is not entirely restricted to the TrkA positive, NGF responsive, sub-population of sensory neurons in each ganglion at P0. Immuno-histochemistry and/or in-situ hybridisation, using probes and antibodies against TrkA and β -CGRP, would help to resolve these possibilities.

Much of the data presented in this chapter came from TrkA/Bax and NT-3/Bax double-null mutant neonatal mice and from single-null mutant Bax^{-/-} neonates. Because these animals are not temporally controlled, conditional null mutants, they lack functional NGF/TrkA and NT-3-dependent signalling and are devoid of Bax from the moment of conception. This has two potentially important consequences on sensory neuron development that must be kept in mind when interpreting results. The differentiation of sensory neurons can effectively be divided into “primary” and “secondary” processes. In the primary differentiation event, proliferating, committed sensory neuron precursors stop dividing and become post-mitotic sensory neurons. In mice, this occurs between E9 and E14, and results in broad sub-classes of presumptive proprioceptive, mechanoreceptive and nociceptive (TrkA-positive) neurons. A second period of differentiation occurs around birth and extends to around P7 in the mouse. During this second period of differentiation, sensory neurons of each broad sub-class become “specialized” into distinct sub-populations that have specific, refined, functional characteristics and sensory modalities (e.g. Molliver et al., 1997). The data comparing gene expression between wild type mice and Bax^{-/-} mice tends to suggest that the differentiation program of sensory neurons is largely unaffected by the lack of Bax expression (figures 3.13 to 3.17). However, it is not clear to what extent the lack of NGF/TrkA- or NT-3-dependent signalling during the embryonic and neonatal period affects either the primary or secondary differentiation of sensory neurons.

This chapter explores the expression of mRNAs that are predominantly restricted to TrkA-positive, peptidergic nociceptive neurons within adult sensory ganglia. Any changes in primary or secondary differentiation, as a result of the loss of NT-3- or TrkA-dependant signalling, may alter the number of TrkA-positive presumptive nociceptive neurons initially generated between E10 and E14 or the number of TrkA-positive peptidergic, nociceptors present within neonatal sensory ganglia at P0. This phenomenon may alter the levels of the mRNAs under investigation in the absence of genuine transcriptional regulation by NT-3 or NGF/TrkA-dependant signalling. The loss of NT-3 has been shown to affect the timing of the “primary” differentiation of DRG neurons (Farinas et al., 1996), and is associated with an increase in the ratio of TrkA-positive neurons to TrkC-positive neurons in developing sensory ganglia of NT-

$3^{-/-}/Bax^{-/-}$ double-null mutants compared to wild-type mice (Genc et al., 2004.)

Sensory ganglia have not been examined in the $TrkA^{-/-}/Bax^{-/-}$ double-null mutant to determine whether there is a change in the number of TrkA-positive neurons initially generated in the absence of NGF/TrkA signalling (this would necessitate using a reporter gene driven by the TrkA promoter, since TrkA is deleted). Neither double-null mutant strain has been examined to determine whether there is an alteration in the secondary differentiation process that may lead to a change in the number of TrkA-positive, peptidergic nociceptors that reside within postnatal sensory ganglia.

The second thing that must be taken into account when interpreting data from the transgenic neonatal ganglia is the possibility that the loss of neurotrophic support during the period of target field innervation, that occurs when TrkA or NT-3 is deleted from conception, may alter the pattern and density of normal target field innervation. Moreover, at older ages the loss of trophic support from NGF or NT-3 may alter the degree of terminal arborisation, particularly in peripheral targets (e.g. Patel et al., 2000; Lentz et al., 1999; Genc et al., 2004). The alteration in target field innervation/arborisation may effectively reduce the accessibility of sensory neurons to other target field-derived trophic factors, and this may in turn affect neuronal gene expression. Thus the deletion of TrkA or NT-3 from conception could again lead to changes in gene expression within neonatal sensory neurons that are not the result of direct transcriptional regulation by NGF or NT-3.

This scenario, described in the above two paragraphs, could theoretically account for some or all of the reduction in the expression of both CGRP mRNAs in sensory ganglia from TrkA/Bax double-null mutant neonates, rather than a direct regulation of CGRP mRNA expression by NGF per se. However, this would seem unlikely given that the *in-vitro* data (see below) and previous publications on adult rodents (Lindsay et al., 1989; Verge et al., 1995; Jiang and Smith, 1995; Price et al., 2005; Ma et al., 1995; Schuligoi and Amann, 1998; Schicho et al., 1999; Tandrup et al., 1999; Price et al., 2005) clearly demonstrate that NGF can directly influence the expression of CGRP. Further experiments using *in-situ* hybridisation and immuno-histochemistry (with probes against well characterized phenotypic markers of different neuronal sub-populations) and/or experiments with temporally controllable TrkA or NGF conditional knockout mouse lines would categorically address this question.

In-vitro cell culture data revealed that NGF is necessary to maintain the expression of α -CGRP mRNA over time in culture in both trigeminal and nodose neurons (figure 3.30. and 3.40 respectively). Dissecting out neurons and culturing them *in-vitro* effectively removes them from target field-derived neurotrophic factor support, and it is this lack of neurotrophic factor support that is largely responsible for the decrease in α -CGRP mRNA expression over time in culture. Sciatic nerve transection in adult mice and rats also removes target field-derived neurotrophic factor support from DRG neurons and leads to a reduction in the expression of α -CGRP mRNA and peptide (Noguchi et al., 1990; Mulder et al., 1997; Sterne et al., 1998; Shi et al., 2001; Shadiack et al., 2001). The fact that NGF can totally restore α -CGRP mRNA levels in cultured trigeminal neurons to levels comparable (in fact slightly greater) to those found at the time of plating, and hence *in-vivo*, suggests that α -CGRP mRNA is predominantly expressed in TrkA expressing NGF-responsive trigeminal neurons at P0. Developing nodose ganglia contain a small sub-population of NGF responsive neurons (Forgie et al., 1999). Since NGF can totally prevent the culture induced down-regulation of α -CGRP mRNA expression in nodose neurons, it is tempting to speculate that it is target field-derived NGF withdrawal that is responsible for the drop in α -CGRP mRNA expression and that α -CGRP is predominantly expressed by the NGF responsive sub-population of nodose neurons at P0.

Interestingly, β -CGRP mRNA expression was sustained in culture at *in vivo* (time 0) levels in trigeminal neurons in the absence of neurotrophic factor support for at least 48 hrs (figure 3.30.). This could suggest that NGF, or indeed other target field-derived neurotrophic factors, is/are not entirely responsible for setting the steady state *in vivo* expression levels of this mRNA at P0. In support of this is the fact that, although β -CGRP mRNA expression is clearly reduced in trigeminal ganglia of transgenic mice lacking functional TrkA/NGF signalling, the reduction is only two-fold and is significantly less than the reduction of α -CGRP expression in the same ganglia. An alternative explanation for the lack of a fall in β CGRP mRNA expression in trigeminal neurons deprived of neurotrophic factor support (and in particular NGF), may be that β CGRP mRNA is a stable mRNA with a long “turn-over” time and so it takes longer for mRNA levels to fall after withdrawal of support from target field

derived NGF. It is curious that whilst 10ng/ml NGF can enhance 24-hour β -CGRP mRNA expression levels to levels significantly above those found *in-vivo*, it cannot maintain this enhanced expression at 48 hours (figure 3.30). All the other mRNAs that showed enhanced *in-vitro* expression levels when trigeminal neurons were cultured for 24 hours with NGF, remained at elevated expression levels at 48 hours (e.g. α -CGRP and see below).

The data presented in this chapter suggests an essential role for NGF/TrkA signalling in regulating the expression of α - and β -CGRP mRNAs in neonatal trigeminal, nodose and DRG neurons. This data compliments the previous study by Patel et al., (Patel et al., 2000) who explored CGRP expression in P0 mouse DRG by immunohistochemistry. The data is also in accordance with numerous studies in the adult rodent highlighting the positive effects of NGF signalling on the expression of CGRP by DRG neurons, as discussed in 2.1.1. (Verge et al., 1995; Jiang et al., 1995; Price et al., 2005; Lindsay et al., 1989; Shadiack et al., 2001; Christensen et al., 1997; McMahon et al., 1995; Schicho et al., 1999; Schuligoj and Amann, 1998; Ma et al., 1995).

In stark contrast to the NGF/TrkA dependent regulation of α - and β -CGRP mRNA expression, NT-3 does not appear to regulate the expression of CGRP mRNAs in any ganglia either *in-vitro* or *in-vivo* (figures 3.24, 3.35 and 3.44). To date, no other data has arisen from other publications to suggest that NT-3 can regulate either of these mRNAs in sensory neurons under other experimental paradigms. This finding is not entirely unexpected given that the cognate NT-3 receptor, TrkC, is not expressed by peptidergic nociceptive neurons. However, NT-3 can, in certain circumstances signal through TrkA and also activates the common neurotrophin receptor p75 (Davies et al., 1995, Huang et al., 1999) which raised the possibility that NT-3 could possibly play a role in regulating gene expression in TrkA expressing, nociceptive sensory neurons (a sub-population that also express p75). Moreover, it has been suggested that NT-3 plays a role in regulating the timing and extent of sensory neuron differentiation from proliferating neuronal precursors in mid-gestation mouse embryos, and may thus in some way regulate the functional phenotype of sensory neurons (Farinas et al., 1996). This published data further suggests that a perturbation in normal NT-3 dependent

signalling during development may effect the expression of certain phenotypic markers that characterise functionally distinct sensory neuron sub-populations. These reasons were the rationale behind measuring the expression of nociceptive sensory neuron specific markers in NT-3^{-/-}/ Bax^{-/-} mice and determining whether NT-3 could regulate their expression in-vitro.

3.4.3. Substance P

On the whole, Substance P (SP) mRNA showed a very similar pattern of regulation to that of α - and β -CGRP mRNAs with which it is co-expressed in the same sub-population of small diameter peptidergic, TrkA positive, adult sensory neurons (Lundberg et al., 1985; Lee et al., 1985; Hokfelt et al., 1975; Cuello et al., 1978; Skofitsch and Jacobowitz, 1985). The expression levels of SP mRNA, however, show little change between E16 and adult in all three sensory ganglia studied, a situation that is markedly different to the significant developmental increase in expression levels observed for CGRP mRNAs (figure 3.6.). SP mRNA levels are, on the whole, markedly lower than those of CGRP mRNAs (especially at postnatal ages), as evidenced by the higher QPCR CT values for SP mRNA compared to CGRP mRNAs and a similar QPCR reaction efficiency (data not shown). The data in chapter 2 revealed that SP mRNA required NGF/TrkA signalling for embryonic induction. In accordance with this, the transgenic studies carried out in this chapter revealed a dramatic loss of SP mRNA in all three sensory ganglia of P0 TrkA^{-/-}/Bax^{-/-} mice compared to P0 Bax^{-/-} mice (figure 3.20A), suggesting that NGF/TrkA signalling is required to establish and maintain the correct *in-vivo* expression levels of SP mRNA in nociceptive sensory neurons of all three ganglia in the neonatal period. It seems that the loss of SP mRNA in the absence of NGF/TrkA signalling is not as dramatic as the loss of CGRP, suggesting that an additional factor may also positively regulate the expression of SP at the transcriptional level. These data differ slightly from those of Patel et al (1990). These authors were unable to detect either SP peptide or mRNA in DRG from TrkA^{-/-}/Bax^{-/-} neonates. This difference may be due to the different mouse strains used in my study (CD1 background) and the Patel study (C57 background) or, more likely, due to the greater sensitivity of RT-QPCR compared to in-situ hybridisation.

It is possible that, as in the case of α - and β -CGRP, the lack of functional NGF/TrkA signalling from the earliest stages of development changes the programme of neuronal differentiation in sensory ganglia and significantly alters the pattern of target field innervation (see above). Such a scenario could theoretically account for some/all of the reduction in SP mRNA expression in sensory ganglia, rather than reflecting a direct regulation of SP mRNA expression by NGF per se. However, this would seem unlikely given that the *in-vitro* data (see below) and previous publications on adult rodents (Lindsay et al., 1989; Zhang et al., 1995; Schuligoi and Amann, 1998; Tandrup et al., 1999) clearly demonstrate that NGF can directly influence the expression of SP. Again, as in the case of CGRP, further experiments with temporally controllable TrkA or NGF conditional knockout mouse lines, as well as detailed *in-situ* hybridisation and/or immuno-histochemical analysis of ganglia from knockout neonates, would definitively address this question.

NGF was able to prevent the rapid decrease in the levels of SP mRNA expressed by cultured neonatal sensory neurons as a result of their removal from a source of target field-derived neurotrophic factors (figures 3.31. and 3.40B.). This result reflects the drop in SP mRNA and peptide levels that are observed in small, peptidergic, L4-L6 DRG neurons of adult rodents following sciatic nerve injury due to a reduction in the availability of foot-pad derived neurotrophic factors (Nielsch et al., 1987; Zhang et al., 1995; Sterne et al., 1998). The results in this chapter show that in the case of trigeminal neurons, 10ng/ml NGF can totally prevent the decrease in SP mRNA expression that occurs over time in culture, suggesting that SP mRNA is predominantly expressed in NGF responsive, P0 trigeminal neurons in a similar manner to adult DRG sensory neurons. In contrast, 10ng/ml NGF can only partially prevent the time-dependent decrease in SP mRNA levels that occurs in cultured nodose neurons, perhaps suggesting that SP mRNA is expressed by both the small NGF-responsive sub-population of nodose neurons and also by another sub-population of non NGF-responsive nodose neurons. *In-situ* hybridisation and/or immuno-histochemistry of neonatal nodose ganglia could determine whether this is the case. Alternatively, NGF may act in conjunction with other trophic factors, in a partially redundant way, to regulate SP mRNA expression in TrkA-positive nodose neurons. Data from culture experiments and RT-QPCR analysis of RNA extracted

from sensory ganglia of NT-3^{-/-}/Bax^{-/-} and Bax^{-/-} mice did not reveal a role for NT-3 in regulating the neonatal expression of SP mRNA, either *in-vitro* or *in-vivo* (figures 3.26A, 3.36, 3.44B).

3.4.4. Nav1.8 and Nav1.9

Messenger RNAs for the two TTX-resistant sodium channels, Nav1.8 and Nav1.9, were expressed at all developmental ages assayed (figures 3.7 and 3.8). The mRNAs for both of these TTX-resistant sodium channels showed a similar, but not identical developmental pattern of expression between the ages of E16 and adult. For both mRNAs, the lowest levels of expression were found at E16 in all three sensory ganglia, and both showed a drop in expression between neonatal ages and adulthood in all ganglia. An exception to this was Nav1.8 mRNA, which showed peak levels of expression in adult nodose ganglia, not neonatal nodose ganglia. These data raise the possibility that Nav1.8 and Nav1.9 mRNAs are regulated *in-vivo* by a similar mechanism, as might be expected given the co-localization of both sodium channels in many nociceptive adult DRG sensory neurons (Roy and Narahashi 1992; Black et al., 1996; Rush et al., 1998; Amaya et al., 2000). Nav1.8 is expressed in most C- and A- δ - (and a few A- β) fibre rodent DRG nociceptive neurons (Sangameswaren et al., 1996; Amaya et al., 2000; Fang et al., 2005). Nav1.9 seems to be more restricted in its expression pattern, being most highly expressed in small diameter, non-peptidergic, IB4-positive, C-fibre nociceptors (Black et al., 1996; Amaya et al., 2000; Benn et al., 2001; Fang et al., 2002).

In chapter 2, I provided data to show that NGF/TrkA signalling is required for the induction and correct regulation of both Nav1.8 and Nav1.9 mRNA expression in mouse sensory neurons during the embryonic period. The results presented in this chapter suggest that the expression of both of these mRNAs is also regulated by NGF/TrkA signalling in P0 trigeminal ganglia and DRG. A significant reduction in the expression levels of both mRNAs was observed in trigeminal and dorsal root ganglia of P0 TrkA^{-/-}/Bax^{-/-} mice compared to Bax^{-/-} mice (figure 3.19). This decrease in TTX-resistant sodium channel expression was not seen in TrkA^{-/-}/Bax^{-/-} nodose ganglia neurons. The number of NGF responsive, TrkA-positive neurons within the

P0 nodose ganglion is small (Forgie et al., 1999), whilst the number of nociceptive neurons that would be expected to express these TTX-resistant sodium channels is likely to be large. Therefore, it is not surprising that NGF does not play a significant role in regulating the expression of Nav1.8 and Nav1.9 mRNAs in the nodose ganglion. The fact that there is absolutely no decrease in the expression of either sodium channel mRNA in the absence of NGF/TrkA signalling (in fact a slight increase was apparent) may be because the NGF responsive sub-population of nodose neurons are not nociceptive, and hence do not normally express nociceptor specific TTX-resistant sodium channel mRNAs. However, this seems unlikely since the CGRP and SP mRNA expression data presented above suggests that NGF responsive nodose neurons are peptidergic and as has been previously discussed peptidergic TrkA-positive neurons are nociceptive in neural crest-derived sensory ganglia. In-situ hybridisation could be used to address this question.

Perhaps a more likely scenario to explain the nodose ganglion data from TrkA/Bax double-null mutant neonates is that other growth factors act (possibly in a partially redundant way) to regulate the expression of TTX-resistant sodium channels in the NGF-responsive sub-population of nodose neurons (see discussion of *in-vitro* data below). BDNF, NT-4 and GDNF have all been demonstrated to have trophic effects on nodose ganglion neurons and are candidates for growth factors that may regulate TTX-resistant sodium channel expression in this ganglion (Davies et al., 1986; Lindsay et al., 1985; Davies et al., 1993; Buj-bello et al., 1994; Cummins et al., 2000; Wiklund et al., 2000; Blum et al., 2002).

Data from P0 neuronal cultures demonstrate that NGF positively regulates the expression of Nav1.8 and Nav1.9 mRNA in trigeminal neurons. Exogenous NGF prevented, to some extent, the rapid drop in TTX-resistant sodium channel mRNA levels that is a consequence of removing trigeminal neurons from their target field-derived source of neurotrophic factor support (figure 3.32). The drop in Nav1.8 and Nav1.9 levels in culture mirrors that seen in L4-L6 DRG neurons following peripheral axotomy or sciatic nerve section (Okuse et al., 1997; Cummins et al., 1997; Tate et al., 1998; Dib-hajj et al., 1998; Novakovic et al., 1998; Sleeper et al., 2000; Decosterd et al., 2002). It is worth noting that although levels of Nav1.8 mRNA are retained at levels close to those at time 0 in the presence of exogenous NGF, Nav1.9 mRNA

expression is only partially rescued by NGF. This could be due to a difference in the expression patterns of these two sodium channels. In the adult, Nav1.8 is expressed by the majority of C- and A-fibre nociceptive rodent DRG neurons, whilst Nav1.9 is more highly expressed by, and more restricted to, the IB4-positive, TrkA-negative population of small-diameter DRG neurons. It has not been determined whether this is the case in neonatal sensory neurons where the majority of presumptive nociceptive neurons are still TrkA-positive. In the adult rodent, the IB4-reactive population of neurons are predominantly responsive to GDNF family members, and in particular GDNF itself, rather than the neurotrophins. It is therefore possible that the presence of both NGF and GDNF in P0 trigeminal neuron cultures might restore expression of Nav1.9 mRNA to that seen at time 0 *in-vivo*. Although a number of TrkA-negative, NGF non-responsive, neurons expressing Nav1.8 mRNA almost certainly exist in the neonatal trigeminal ganglion, NGF appears to be able to almost completely maintain the levels of Nav1.8 mRNA in cultured trigeminal neurons. It is possible that the addition of exogenous NGF at a concentration of 10ng/ml can increase Nav1.8 mRNA expression to levels higher than those found *in-vivo* in TrkA-positive cultured trigeminal neurons, hence giving the illusion that NGF is regulating Nav1.8 mRNA expression in cultured TrkA-negative nociceptors.

Interestingly, NGF appears to be able to prevent part of the decrease in Nav1.8 and Nav1.9 mRNA expression levels in nodose neurons that occur as a consequence of *in-vitro* culture. Although the efficacy of NGF in maintaining TTX-resistant sodium channel expression in nodose neurons is small, it is statistically significant (figure 3.41). As it is such a small effect it is likely that this represents the ability of NGF to maintain levels of these sodium channel mRNAs in the small sub-population of TrkA-positive nodose neurons, rather than acting through p75 to promote Nav1.8 and Nav1.9 mRNA expression across the nociceptive nodose neuron population as a whole. A question that arises from the *in-vitro* data is why nodose ganglia from P0 TrkA^{-/-}/ Bax^{-/-} mice do not show a reduction in TTX-resistant sodium channel mRNA expression compared to nodose ganglia of P0 Bax^{-/-} mice when NGF appears to be able to positively regulate the expression of both sodium channel mRNAs in culture? In fact, nodose ganglia from double-null mutant neonates appear to express higher levels of these mRNAs, although this is not statistically significant (figure 3.19). As alluded to above, it is possible that there is a redundancy inherent in the regulation of

TTX-resistant sodium channel expression in NGF-responsive nodose neurons that could account for this discrepancy. If NGF and another growth factor can both promote Nav1.8 and Nav1.9 mRNA expression in NGF-responsive nodose neurons, the loss of TrkA by these neurons may not reduce the expression of either sodium channel mRNA, as the second growth factor would be sufficient to maintain expression. Alternatively, the lack of NGF/TrkA signalling from conception raises the possibility that neuronal differentiation is altered in the TrkA/Bax double-null mutant mouse line with the consequence that the NGF responsive nodose neuron sub-population do not arise but are replaced by neurons of a different phenotype. If this is correct and the “replacement” neurons express higher levels of Nav1.8 and Nav1.9 mRNAs than the “normal” TrkA expressing sub-population, this would account for the data obtained in the *in-vivo* study. Careful analysis of nodose ganglia from TrkA^{-/-}/Bax^{-/-} and Bax^{-/-} neonates using a combination of in-situ hybridisation and immunohistochemistry and/or further experiments with a temporally controllable, TrkA conditional knockout mouse line would address this possibility. As in the case of CGRP and SP mRNAs, RT-QPCR data from transgenic animals and *in-vitro* cultures suggests that NT-3 plays no role in regulating the expression of Nav1.8 and Nav1.9 mRNAs in sensory ganglia of neonatal mice (figures 3.25, 3.37 and 3.45).

3.4.5. Galanin

The expression of galanin mRNA was investigated in TrkA/Bax double-null mutant and NT-3/Bax double-null mutant transgenic neonatal mice. However, since galanin mRNA could not be reliably quantified in sensory neuron cultures, no culture data is presented in this chapter. This is most likely due to low-level expression of galanin in the neuronal cultures combined with the small amount of total RNA that can be extracted and purified from cultured neurons. In addition, the galanin QPCR reaction was not as efficient, or sensitive as many of the other QPCR reactions.

The developmental expression pattern of galanin mRNA was similar in both trigeminal and dorsal root ganglia, with highest expression levels at E16 and levels gradually decreasing as development proceeds (figure 3.9). Once again, as with Nav1.8 and Nav1.9 mRNAs, the developmental expression pattern was different in

nodose ganglia compared to the other two sensory ganglia. In the case of nodose ganglia, there was no significant difference in the levels of galanin mRNA expressed from E16 to adult, suggesting that a similar number of nodose ganglion neurons express the message for this neuropeptide at all developmental stages (figure 3.9). The data from trigeminal and dorsal root ganglia is broadly in agreement with previous studies, and is indicative of an important role for galanin in developing sensory neurons (Xu et al., 1996; Ma et al., 1999). It is, perhaps, a little strange that the drop in galanin mRNA expression levels that occurs between neonatal ages and adult in both trigeminal and dorsal root ganglia was not greater than that observed, as previous research has shown that whilst the majority of developing sensory neurons from these ganglia express galanin, only 5% of adult neurons continue to express mRNA for this neuropeptide (Xu et al., 1996; Ma et al., 1999). It can only be assumed that this small population of neurons that retain expression of galanin mRNA significantly up-regulate the expression of this mRNA between birth and adulthood.

There was a significant decrease in galanin mRNA expression in the DRG of $TrkA^{-/-}/Bax^{-/-}$ neonates compared to $Bax^{-/-}$ neonates (figure 3.21A). A small decrease in the expression of this neuropeptide mRNA was also observed in the trigeminal ganglia of $TrkA^{-/-}/Bax^{-/-}$ neonates compared to $Bax^{-/-}$ neonates, although this decrease was not statistically significant. These data suggest a role for NGF as a positive regulator of galanin mRNA expression within neonatal, neural crest-derived sensory neurons. However, since the reduction in galanin mRNA in trigeminal and dorsal root ganglia of $TrkA^{-/-}/Bax^{-/-}$ neonates was small, it would appear that NGF/TrkA signalling is not absolutely essential for maintaining galanin mRNA expression at this age, and other factors have a role in the regulation of this neuropeptide in these ganglia. This is not surprising in light of the fact that galanin mRNA is expressed in the majority of neonatal trigeminal and DRG neurons, whereas TrkA expression is becoming more restricted by this stage to presumptive peptidergic, nociceptive neurons. Indeed, GDNF and BDNF (Wang et al., 2003; Kerekes et al., 1995 respectively) have both been shown to regulate galanin mRNA expression in adult rat DRG (albeit in a negative manner). Receptors for these neurotrophic factors (especially TrkB) are expressed on neonatal DRG and trigeminal neurons, therefore a role for these neurotrophic factors in regulating galanin expression in neonatal neural crest-derived sensory neurons is also possible. The levels of galanin mRNA expressed by nodose

neurons from $TrkA^{-/-}/Bax^{-/-}$ neonates were similar to those found in nodose neurons from $Bax^{-/-}$ neonates. This would suggest that either galanin is not expressed in the small TrkA-positive sub-population of nodose neurons, or that, if it is, NGF plays no role in regulating its expression at neonatal ages.

The apparent positive regulatory effects of NGF on galanin mRNA expression conflicts with previous work on adult, rodent sensory neurons that suggested a negative role for NGF in regulating galanin mRNA and peptide expression (Verge et al., 1995; Kerekes et al., 1997; Ozturk and Tonge; Shadiack et al., 2001). There may be several reasons why the results from transgenic animals presented in this chapter suggest a positive rather than a negative regulatory role for NGF in modulating neonatal galanin expression. The simplest explanation is that neonatal rodent neurons respond differently to NGF compared to adult neurons. The widespread expression of galanin in developing sensory ganglia and its subsequent restriction to a very small subset of neurons in the adult (Xu et al., 1996; Ma et al., 1999), suggests that galanin plays an important role in the survival and/or development of sensory neurons, a role that becomes redundant in the adult. Differential regulation of this neuropeptide mRNA in developing and adult neurons may therefore be anticipated. The data from transgenic animals presented in this chapter was obtained from intact, non-lesioned neurons. The negative effects of NGF on modulating galanin expression that were observed in previously published research appears to have been restricted to damaged neurons. For example, exogenous NGF is unable to decrease the expression of galanin in non-injured lumbar DRG neurons following partial sciatic nerve lesion in adult rodents, but it can prevent the up-regulation of galanin expression in neighbouring injured neurons (Verge et al., 1995). Postnatal culture data from P0 sensory neurons, a situation that effectively mimics injured/axotomised neurons, would have proved informative in determining whether the ability of NGF to inhibit galanin mRNA expression is restricted to damaged sensory neurons in the neonatal rodent in the same way as it appears to be in the adult. Unfortunately, this data could not be obtained due to a lack of sensitivity of the PCR reaction and the small amount of total RNA that can be extracted and purified from cultured sensory neurons.

Once again, it is also possible that the lack of functional NGF/TrkA signalling from conception in the $TrkA/Bax$ double null mutants affects the differentiation of sensory

neurons into different functional sub-populations. This raises the possibility that the reduction of galanin expression in the absence of TrkA reflects an alteration in the composition of sensory ganglia, with regards to the numbers of neurons with different functional modalities, rather than a direct effect of NGF/TrkA signalling on galanin mRNA regulation. Further experiments with temporally controllable TrkA or NGF conditional knockout mouse lines would address this possibility, as would a detailed in-situ hybridisation and immuno-histochemistry analysis of sensory ganglia from TrkA/Bax double-null mutants using antibodies and probes against galanin and other markers that characterise functionally distinct neuronal-sub-populations.

It is also possible that the lack of functional NGF/TrkA signalling throughout development reduces the target field innervation density of sensory neurons and/or leads to inappropriate target field innervation patterns. This may affect the accessibility of sensory neurons to additional target field-derived trophic factors that normally positively regulate galanin mRNA expression during embryonic development and in the neonatal period.

Galanin has been shown to have survival promoting effects on developing, mainly nociceptive, sensory neurons. Analysis of the adult galanin null mutant mouse revealed a 13% reduction in, predominantly, small DRG neurons. This loss of neurons equated to a 24% decrease in TrkA-positive neurons that express SP, i.e. peptidergic nociceptive neurons (Holmes et al., 2000). Since NGF/TrkA signalling appears to promote the expression of galanin mRNA in developing sensory neurons, it is intriguing to propose that some of the sensory neuron cell loss observed in NGF and TrkA single knockout embryos/neonates occurs as a result of a galanin deficiency that is as consequence to the lack of functional NGF/TrkA signalling.

The data obtained from RT-QPCR analysis of RNA extracted from sensory ganglia of NT-3^{-/-}/Bax^{-/-} and Bax^{-/-} neonatal mice suggests that NT-3 may play a role in regulating galanin mRNA expression in neonatal trigeminal neurons, but not nodose or DRG neurons (figure 3.27B). Trigeminal ganglia from NT-3^{-/-}/Bax^{-/-} neonates contained significantly higher levels of galanin mRNA than trigeminal ganglia from Bax^{-/-} mice, suggesting that NT-3 normally acts to suppress galanin mRNA expression. Since the deletion of TrkA resulted in a decrease in galanin mRNA

expression in trigeminal ganglia, it is unlikely that NT-3 signals through TrkA to suppress galanin mRNA expression. Unlike DRG, trigeminal ganglia do not contain a sub-population of proprioceptive neurons expressing full-length functional TrkC in the neonatal period (these reside in the trigeminal mesencephalic nucleus) and therefore probably contains very few neurons expressing functional TrkC (Ernfors et al., 1992). This raises the possibility that NT-3 suppresses galanin mRNA expression by signalling through the common p75 neurotrophin receptor. NT-3 can, under certain circumstances signal via TrkB (Davies et al., 1995; Huang et al., 1999), so it is also possible that NT-3 signals through TrkB to inhibit galanin mRNA expression in the trigeminal ganglion. As mentioned previously, study of null mutant mice has shown that a lack of NT-3 expression from conception can also alter the timing and dynamics of sensory neuron differentiation (Farinas et al., 1996). It is therefore possible that the increased expression of galanin mRNA within trigeminal ganglia in the absence of NT-3 observed here, reflects a change in the number of trigeminal neurons that differentiate into galanin-positive neurons rather than an indication of direct regulation of galanin mRNA expression by NT-3. Alternatively, the marked drop in GAPDH mRNA expression observed in trigeminal ganglia of NT-3^{-/-}/Bax^{-/-} neonates (3.23A) (see explanation above) may give the impression that galanin mRNA expression is higher in trigeminal ganglia from these animals compared to ganglia from Bax^{-/-} mice after normalisation, when in reality the galanin mRNA levels per galanin-positive neuron may be very similar in ganglia from both genotypes. Once again, a detailed immuno-histochemistry and in-situ hybridisation analysis of sensory ganglia from NT-3/Bax double-null mutant neonates would prove useful in addressing these questions. In addition, further experiments using p75 and TrkB null mutants, or a temporally controllable, conditional NT-3 null mutant would help to determine which of the above mechanisms operate to alter the levels of galanin mRNA expression within the neonatal trigeminal ganglion in the absence of NT-3. Culture data from experiments where trigeminal neurons were grown in the presence or absence of NT-3 would also shed light on this question, however, the limited sensitivity and efficiency of the galanin mRNA RT-QPCR reaction preclude this approach at present.

3.4.6. PACAP

The developmental time-course of PACAP mRNA expression is identical in all three sensory ganglia studied. In all three ganglia, there is a 2- to 3-fold increase in the expression of this neuropeptide mRNA between E16 and P0, followed by a drop back to E16 levels by P5. PACAP mRNA levels are maintained at P5 levels until adulthood in all ganglia (figure 3.10). This conflicts, somewhat, with previous data that shows a significantly more widespread expression of PACAP mRNA and peptide in embryonic sensory ganglia compared to adult (Shuto et al., 1996; Waschek et al., 1998; Jiang et al., 2003). This result might therefore suggest that as the expression of PACAP mRNA becomes more restricted, the levels of mRNA expression increase in those cells that retain expression.

No difference in the expression of PACAP mRNA was observed between sensory ganglia isolated from either *TrkA*^{-/-}/*Bax*^{-/-} or *Bax*^{-/-} neonates, suggesting that NGF/*TrkA* signalling plays no role in regulating PACAP mRNA expression perinatally (figure 3.20). This conclusion would seem to be confirmed by the results obtained from P0 trigeminal and nodose neuron cultures. PACAP mRNA expression levels increased significantly over the first 24 hrs in culture for both trigeminal and nodose neurons cultured in the absence of neurotrophic factor support (figures 3.33B and 3.42). This increase mirrors that seen following axotomy of adult DRG neurons and probably reflects the removal of target field derived neurotrophic support from neurons (Jongsma Wallin et al., 2001). Whilst PACAP mRNA levels fell after 24 hrs in trigeminal neuron cultures, they still remained above *in-vivo* levels at 48 hrs. The addition of 10ng/ml NGF to cultures had no statistically significant effect on the levels of PACAP mRNA expressed by either nodose or trigeminal ganglia neurons (figure 3.33B and 3.42). The *in-vitro* and *in-vivo* data presented in this chapter conflicts with data from previous research using adult rodents which demonstrated that PACAP mRNA expression is positively regulated by NGF in both intact and injured DRG neurons, and following inflammation (Jongsma-Wallin et al., 2001, 2003).

The simplest explanation of the discrepancy between the *in-vivo* data presented here and the data published previously, is that the regulation of PACAP mRNA expression

is fundamentally different in neonatal sensory neurons compared to adult sensory neurons. PACAP mRNA and peptide are reported as widely expressed in embryonic and neonatal sensory ganglia, whereas in adult sensory ganglia, expression is predominantly restricted to a small sub-population of peptidergic nociceptors (Sheward WJ et al., 1998; Shuto et al., 1996; Waschek et al., 1998; Jiang et al., 2003; Moller et al., 1993; Mulder et al., 1994). The identity of the neuronal sub-populations expressing PACAP mRNA in neonatal sensory ganglia has not been established. However, it is possible that the majority of PACAP mRNA positive neonatal sensory neurons do not express TrkA, therefore the deletion of TrkA would not be expected to significantly alter the levels of PACAP mRNA in neonatal sensory neurons. Alternatively, it is possible that NGF regulates the expression of PACAP mRNA in neonates and adults by signalling through the common neurotrophin receptor, p75, and not TrkA, a scenario that is consistent with both the *in-vivo* data presented here and previously published *in-vivo* data from adult rats. Indeed, NGF has been reported to reduce the expression of PACAP mRNA in adult proprioceptive, TrkA negative, rat DRG neurons following nerve transection (Jongsma Wallin et al., 2001). Whilst this may reflect a paracrine effect of NGF on proprioceptive neurons, via actions on TrkA positive peptidergic nociceptors, it may also indicate that NGF can regulate PACAP mRNA expression via p75-dependent signalling (data suggesting that lesioned proprioceptive neurons start expressing TrkA has not emerged). Additional analysis of PACAP expression in neonatal and adult NGF/Bax and p75/Bax double-null mutants would determine whether NGF regulates PACAP mRNA expression in sensory neurons *in-vivo* via TrkA or p75 dependent signalling.

Another possible explanation for the unexpected data from TrkA/Bax double-null mutants stems from the observation that NT-3 appears to decrease PACAP mRNA and peptide expression selectively in TrkA-expressing nociceptive neurons in intact, non-lesioned adult rats (Jongsma-Wallin et al., 2001). If the actions of NT-3 are mediated by TrkA (and not p75), PACAP mRNA expression in peptidergic nociceptors would appear to be regulated in an antagonistic manner by NGF and NT-3 both signalling via TrkA. If the regulatory influences of both neurotrophins were of similar magnitude, the loss of TrkA expression in TrkA/Bax double-null mutants would not be expected to significantly alter the expression levels of PACAP mRNA.

Based on the results of previous *in-vivo* experiments, one would perhaps predict that NGF would up-regulate the expression of PACAP mRNA in cultured neural crest-derived neonatal sensory neurons. Figure 3.33B clearly shows that this is not the case. This unexpected data can be explained in a number of alternative ways, none of which are mutually exclusive. First, it has been widely reported that axotomy, a situation that is largely mimicked by placing neurons in culture, leads to an increase in PACAP mRNA expression in large, non-nociceptive TrkC/TrkB expressing neurons that do not express TrkA (Zhang et al., 1995; Zhang et al., 1996; Jongsma-Wallin et al., 2001). The up-regulation of PACAP mRNA in cultured sensory neurons is clearly evident from the data presented in figures 3.33B, 3.38B, 3.42 and 3.46. Since the majority of sensory neurons expressing PACAP mRNA in culture do not express TrkA (particularly in the case of the nodose ganglion), it is perhaps not surprising that NGF does not appear to regulate the expression of this peptide in culture. *In-vivo* data showing that NGF up-regulates the expression of PACAP mRNA and peptide in adult DRG neurons has been based on in-situ hybridisation and immuno-histochemistry, techniques that allow analysis of gene expression at the level of the individual neuron. The RT-QPCR assay used in this thesis reveals the changes in gene expression across the whole population of neurons within sensory ganglia. If the number of neurons expressing TrkA is small in the neuronal population from which total RNA for RT-QPCR analysis was extracted, and at the same time the number of neurons expressing PACAP message is large, NGF induced changes in PACAP mRNA levels in the small NGF-responsive sub-population will be unlikely to be detected. This explanation, however, cannot be the whole answer, as data presented in chapter 4 clearly show that NGF down-regulates PACAP mRNA expression in cultured adult DRG neurons where the majority of neurons are likely to be TrkA-negative.

Second, it is possible that the *in-vitro* regulation of PACAP mRNA expression does not reflect the *in-vivo* regulation of expression. All the data on the regulation of PACAP mRNA published to date has come from *in-vivo* experiments, predominantly sciatic nerve lesion. It appears that for most genes investigated in this thesis, placing neurons in culture mimics the effects of sciatic nerve lesion with regard to changes in mRNA expression. It also appears that for most genes investigated the regulation of mRNA expression by trophic factors *in-vitro* mirrors that seen after sciatic nerve lesion following infusion of trophic factors. It may be that PACAP is just an

exception to this apparently general rule. Placing neurons in culture alters the expression of a large number of mRNAs and proteins. Perhaps key elements of the signalling pathways regulating PACAP mRNA expression show aberrant expression/phosphorylation *in-vitro* and this perturbs the normal regulation of mRNA expression. Alternatively, it may be that NGF normally regulates PACAP mRNA expression *in-vivo* in a co-operative manner with additional trophic factors. Exogenous NGF therefore may not have the expected effects on PACAP mRNA expression in the absence of these additional trophic factors in the culture medium.

The third possible explanation to account for the discrepancy between my culture data and the previously published results of *in-vivo* experiments concerns the fact that all the latter data has come from the analysis of DRG neurons. It may simply be that neurotrophic factors regulate PACAP mRNA expression in trigeminal neurons in a different manner to DRG neurons. However, since NGF is shown to repress the expression of PACAP mRNA by cultured adult DRG neurons, in a dose-dependent manner, in chapter four of this thesis, this hypothesis seems unlikely. The fourth possible explanation for the inability of NGF to positively regulate PACAP mRNA expression in cultured neonatal, P0, trigeminal neurons concerns the fact that all previous *in-vivo* experiments have been performed on the rat, whereas my data is from mouse sensory neurons. It is possible that the regulation of PACAP mRNA expression differs between rat and mouse sensory neurons. There is a precedent for this in data from previous work. For example, it has been observed that the regulation of expression of the mRNAs encoding the NT-3 receptor, TrkC, and the MSP receptor, RON, are fundamentally different in cultured rat sensory neurons compared to cultured mouse sensory neurons (S. Wyatt unpublished data). This explanation is also consistent with the data on PACAP mRNA expression presented in chapter 4.

One interesting observation from the culture data is that PACAP mRNA levels increase significantly more in cultured nodose neurons (approximately nine-fold) compared to cultured trigeminal neurons (approximately four-fold) over the first 24 hours after plating (figures 3.33B, 3.38B, 3.42 and 3.46). This raises the possibility that PACAP plays a particularly important role in regeneration following lesion of the vagal nerve or other trauma to nodose neurons. Unfortunately, the lesion-induced dynamics of gene expression changes in nodose neurons *in-vivo* have not been

investigated, so it is not known whether the dramatic up-regulation of PACAP mRNA expression occurs in nodose neurons following trauma.

Trigeminal ganglia from P0 NT-3^{-/-}/Bax^{-/-} neonates displayed a small but statistically significant increase in the expression levels of PACAP mRNA compared to ganglia from Bax single-null mutant neonates (figure 3.26B). An increase in the levels of PACAP mRNA was also observed in DRG obtained from double-null mutants compared to those from Bax^{-/-} neonates, although in this case the increase was not quite statistically significant. These data would suggest a role for NT-3 as a negative regulator of PACAP mRNA expression in neonatal sensory neurons. A similar role for NT-3 has been shown in adult sensory neurons. In the adult rat, NT-3 infusion down-regulates the expression of PACAP mRNA in non-lesioned peptidergic nociceptors residing within lumbar DRG, and prevents the increased expression of PACAP mRNA by large, predominantly proprioceptive, DRG neurons following sciatic nerve lesion (Jongsma-Wallin et al., 2001). In contrast to trigeminal and DRG neurons, the data in figure 3.26 shows that NT-3 does not regulate PACAP mRNA expression in neonatal nodose neurons in-vivo. NT-3 also does not regulate PACAP mRNA expression in cultured neonatal nodose neurons (figure 3.46). These data provide another example of gene expression being regulated in a different manner in placode-derived sensory neurons compared to neural crest-derived sensory neurons.

Previously published data from experiments with adult rodents, and the data I obtained from NT-3/Bax double-null mutant neonates, indicate that NT-3 normally acts to suppress the expression of PACAP mRNA in neural crest-derived sensory neurons. However the results from my culture experiments with P0 trigeminal neurons suggest the opposite. As shown in figure 3.38B, NT-3 significantly increases the amount of PACAP mRNA expressed by neonatal trigeminal neurons after 48 hours in culture. This somewhat surprising result is difficult to explain. Neurons placed in culture can often show aberrant gene expression. An unpublished observation that has been made previously in our lab, is that early embryonic mouse trigeminal sensory neurons rapidly down-regulate expression of the full-length functional form of the cognate NT-3 receptor, TrkC, when placed in culture. Although it is not clear whether the same phenomenon occurs in the case of neonatal trigeminal neurons, this may explain why NT-3 cannot prevent the increase in PACAP mRNA

expression that occurs in predominantly larger, non-nociceptive (TrkC/TrkB expressing), sensory neurons when target field derived neurotrophic factor influences are removed. This does not, however, explain why NT-3 appears to enhance PACAP mRNA expression in trigeminal neuron cultures. Some or all of the explanations in the previous paragraph regarding the unexpected effect of NGF in regulating the *in-vitro* expression of PACAP mRNA may apply to the unexpected effects of NT-3. As mentioned previously, adult trigeminal ganglia do not contain a sub-population of TrkC positive proprioceptive neurons and neonatal trigeminal ganglia contain few full-length TrkC-positive neurons (Ernfors et al., 1992). This would suggest that the effects of NT-3 in increasing PACAP mRNA expression in cultured trigeminal neurons are mediated via TrkA, TrkB or p75.

3.4.7. VR1 (TRPV1)

The developmental pattern of VR1 mRNA expression is similar for both trigeminal and dorsal root ganglia, although expression levels are significantly higher at all ages in DRG compared to trigeminal ganglia (figure 3.11 A and C). In both ganglia, the highest expression levels of VR1 mRNA are found at E16 and there is a gradual, but highly significant, drop in the expression of this ion channel from E16 to adulthood. This is in agreement with the observation that virtually all newly “born” mouse TrkA-positive, presumptive nociceptive sensory neurons express VR1 mRNA and protein (Patrik Ernfors, Karolinska Institute, personal communication). During the late embryonic period and first post-natal week many of these VR1 positive sensory neurons lose expression of this ion channel, especially the sub-population that become IB4-positive c-fibre nociceptors (P Ernfors, personal communication). The expression data presented in figure 3.11A and C is also in agreement with previously published data. In the adult mouse, VR1 expression is predominantly restricted to TrkA-positive, peptidergic nociceptive neurons (Zwick et al., 2002). During the first post-natal week around half of the population of nociceptive neurons down-regulate expression of TrkA and begin to express receptors for the GDNF family of trophic factors (Molliver et al, 1997).

Once again, the data presented in figure 3.11 B (and figure 3.19B) reveals that the regulation of nociceptive neuron markers differs in the predominantly placode-derived nodose ganglia, compared to the neural-crest derived sensory ganglia. E16 nodose ganglia express significantly lower levels of VR1 mRNA than trigeminal and dorsal root ganglia. Expression of this ion channel mRNA increases significantly in the nodose ganglion between E16 and P0, and then between P0 and P5, so that by P5 the levels of VR1 mRNA are markedly higher in the nodose ganglia than they are at E16 in trigeminal and dorsal root ganglia. This would suggest that VR1 mRNA is not restricted to the small TrkA-positive, apparently peptidergic, sub-population of nodose neurons during development, as discussed below. Between P5 and adulthood, there is a dramatic drop in the levels of VR1 mRNA expressed by nodose neurons, although the adult levels of this ion channel mRNA are still higher than those in adult trigeminal and dorsal root ganglia. This raises the possibility that VR1 mRNA expression become largely restricted to TrkA-positive peptidergic neurons in the adult mouse nodose ganglion in the same way as it is in adult neural-crest derived sensory ganglia (although it is not certain whether a TrkA positive, peptidergic sub-population of neurons exists in the adult mouse nodose ganglion). This would not appear to be the case according to a previous publication from the rat (Michael and Priestley, 1999). In-situ hybridisation and/or immuno-histochemistry would determine whether VR1 expression patterns are the same in adult mouse and rat placode-derived sensory ganglia.

The data from neonatal trigeminal ganglia cultures, clearly demonstrates that NGF can significantly prevent the drop in VR1 mRNA expression that occurs when trigeminal neurons are removed from their source of target-field derived neurotrophic factor support (figure 3.33A). This drop in VR1 mRNA expression mirrors the changes in the expression of VR1 mRNA within adult rodent DRG neurons following sciatic nerve lesion (Michael and Priestley, 1999; Michael and Priestley 2002; Fukuoka T et al., 2002; Wendland et al, 2003). Interestingly, this drop in VR1 mRNA expression over time in culture is dependent on culture conditions and/or neuron density. Trigeminal neurons from neonatal rats and mice do not show a reduction in VR1 mRNA or protein expression when they are cultured at a high density, in serum containing media, in the absence of added growth factors (Simonetti et al., 2006). Presumably under these conditions the culture media contains sufficient levels of

NGF (and/or other growth factors), either as a result of autocrine/paracrine production by neurons or non-neuronal cells, or from the serum itself, to prevent a decrease in VR1 mRNA expression. My data clearly shows that in the absence of exogenous NGF, VR1 mRNA is virtually undetectable in RNA extracted from trigeminal neurons that have been cultured for 48 hours, at low density, in defined medium (figures 3.33A and 3.38A). 10ng/ml NGF can restore VR1 mRNA levels to those found *in-vivo* (figure 3.33A). This would suggest that VR1 mRNA, a known marker of nociceptive neurons, is predominantly expressed in TrkA-positive, nociceptive, neonatal mouse trigeminal neurons (at this age the majority of presumptive nociceptive neurons express TrkA, although the IB4-positive, TrkA-negative nociceptive population are beginning to appear (Molliver et al, 1997)). Indeed, in the adult mouse, VR1 is predominantly a marker of TrkA-positive, peptidergic nociceptors in the same way as substance P and CGRP (Zwick et al., 2002).

The *in-vitro* NGF data presented in this chapter are in agreement with previous *in-vitro* and *in-vivo* data from adult rodent DRG neurons showing that NGF can promote VR1 mRNA expression following both sciatic nerve lesion and in culture, and also data showing that sequestration of NGF with an antibody reduces the capsaicin sensitivity of naive DRG neurons (McMahon et al., 1995; Michael and Priestly, 1999; Shu and Mendell, 1999). Moreover, an increase in VR1 expression, driven by an inflammation associated increase in NGF expression, has been observed in TrkA-positive nociceptive neurons within rat lumbar DRG following CFA injection to the rat hindpaw. This increase in VR1 expression can be prevented by the concomitant administration of an NGF blocking antibody (Amaya et al., 2004).

No difference in VR1 mRNA expression was observed in neural crest-derived sensory ganglia dissected from TrkA^{-/-}/Bax^{-/-} mice, compared to those from Bax^{-/-} mice (figure 3.21B). This data is somewhat surprising in light of the *in-vitro* data obtained from P0 trigeminal neuron cultures (figure 3.33A) and the substantial evidence indicating that NGF can up-regulate the *in-vitro* and *in-vivo* expression of VR1 mRNA in adult DRG neurons (including the data presented in chapter 4 of this thesis). Results presented in this chapter from NT-3^{-/-}/Bax^{-/-} mice, however, may provide an explanation for this observation (figure 3.27 A). An increase in VR1 mRNA levels was observed in trigeminal ganglia and DRG of NT-3^{-/-}/Bax^{-/-} neonates, suggesting that NT-3 may

normally act in the neonatal period to repress VR1 mRNA expression *in-vivo* (although see alternative explanations below). Since VR1 is predominantly expressed by TrkA positive, peptidergic neurons in the adult mouse, and not those expressing TrkC (or the other possible NT-3 receptor, TrkB), it is quite possible that the negative effects of NT-3 on neonatal VR1 mRNA expression are predominantly mediated through its non-preferred receptor TrkA. If one hypothesised that NGF and NT-3 are both signalling via TrkA to regulate VR1 expression in opposite directions during the neonatal period, deleting TrkA may therefore not be expected to significantly alter VR1 expression in neonatal ganglia. However, although a feasible explanation, it should be borne in mind that the time course data suggested that VR1 mRNA is likely to be more widely expressed in neonatal trigeminal and DRG neurons and not entirely restricted to TrkA-positive cells (see above), and it is also possible that NGF and/or NT-3 can regulate VR1 expression via p75 dependent signalling. Analysis of VR1 mRNA expression in neural crest-derived sensory ganglia from TrkC/Bax double-null mutant, NGF/Bax double-null mutant and p75/Bax double-null mutant mice would help to determine whether this hypothesis is correct. Such analysis is, however, beyond the scope of this thesis.

It is possible that peripheral target fields contain trophic factors, apart from NT-3, that normally act to suppress VR1 mRNA expression. Target field innervation is seriously compromised in TrkA/Bax double-null mutant animals (Patel et al, 2000) thus preventing sensory neurons from being exposed to putative target field-derived negative regulators of VR1 mRNA expression. If this hypothesis were correct, neonatal TrkA^{-/-}/Bax^{-/-} sensory neurons would be deprived of exposure to positive and negative regulators of VR1 mRNA expression and the net result may be no change in VR1 mRNA expression levels.

An alternative hypothesis to explain the TrkA/Bax double-null mutant data for trigeminal and dorsal root ganglia is that VR1 mRNA expression is positively regulated by a number of different trophic factors in a partially redundant way in neonatal mice. The data from chapter 4 of this thesis support this idea. NGF, MSP and artemin can all positively regulate VR1 mRNA expression in cultured adult mouse DRG neurons (figures 4.9C, 4.15B, 4.23B). If all these trophic factors play a role in positively regulating VR1 mRNA expression in neonatal neural crest-derived sensory

neurons, the loss of NGF/TrkA signalling may not lead to a decrease in VR1 expression if MSP and/or artemin (or possibly additional, as yet unidentified, factors) can compensate for its loss. Determining the validity of this hypothesis would require a careful analysis of VR1 mRNA expression in sensory ganglia of double- and triple-null mutants for TrkA, GFR- α 3 and RON, preferably as temporally controllable conditional null mutants. This is clearly beyond the scope of this thesis.

Once again, it is possible that the lack of functional NGF/TrkA signalling from conception perturbs the programme of “primary” and/or “secondary” differentiation in neural crest-derived sensory ganglia in such a way as to result in the loss, or reduction, of the sub-population of neurons that ordinarily become neonatal TrkA-positive peptidergic nociceptors. This functional sub-group may, under these circumstances, be partly or wholly replaced by other sub-populations of VR1 mRNA-positive neurons that are not responsive to NGF. Careful immuno-histochemical analysis of sensory ganglia from TrkA/Bax double-null mutants and Bax null mutant neonates and/or analysis of VR1 mRNA in temporally-controlled TrkA conditional null mutants would determine whether this hypothesis is valid.

Interestingly, VR1 mRNA levels are significantly increased in nodose ganglia of TrkA^{-/-}/Bax^{-/-} neonates compared to nodose ganglia of Bax^{-/-} mice (figure 3.21 B). The simplest explanation for this is that NGF/TrkA signalling negatively regulates the expression of VR1 mRNA in the small NGF-responsive sub-population of neonatal nodose neurons. This explanation makes the assumption that VR1 mRNA expression is predominantly restricted to TrkA-positive, peptidergic, placode-derived neonatal mouse sensory neurons in the same way as it is in adult mouse neural crest-derived sensory neurons. The high levels of VR1 mRNA expressed by neonatal nodose ganglia, in comparison to the levels of VR1 mRNA in adult trigeminal and dorsal root ganglia (figure 3.11), and the observation that VR1 is co-expressed with TrkB in adult rat nodose ganglia (Michael and Priestley, 1999) would tend to argue against this possibility, however, as mentioned for other genes, expression patterns differ between rat and mouse in adult neural crest-derived sensory ganglia as could be the case for VR1. In-situ hybridisation and/or immuno-histochemical analysis of neonatal nodose ganglia would address this point.

Alternatively, if VR1 mRNA is indeed expressed by a large proportion of neonatal nodose neurons, most of which are TrkA-negative, it is possible that NGF/TrkA signalling normally represses VR1 mRNA expression in these neurons by an indirect, paracrine mechanism. For example, NGF activation of TrkA expressed on a small sub-population of nodose neurons may result in the release of a second trophic molecule that can then down regulate VR1 mRNA expression in TrkA-negative nodose neurons. Determining whether NGF can down-regulate VR1 mRNA expression, in low-density cultures of neonatal nodose neurons, would be useful to show whether NGF has the ability to decrease VR1 mRNA expression under conditions where paracrine mechanisms are likely to be inefficient. Unfortunately, reliable quantitative data on VR1 mRNA expression in cultured nodose neurons could not be obtained due to a combination of the small amount of total RNA that can be extracted from nodose neuron cultures and the relative inefficiency of the VR1 QPCR reaction. Once again, a perturbation in primary and secondary neuronal differentiation may account for the data from TrkA/Bax double-null mutant animals, especially if this results in the expansion of a sub-population of TrkA-negative neurons that express high levels of VR1 mRNA.

Although the data from NT-3/Bax double-null mutant neonates suggest that NT-3 acts during the perinatal period to suppress VR1 mRNA expression in neural crest-derived sensory ganglia, but not nodose sensory ganglia, the *in-vitro* data does not conclusively support this hypothesis (figure 3.38A). The VR1 mRNA levels expressed by P0 trigeminal neurons show a significant reduction after 24 hours in culture in the absence of neurotrophic factor support. It appears that 10ng/ml NT-3 may accentuate this reduction, however, the effect of NT-3 does not reach statistical significance. By 48 hrs in culture, VR1 mRNA can no longer be detected in trigeminal neurons, therefore any further reduction in VR1 mRNA expression as a result of exogenous NT-3 are impossible to detect. More detailed time-course experiments (with readings at 6, 12, 18 and 24 hrs) may help to determine whether NT-3 can down-regulate VR1 mRNA expression in cultured P0 trigeminal neurons.

The *in-vivo* data presented in figure 3.27A is in agreement with a recent publication documenting the role of NT-3 in regulating VR1 mRNA expression following chronic

constriction injury (CCI) of the adult rat sciatic nerve. Although there is a large decrease in VR1 mRNA expression in injured nociceptive neurons following CCI, non-injured nociceptive neurons and a few large mechanoreceptive neurons display greatly increased expression of VR1 mRNA that can be prevented by NT-3 infusion. NT-3 infusion also concomitantly decreased injury associated hyperalgesia (Wilson-Gerswing et al., 2005). It has been proposed by the authors that the effects of NT-3 are mediated through TrkA rather than TrkC.

3.4.8. Summary

This chapter has focussed on the regulation of gene expression in neonatal trigeminal, nodose and dorsal root ganglia. The regulation of gene expression by the neurotrophic factors NGF and NT-3 was explored both *in-vivo*, using transgenic mice, and *in-vitro*, using cultured neurons.

The data for α -CGRP, SP, Nav1.8 and Nav1.9 mRNAs in neural crest derived-sensory ganglia was broadly in line with previously published *in-vitro* and *in-vivo* data from adult rodents, and demonstrated that NGF/TrkA, but not NT-3, signalling plays an important role in regulating the expression of these mRNAs in the neonatal period. In fact, the data for α -CGRP, SP and Nav1.8 mRNAs, in particular, would suggest that NGF alone is the primary positive-regulator of transcriptional activity for these genes in neonatal neural crest-derived sensory neurons. Whilst β -CGRP and Nav1.9 mRNAs are clearly regulated by NGF/TrkA signalling in neonatal, neural crest-derived sensory ganglia, the data suggest that other factors co-operate with NGF in regulating the expression of these mRNAs.

Much of the data presented on the regulation of CGRP, substance P and TTX-resistant sodium channel mRNAs in the neonatal period is novel, since most previous investigations have concentrated on their regulation in the adult, predominantly in models of nerve-lesion and inflammation. This neonatal data has important implications, since all of these mRNAs encode proteins and peptides that play important roles in setting sensory thresholds. Changes in their expression are also

associated with neuropathic pain syndromes as detailed in section 1. The data suggests that the regulation of these genes following nerve-trauma (a situation that often leads to neuropathic pain) will be similar in neonatal and juvenile mice compared to adult mice. Extrapolation of this data to the human may be useful in future studies on chronic pain conditions in babies and children.

Analysis of the regulation of galanin, PACAP and VR1 mRNA expression in neural crest-derived sensory neurons produced some results that are in accordance with the regulation of these mRNAs in adult rodent neural-crest derived sensory neurons. However, some of the data highlights important differences in the regulation of these mRNAs in the neonatal period compared to the adult. One thing these three mRNAs have in common is that they are much more widely expressed amongst different sensory neuron sub-populations in the developing mouse nervous system than they are in the adult (in the adult mouse, these mRNAs are predominantly expressed in a sub-population of TrkA-positive, peptidergic, nociceptive neurons). This fact alone may account for much of the unexpected data that arose during my investigation of their neonatal expression.

The existing published literature has established that galanin mRNA expression is not affected by exogenous NGF in intact adult sensory neurons. In contrast, NGF appears to suppress the expression of galanin in damaged neural crest-derived sensory neurons (Hokfelt et al., 1987; Villar et al., 1989; Wiesenfeld-Hallin., 1992; Zhang et al., 1998; Holmes et al., 2005). Surprisingly, my data suggests that NGF positively regulates the expression of galanin mRNA in neonatal trigeminal and DRG neurons, probably in conjunction with other neurotrophic factors. In contrast, NT-3 appears to be able to suppress the expression galanin mRNA in neonatal trigeminal neurons. A number of caveats have to be born in mind when interpreting the *in-vivo* data presented in this chapter, and alternative explanations of the data, other than direct transcriptional regulation of the various mRNAs by NGF and NT-3, are possible (see above). However, if galanin transcription is directly regulated by NGF and NT-3 in undamaged neonatal, neural crest-derived neurons, this data may have important implications for the study of pathological pain states in babies and children given the anti-nociceptive effects of galanin in the adult rodent (Hao et al., 1999; Yu et al.,

1999; Liu and Hokfelt 2000; Blakeman et al., 2001; Hygge-Blakeman et al., 2004; reviewed in Wiesenfeld-Hallin et al., 2005).

The existing literature, based predominantly on *in-vivo* studies of the adult rat, indicates that NGF positively regulates the expression of PACAP mRNA in neural crest-derived sensory neurons (Jongsma-Wallin et al., 2001, 2003). My data, however, suggests that NGF does not regulate the expression of PACAP mRNA in neonatal sensory neurons. Whilst a number of hypotheses could account for this discrepancy (described in detail above), the data may reflect a genuine difference in the regulation of PACAP mRNA expression between neonatal and adult sensory neurons. The data presented in this chapter indicates that NT-3 negatively regulates PACAP mRNA expression in neonatal neural crest-derived mouse sensory neurons in a similar manner to its action on PACAP mRNA expression in the adult rat (Jongsma-Wallin et al., 2001).

The *in-vitro* data presented in this chapter shows that NGF can regulate the expression of VR1 mRNA in cultured neonatal, neural crest-derived sensory neurons. This data is in agreement with data from adult rodents and the observations made in chapter 4 of this thesis (McMahon et al., 1995; Michael and Priestly, 1999; Shu and Mendell, 1999). In contrast to the *in-vitro* data, no loss of VR1 mRNA expression was observed in either the trigeminal or dorsal root ganglia of TrkA/Bax double-null mutant neonates. This may reflect a combination of the widespread expression of VR1 mRNA in neonatal sensory ganglia together with a redundancy in the regulation of VR1 mRNA by a combination of growth factors. The data presented in chapter 4 of this thesis supports the validity of the latter hypothesis. It is not known to what extent other growth factors positively regulate the expression of VR1 mRNA in adult rodent sensory ganglia *in-vivo*. An analysis of VR1 mRNA expression in either TrkA/Bax or NGF/Bax double-null mutant adult mice (preferably temporally controlled conditional knockouts) may reveal a similar redundancy in the regulation of VR1 mRNA expression in adult sensory neurons. Given the importance of VR1 in setting sensory thresholds and its implication in the aetiology of neuropathic pain, determining whether several factors can regulate the expression of VR1 mRNA in a redundant way, in both neonates and adults, is an important question. In agreement with data from adult rodents, NT-3 appears to have a negative regulatory influence on VR1

mRNA expression in neonatal, neural crest-derived, mouse sensory neurons. The negative regulatory effects of NT-3 on galanin, PACAP and VR1 mRNA expression within neonatal and adult sensory neurons (data presented here and Wilson-Gerswing et al., 2005; Jongsma-Wallin et al., 2001) may warrant a thorough investigation into the efficacy of NT-3 in treating neuropathic pain.

To date very little has been known about the regulation of expression of nociceptive neurons markers in the neurogenic placode derived nodose ganglion, either under normal conditions or following nerve lesion/trauma. The data presented in this chapter demonstrates that developmental expression patterns and transcriptional regulation differs between placode-derived, neonatal sensory neurons and neural-crest derived, neonatal sensory neurons for many of the mRNAs investigated. Whilst this may partly reflect the small population of TrkA-positive nociceptors that reside within nodose ganglia, it seems likely that there are some genuine differences in the growth factor control of nociceptive neuron marker mRNA expression. The data presented in this chapter suggests that α -CGRP is almost exclusively restricted to the NGF-responsive population of nodose neurons whereas substance P and β -CGRP mRNAs are expressed in this neuronal subset and another, probably small, subset of nodose neurons. Nav1.8 and 1.9 mRNAs would appear to be expressed widely in the nodose ganglion. NGF can positively regulate the expression of all these mRNAs, with the largest regulatory effect apparent for α -CGRP mRNA in accordance with the apparently exclusive expression of this mRNA within NGF-responsive nodose neurons. Of particular note, is the observation that NT-3 appears to be able to negatively regulate galanin, PACAP and VR1 mRNA expression in neural crest-derived sensory neurons, but not in nodose ganglion neurons. This may reflect a lack of functional NT-3 receptors on neonatal nodose neurons, or a genuine difference in the regulation of these mRNA between placode-derived and neural-crest derived sensory neurons. Further work will be needed to determine which of these two possibilities are correct and whether the same situation exists in the adult. Given the importance of all three of these molecules in nociception, and their potential involvement in the generation of neuropathic pain, these are important questions to answer. The most striking difference between neonatal nodose- and neural crest-derived sensory neurons is in the regulation of VR1 mRNA expression by NGF. NGF

positively regulates the expression of VR1 mRNA in both cultured neonatal and adult neural-crest derived sensory neurons *in-vivo*. In marked contrast, NGF appears to repress the expression of VR1 mRNA in neonatal nodose neurons *in-vivo*, possibly via a paracrine mechanism. A number of possible alternative explanations exist for the data suggesting this, especially in the absence of *in-vitro* culture data showing a direct down-regulation of VR1 mRNA by NGF. However, if NGF really is a negative regulator of VR1 mRNA expression in placode-derived sensory neurons this has important implications for researchers working on the role of VR1 in the aetiology of neuropathic pain and visceral pain states, especially if VR1 mRNA expression is regulated in a similar way by NGF in adult nodose neurons. Further experimental work is needed to clarify whether NGF is a true negative regulator of VR1 mRNA expression in neonatal and adult mice.

3.5. Results in Brief

Chapter 3 focussed upon the expression of particular mRNAs of interest, and their neurotrophic regulation in the postnatal mouse. Both transgenic mice and neuronal cultures were used and expression of mRNAs of interest were quantified using real-time Q-PCR. The key findings can be outlined as below:

α -CGRP (and also β -CGRP and SP):

- NGF/TrkA signalling is required for expression of α - and β -CGRP, and SP mRNAs in trigeminal, nodose and dorsal root ganglia.
- NT-3 produces no effect on the expression of these mRNAs.

Nav1.8 (and Nav1.9):

- NGF/TrkA signalling is required for expression of both Nav1.8 and Nav1.9 mRNAs in trigeminal ganglia and DRG, but results suggest this is not the case for cells of the nodose ganglia.
- NT-3 produces no effect on the expression of these mRNAs.

PACAP:

- Results show no apparent effects of NGF on PACAP mRNA expression despite previous research showing a positive role for NGF/TrkA signalling on PACAP mRNA and protein expression in DRG.
- NT-3 appears to show an inhibitory effect on PACAP mRNA expression *in vivo*, and this effect could explain why no alteration in expression, either positive or negative is observed in TrkA^{-/-}/Bax^{-/-} mice. If both the inhibitory

effects of NT-3 and the positive effects of NGF are produced via TrkA, then the antagonistic effects of both factors will be lost in the transgenic knockout.

- In contrast to the above finding, NT-3 seems to show a positive effect on PACAP mRNA *in vitro*.

Galanin:

- A significant down-regulation in galanin mRNA in trigeminal ganglia of TrkA^{-/-}/Bax^{-/-} mice in comparison to Bax^{-/-} mice, and a notable, but not significant, down-regulation in DRG of TrkA^{-/-}/Bax^{-/-} was observed in comparison to Bax^{-/-} mice. These results suggest a role for NGF as a positive regulator of galanin mRNA within neural crest derived sensory neurons, however, as effects were only small it suggests that NGF is not exclusively responsible for the regulation of galanin mRNA expression in these ganglia at this age.
- No effect on galanin mRNA expression was observed in neurons of the nodose ganglia, suggestive that either galanin is *not* expressed in small TrkA positive subpopulation of nodose neurons, or that, if it is, NGF plays no role in regulating its expression at neonatal ages.
- NT-3 may act to suppress galanin mRNA expression in the trigeminal, but not in the nodose or dorsal root ganglia, however results from the TrkA^{-/-}/Bax^{-/-} mouse suggest that this effect is not mediated via TrkA. Since trigeminal ganglia do *not* contain a subpopulation of neurons that express functional TrkC receptors, this raises the possibility that NT-3 suppresses galanin mRNA expression by signalling through the common p75 neurotrophin receptor.

VR1:

- NGF was found to positively regulate the expression of VR1 mRNA in cultured trigeminal ganglia neurons.

- BUT no difference in VR1 mRNA in trigeminal or dorsal root ganglia was observed in TrkA^{-/-}/Bax^{-/-} mice vs Bax^{-/-} mice.
- NT-3 could suppress expression of VR1 mRNA in trigeminal and dorsal root ganglia, as revealed by study of NT-3^{-/-}/Bax^{-/-} mice. This may explain why no positive effects of NGF/TrkA signalling were observed in TrkA^{-/-}/Bax^{-/-} mice. If both the negative effects of NT-3 and the positive effects of NGF are mediated via TrkA, then no alterations in VR1 mRNA expression would be anticipated in mice lacking functional TrkA receptors.
- Other explanations for results observed in TrkA^{-/-}/Bax^{-/-} mice include:
 - Compensation by other neurotrophic factors
 - Lack of TrkA from conception could alter the differentiation of a subset of neurons, such that those that express TrkA no longer express VR1
- Study of results for nodose neurons suggests NGF/TrkA signalling negatively regulates the expression of VR1 mRNA in nodose neurons. However nodose neurons tend to express TrkB rather than TrkA, so results might suggest NGF/TrkA signalling can repress VR1 mRNA expression via a paracrine mechanism.

Chapter 4

Neurotrophic Factor Regulation of Gene Expression in Adult Sensory Neurons

4.1 Introduction

In chapters 2 and 3 of this thesis, I investigated the developmental transcriptional regulation of a number of proteins and peptides that are markers of peptidergic, nociceptive sensory neurons in the adult rodent. The developmental mRNA expression profile for each of these markers was determined in neural crest- and placode-derived sensory neurons. In addition, the role that the neurotrophic factors NGF and NT-3 play in initiating and regulating the transcriptional expression of each mRNA was investigated using *in-vivo* and *in-vitro* approaches. In this chapter, I investigate the transcriptional regulation of the same mRNAs in cultured adult DRG neurons. In addition to the genes examined in previous chapters, I also examine the transcriptional regulation of a number of other genes that may play important roles both in determining normal nociceptive thresholds, and in the generation of inflammatory and neuropathic pain conditions. These additional genes are the non-peptidergic nociceptive neuron marker, P2X3; the TTX-sensitive sodium channels, Nav1.6 and Nav1.7; and two proteins whose expression is associated with neuronal damage, DINE and ATF3. The previous two chapters have concentrated on examining the transcriptional regulation of nociceptive neuron markers by NGF and NT-3. I extend the study in this chapter to include an investigation of the effects that the neurotrophic factors artemin, MSP and LIF have on the regulation of gene expression at the transcriptional level.

Under normal circumstances, the mRNA levels of all the genes examined in this chapter are likely to be fairly static within each adult sensory neuron sub-population (in the case of DINE and ATF3 expression is effectively absent, as described below). Since all these genes have important roles in determining nociceptive thresholds, it is

important to establish the neurotrophic growth factor, or combination of growth factors, that set these steady state expression levels. This of particular importance, since changes in the expression of these genes that occur during inflammation and following nerve trauma, and which may be causally related to pathological pain conditions, are likely to be driven, at least in part, by a change in the availability of neurotrophic factors that normally regulate their steady state levels in the “normal” adult. For example, inflammation is associated with an increase in the levels of NGF and GDNF in tissues surrounding nerves, Schwann cells and within DRG themselves. These increases in neurotrophic factor expression drive changes in neuronal gene expression, either directly or indirectly, that can lead to mechanical and thermal hyperalgesia (e.g. Amaya et al., 2004; Ji et al., 2002; Hefti et al., 2006). Conversely, neuronal damage reduces the accessibility of neurons to target field-derived neurotrophic factors and leads to changes in gene expression in damaged neurons that may contribute to the development of neuropathic pain. The reduced ability of damaged neurons to sequester and retrogradely transport target field-derived neurotrophic factors effectively increases the availability of these factors to undamaged sensory neurons innervating the same target field. This in turn leads to changes in the expression of functionally important genes in these “spared” neurons, which in itself may drive the generation of pathological pain (e.g. Hudson et al., 2001; Fukuoka et al., 2002; Winston et al., 2001; Ogun-Muyiwa et al., 1999; Wendland et al., 2003). Quite clearly, therefore, it is vital to fully understand the neurotrophic factor/factors that regulate the expression of functionally important genes within adult sensory neurons, as this will lead to an increased understanding of how inflammatory and neuropathic pain develops. Such an increased understanding of gene regulation may lead future research down fruitful therapeutic avenues.

The most direct way of determining which neurotrophic factors regulate the expression of sensory neurons genes in the adult would be to use transgenic animals that have null deletions of either the neurotrophic factors, or their receptors. This approach was used, successfully, in chapter 2 to determine whether NGF/TrkA signalling was important for the induction of a number of genes that are nociceptive neuron markers. In chapter 3, double-null-mutant mice, that contained a deletion of either TrkA or NT-3 in addition to a deletion of the pro-apoptotic protein, Bax, were used to examine the regulation of sensory neuron gene expression in neonatal sensory

ganglia. The double-null mutant approach was necessary to ensure that results were not confounded by widespread loss of specific neuronal sub-sets in the absence of neurotrophic factor signalling during the period of naturally occurring neuronal death. Despite the deletion of Bax and the concomitant prevention of neuronal cell death, there are a number of caveats to the interpretation of data from chapter 3 regarding the effects of trophic factor/receptor deletion on neuronal differentiation and target field innervation (discussed in detail in chapter 3). For this reason, it was necessary to validate data from the transgenic mice with cell culture data. Such cautionary interpretation of data is likely to be more important in the case of adult transgenic mice, so, although null mutants of artemin, NGF, MSP and LIF receptors are available, it was decided to carry out the entire study on gene regulation in adult sensory neurons using an *in-vitro*, cell culture approach.

Ideally, a temporally and spatially controlled knockout of neurotrophic factor receptor genes, using a cre-ERT2/loxP-site system, would have been used to generate *in-vivo* gene regulation data, thus avoiding the pitfalls of aberrant differentiation and/or target field innervation affecting the results. However, whilst suitable nociceptive neuron-specific cre-recombinase (Agarwal et al., 2004) and floxed LIF and MSP receptor (Betz et al., 1998; Waltz et al., 2001) mouse lines exist, mouse strains containing loxP flanked TrkA and GFR- α 3 have not been generated to date. Using gene targeting approaches to generate floxed TrkA and GFR- α 3 alleles is both time-consuming and costly and far beyond the scope of this thesis. Another alternative approach that could be used to, determine the effects that neurotrophic factors have on regulating gene expression in the adult, is intraplantar injection of exogenous neurotrophic factors, function blocking antibodies or receptor-Fc constructs into one hindpaw footpad of mice over a number of days. Hindpaw footpads receive their sensory innervation from L4, 5 and 6 lumbar DRG. An analysis of mRNA expression from ipsilateral L4-L6 DRG and contralateral L4-L6 DRG (as controls) would allow analysis of the effects that increasing (neurotrophic factors) or decreasing (blocking antibodies and receptor-Fc) target field neurotrophic factor levels has on the transcriptional regulation of the genes in question. This approach, however, has two major drawbacks. The first is the cost of the large amounts of neurotrophic factors and blocking reagents required to successfully alter gene expression in L4-L6 DRG of a number of animals. The second

drawback is the possibility that any effects that exogenous neurotrophic factors and blocking reagents have on changing DRG mRNA expression may be via a paracrine effect, on other tissues expressing receptors for the neurotrophic factors, and hence not reflect the direct effects of neurotrophic factor signalling on DRG neurons. For these two reasons, I decided not to pursue this approach.

Since adult rodent DRG neurons can survive in culture independently of neurotrophic factors (Lindsay, 1988), they allow a direct comparison of the effects of specific neurotrophic factors on regulating gene expression as a true 'no neurotrophic factors control' can be set up without the need for caspase inhibitors to prevent apoptosis. In many respects, culturing neurons can be regarded as a model of axotomy and/or peripheral nerve injury. In culture, and following peripheral nerve injury, neurons have their processes damaged and are deprived of their usual source of target field-derived neurotrophic factors. In both cases, neurotrophic factor deprivation leads to significant changes in the expression of a number of functionally important proteins and peptides including CGRP, SP, Nav1.8, Nav1.9, PACAP and VR1 (Lindsay et al., 1989; Verge et al., 1995; Jiang and Smith, 1995; Price et al., 2005; Lindsay et al., 1989; Zhang et al., 1995; Aguayo and White, 1992; Black et al., 1997; Fjell et al., 1999; Jongsma Wallin et al., 2001; McMahan et al., 1995; Winston et al., 2001; Ogun-Muyiwa et al., 1999; Wendland et al., 2003). Indeed, adult DRG cultures have been extensively used in the fields of inflammatory and neuropathic pain research to examine the regulation of a number of genes that have previously been implicated in the aetiology of neuropathic pain following *in-vivo* nerve lesion/axotomy/crush models (Nielsch et al., 1987; Zhang et al., 1995; Sterne et al., 1998; Zhang et al., 1996; Jongsma-Wallin et al., 2001; Armstrong et al., 2003; Hokfelt et al., 1987; Villar et al., 1989; Noguchi et al., 1993; Nahin et al., 1994; Ma et al., 1997; Shi et al., 1999; Michael and Priestly, 1999; Michael and Priestly 2002; Fukuoka T et al., 2002; Waxman et al., 1994; Cummins and Waxman, 1997; Sleeper et al., 2000; Kim et al., 2001; Abe et al., 2002; Chung et al., 2004; Okuse et al., 1997; Cummins et al., 1997; Tate et al., 1998; Dib-hajj et al., 1998; Novakovic et al., 1998; Sleeper et al., 2000; Decosterd et al., 2002). For this reason, the data generated in this chapter will largely be interpreted in the context of its relevance to studies on inflammatory and neuropathic pain.

The main introduction in chapter one includes a detailed account of the biology of the neurotrophic factors; NGF, artemin, MSP, and their respective receptors LIF was mentioned briefly and is described in more depth further in this chapter. The introductions to chapters 2 and 3 discuss the function, significance and regulation of: the neuropeptides SP, galanin, PACAP, and α - and β -CGRP; the TTX-resistant sodium channels Nav 1.8 and Nav 1.9; and the TRP ion channel, VR1. The remainder of this introduction will give some background to the new genes studies in this chapter, namely; P2X3, Nav 1.6, Nav 1.7, DINE and ATF3.

4.1.1. Damage-induced neuronal endopeptidase (DINE)

Damage-induced neuronal endopeptidase (DINE) is a membrane-bound enzyme belonging to the family of Zn-metalloproteases, which includes: neural endopeptidase (NEP); Kell blood group antigen (KELL); the endothelin-converting enzymes, ECE-1 and ECE-2 and PEX (See Turner et al., 1997 for a review; Valdenaire et al., 2000). Two independent groups, using different techniques, initially discovered DINE. Valdenaire et al., (Valdenaire et al., 1999) screened human caudate nucleus and spinal cord cDNA libraries, whilst Kiryu-Seo et al., (Kiryu-Seo et al., 2000) used differential display PCR on reverse transcribed RNA that had been extracted from either lesioned or non-lesioned hypoglossal nuclei.

In the developing rat, DINE mRNA expression is first apparent in the neural tube at E12-E13. DINE mRNA expression is restricted to differentiated neurons and is absent from neural precursors, glial precursors and glial cells (Nagata et al., 2006). At early developmental stages (E12-E14), most differentiated neurons of the neural tube express DINE mRNA. However, as these cells migrate during development, DINE mRNA expression becomes increasingly restricted to several specific brain nuclei that characteristically contain cholinergic and/or peptidergic neurons. DINE mRNA expression is significantly more widespread in the embryonic and neonatal CNS compared to the adult CNS. In the developing PNS, DINE mRNA is only expressed transiently in sensory ganglia shortly before birth. In contrast, DINE expression is maintained in sympathetic ganglia in the adult (Nagata et al., 2006). DINE mRNA

expression is highest in the hypothalamus; large cholinergic cells within the striatum and some cranial motor nerve nuclei within the adult rodent CNS (Valdenaire et al., 1999; Kiryu-Seo et al., 2000; Nagata et al., 2006).

Whilst DINE protein and mRNA are only detectable at very low levels in the adult nervous system under normal circumstances, they are dramatically up-regulated in response to nerve injury (Kiryu-Seo et al., 2000; Kato et al., 2002; Ohba et al., 2004). For example, sciatic nerve lesion induces DINE mRNA expression in predominantly IB4-negative, TrkA-positive neurons, in a similar pattern to that of the neuropeptide galanin (Kato et al., 2002). The dramatic increase in DINE expression is only observed in neurons, with no detectable expression in injured glial cells (Kiryu-Seo et al., 2000; Kato et al., 2002; Ohba et al., 2004; Nagata et al., 2006).

To date, no specific substrate for the enzymatic activities of DINE has been identified. However, since the expression of many neuropeptides, growth factors and transcription factors are increased following nerve injury, it is possible that DINE processes some of these molecules to activate them (Nagata et al., 2006). This may be particularly true in the case of neuropeptides, since DINE is largely restricted to the endoplasmic reticulum, which is the major site of neuropeptide processing (Benoit et al., 2004). Within motor neurons, at least, DINE is the only known protease that is significantly up-regulated in response to nerve injury, suggesting it may modulate the cellular response to injury and act in a neuroprotective manner (Kiryu-Seo et al., 2000). In support of this hypothesis, DINE can partially inhibit C2-ceramide-induced apoptosis in COS cells, possibly by enzymatically activating enzymes such as Mn-superoxide dismutase and Cu/Zn-superoxide dismutase (Kiryu-Seo et al., 2000).

A CNS neuroprotective role for DINE is also suggested following ischemic injury. A late-onset, prolonged expression of DINE mRNA is detected in the peri-infarct cortex and specific nuclei of the thalamus following middle cerebral artery occlusion in adult rats (Ohba et al., 2004). It has been suggested that this up-regulation could stimulate antioxidant activity that in turn promotes neuroprotective effects. This hypothesis is in accordance with the observation that middle cerebral artery occlusion leads to increased neuronal cell death, as a result of increased oxidative stress, in mice

deficient in Mn-superoxide dismutase or Cu/Zn-superoxide dismutase (Kondo et al., 1997).

A DINE knockout mouse has been generated to further investigate the biological roles of this enzyme. Homozygous null-mutant mice die shortly after birth from respiratory failure, due to a lack of lung ventilation (Schweizer et al., 1999; Valdenaire and Schweizer, 2000). Histological analysis of homozygous null-mutant neonates revealed that all other tissues and organs developed normally (Schweizer et al., 1999; Valdenaire and Schweizer, 2000). The restricted expression of DINE in the nervous system and the lethal phenotype of the knockout mouse would, therefore, suggest a vital role for DINE in the nervous system control of respiration.

In addition to its proposed neuroprotective role, and a role in regulating respiration, recent experimental evidence has suggested a possible role for DINE in the regulation of the sleep cycle. The expression of DINE mRNA is significantly suppressed in a subgroup of anterior pituitary cells in animal models of extreme fatigue. Furthermore, DINE mRNA expression continues to decrease in these cells if the period of sleep disturbance is extended (Ogawa et al., 2005). No alterations in DINE mRNA were observed in other brain areas. This effect is reversible, although the recovery is slow, taking approximately 72 hours for DINE mRNA levels to return control levels (Ogawa et al., 2005).

The nerve trauma induced up-regulation of DINE mRNA expression by adult rat DRG neurons has previously been studied using both in-vitro and in-vivo approaches (Kato et al., 2002). It appears as if DINE mRNA induction within adult DRG neurons is the consequence of a combination of target field-derived NGF withdrawal and increased exposure to LIF that is generated by Schwann cells at the site of lesion.

4.1.2. Activating Transcription Factor 3 (ATF3)

Activating transcription factor 3 (ATF3), also known as LRF-1, LRG-21 or CRG-5, is a member of the ATF/CREB family of transcription factors. The name ATF was assigned in 1987 to refer to proteins that could bind to the adenovirus early promoters

E2, E3 and E4 at a specific core sequence 'CGTCA' (Lee et al., 1987). The specific consensus sequence for binding was later defined as TGACGT(C/A)(G/A) (Lin and Green, 1988). The cAMP responsive element binding protein (CREB) was named in a similar manner to define a category of proteins that could bind to the cAMP responsive element (CRE), TGACGTCA, on the somatostatin promoter (Montminy and Bilezikjian, 1987). The unexpected discovery of an identical consensus sequence for both subfamilies led to the amalgamation of both families to create the ATF/CREB family.

ATF3 regulates transcription by binding, through a leucine zipper region, to its consensus DNA binding sites. ATF3 is an unusual member of the ATF family, as it has the ability to both activate and suppress transcription. As a homo-dimer, ATF3 represses transcription (Chen et al., 1994), but upon forming a heterodimeric complex with Jun proteins it becomes an activator of transcription (Hai and Curran, 1991; Hsu et al., 1991). Multiple splice variants of ATF3 mRNA have been identified that encode different variants of the protein (e.g. Hashimoto et al., 2002; Hua et al., 2006).

The rapid, marked induction of ATF3 expression has become a well-established marker of stress and injury in a number of cells and tissues. Examples of injuries that induce ATF3 expression include: mechanical and toxin-induced injury to the rodent liver; ischemia in the rat heart and kidney; the action of pro-inflammatory cytokines on β cells; excito-toxic brain seizure; diabetes-induced peripheral neuropathies; exposure to high concentrations of nitric oxide (NO) (Chen et al., 1996; Yin et al., 1997; Hai et al., 1999; Allen-Jennings et al., 2002; Hartman et al., 2004; Wright et al., 2004). ATF3 is also dramatically up-regulated in DRG neurons following axotomy, with 82% of L4 DRG neurons becoming immuno-reactive for ATF3 following sciatic nerve transection; whilst in control rats no immuno-reactivity for ATF3 is observed (Wang et al., 2003; Averill et al., 2004). In partial nerve injury paradigms, ATF3 mRNA and protein are only induced in damaged neurons, in a pattern that corresponds to the expression of phosphorylated c-Jun, and remains absent from neighbouring "spared neurons" (Tsujino et al., 2000; Tsuzuki et al., 2001; Obata et al., 2003; Wang et al., 2003). An up-regulation of ATF-3 mRNA expression has also been demonstrated in cultured trigeminal ganglion neurons, a further indication that

culturing neurons can be regarded as a model of injury/axotomy (Dussor et al., 2003). Interestingly, ATF3 and its dimerisation partner, c-Jun, are also dramatically up-regulated in Schwann cells ensheathing peripheral nerves following nerve lesion. The timing of Schwann cell ATF3/c-Jun induction appears to coincide with the onset of Wallerian degeneration (Hunt et al., 2004).

Although the functional significance of ATF3 induction in response to injury in non-neuronal cells is still unclear, its up-regulation following insult and injury suggests that it may play a role in protecting cells from stress. Indeed, a role in cell survival has been implicated by data from Kawauchi et al. (Kawauchi et al., 2002). They found that the adenoviral mediated over-expression of ATF3 in human umbilical vein endothelial cells could protect these cells from TNF- α induced apoptosis by suppressing the expression of the pro-apoptotic protein p53. Interestingly, p53 itself may induce the expression of ATF3 in human cell lines following cell trauma (Zhang et al., 2002). Conversely, however, several laboratories have suggested that the induction of ATF3 in liver cells, following stress from ischemia (Haber et al., 1993), partial hepatectomy (Hsu et al., 1991) or overexposure to toxins such as alcohol or acetaminophen (Chen et al., 1996), may actually exacerbate cell damage and promote apoptosis. Data from transgenic mice supports this latter interpretation of the role of ATF3 in non-neuronal cells following traumatic stimuli. Mice over-expressing ATF3 display many symptoms of liver dysfunction, including; enhanced levels of; serum bilirubin, bile acids, alkaline phosphatase, alkaline transaminase and aspartate transaminase. Furthermore, the over-expression of ATF3 leads to a repression of a key enzyme in the gluconeogenic pathway, phosphoenolpyruvate carboxy-kinase, (PEPCK) leading to alterations in glucogenesis and subsequent detrimental effects on glucose homeostasis (Allen-Jennings et al., 2002). In addition to this, mice over-expressing ATF-3 in the heart display contractile dysfunction and conduction abnormalities (Okamoto et al., 2001).

Micro-array analysis of MDA-1986 cells treated with the tumour repressor curcumin has suggested that ATF3 plays a part in mediating the beneficial effects of this drug (Yan et al., 2005). Curcumin treatment causes an increase in ATF3 mRNA expression that appears to be casually linked to the pro-apoptotic, anti-tumour effects of

curcumin (Yan et al., 2005). It has been suggested that induced ATF3 may act by blocking the ubiquitination and subsequent degradation of the tumour suppressor, p53 (Yan et al., 2005). In contrast to the data from MDA-1986 cells, ATF3 has been causally implicated in the malignant growth of Hodgkin/Reed-Sternberg (HRS) cells, which underlie classical Hodgkin lymphoma (cHL). Micro-array analysis of Hodgkin and non-Hodgkin cell lines initially demonstrated that ATF3 mRNA was much more highly expressed in the former cell lines (Janz et al., 2005). Subsequent RNA interference experiments, in which ATF3 expression was selectively “knocked down”, significantly reduced the viability of Hodgkin cells, suggesting that over-expression of ATF3 contributes to the malignant growth of Hodgkin cells.

In-vitro and *in-vivo* experiments investigating the role of ATF3 in the nervous system suggest that ATF3 may be both neuroprotective and enhance nerve regeneration following nerve injury. For example, co-expression of ATF3 with its potential dimerisation partner, c-Jun enhances neurite outgrowth in both PC12 and Neuro2a cell cultures (Pearson et al., 2003). Moreover, inhibition of c-Jun phosphorylation, by inhibition of JNK (c-Jun N-terminal Kinase) dramatically reduces axonal outgrowth, and ATF-3 induction, in nodose, dorsal root, and superior cervical ganglia explant cultures. The same effect is observed in dissociated cultures of nodose and DRG neurons (Lindwall et al., 2004; 2005). In accordance with these observations, JNK activation, c-Jun phosphorylation and ATF3 induction are associated with regenerating sensory neurons *in-vivo* following sciatic or vagal nerve transection (Lindwall et al., 2004). DNA micro-array analysis has revealed that JNK activation (and hence c-Jun phosphorylation) in combination with the over-expression of ATF3 induces the expression of the heat shock protein Hsp27 in PC12 cells (Nakagomi et al., 2003). Over-expression of Hsp27 itself can block JNK-mediated apoptosis of cultured SCG neurons and PC12 cells, apparently by phosphorylating and activating Akt. It is the activation of Akt that enhances neurite outgrowth from neuronal cells (Nakagomi et al., 2003). Taken together, this data suggests that the JNK mediated phosphorylation of c-jun promotes the induction of ATF-3 expression in neurons. ATF-3/c-Jun heterodimers, in turn, induce Hsp27 expression, which in turn phosphorylates and activates Akt to both prevent JNK-mediated apoptosis and to promote neurite outgrowth. The hypothesis that ATF3 plays a neuroprotective role following neuronal trauma finds support in the observation that adenovirus mediated

over-expression of ATF3 in adult rat hippocampal neurons prevents excitotoxic cell death following intra-hippocampal injection of kainic acid (Francis et al., 2004).

Regulation of ATF-3 expression by neurotrophic factors

The dramatic increase in ATF-3 protein expression that occurs within adult rat DRG neurons following sciatic nerve transection can be partly ameliorated by infusion of NGF or GDNF. NGF appears to suppress ATF3 expression in predominantly small neurons, whereas GDNF exerts its effects on both small and large neurons (Wang et al., 2003; Averill et al., 2004). It is not clear whether the effects of the two trophic factors in ameliorating the induction of ATF3 are partially or totally additive. Exogenous GDNF can also partially block the increase in ATF3 expression that occurs in adult mouse facial motor neurons following facial nerve transection (Parsadanian et al., 2006). The experimental paradigms used in these studies cannot rule out the possibility that the effects of GDNF and/or NGF in suppressing ATF3 expression occur as a result of a paracrine action of the neurotrophic factors on other cell types rather than a direct action on neurons. This may explain why a previous study failed to demonstrate that NGF regulates the expression of ATF3 mRNA in cultured adult rat trigeminal neurons (Dussor et al., 2003). Alternatively, the regulation of ATF3 mRNA and protein expression may differ in trigeminal neurons compared to motor neurons and DRG neurons. To date, there are no other reports in the literature describing the regulation of ATF3 mRNA or protein expression by other neurotrophic factors.

4.1.3. P2X3

ATP has many roles within both the PNS and CNS, functioning as an energy source, a neurotransmitter, a neuromodulator and a transmitter of pain (Bean, 1992; Gallighan and Bertrand, 1994). In the latter case, the source of ATP that activates sensory neurons to signal pain is usually ATP that is released from damaged cells following inflammation or physical trauma (reviewed in North, 2002 and 2003). ATP mediates its effects by binding to purinoceptors of two families, the P2X and the P2Y family (for reviews see Burnstock, 2000 or North, 2002). Upon binding ATP is rapidly

degraded by cell surface phosphatases to adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine.

The P2X3 receptor protein is one of seven cloned P2X receptor subunits (P2X1 – P2X7) that are between 384 and 595 a.a. in length and act as cell surface, ATP-gated ion channels. Each P2X protein has two membrane spanning hydrophobic regions separated by a large extra-cellular region. Carboxyl and amino terminal domains are located within the cytoplasm (North, 2002 and 3003). Whilst it is certain that functional channels comprise many subunits interacting with each other, in either a homo- or hetero-multimeric way, the precise topology of native functional P2X ATP-gated ion channels has remained elusive.

Two separate laboratories originally cloned P2X3 from a sensory neuron cDNA library in 1995 (Chen et al., 1995; Lewis et al., 1995). P2X3 expression appears to be almost entirely restricted to primary afferent sensory neurons in adult rodents. In particular, P2X3 mRNA and protein expression is mainly localised to a sub-population of C-fibre nociceptive neurons that are predominantly non-peptidergic and IB4-positive, and, in the case of DRG neurons, whose central projections terminate in the inner region of lamina II of the dorsal horn (Vulchanova et al., 1997 and 1998; Bradbury et al., 1998; Ramer et al., 2001). Interestingly, the more rostral DRG at cervical levels contain a greater number of P2X3-positive neurons than those at lower levels. In particular, cervical DRG contain a sub-population of IB4-negative, A- δ fibre (myelinated) peptidergic nociceptive neurons that express P2X3 (Ramer et al., 2001). This is in accordance with a study reporting that 40% of RT-97-positive, myelinated, trigeminal ganglion neurons (the most rostral neural crest-derived sensory ganglia) express protein for this ion channel in addition to virtually all IB-4 positive C-fibre neurons (Eriksson et al., 1998). P2X3 protein expression is found in the central and peripheral terminal arbors of nociceptive neurons as well as their cell bodies (North et al., 2003). In addition, P2X3 expression often coincides with the expression of the TRP ion channel, VR1, in the rat (Petruska et al., 2000 a and b and 2002).

In contrast to the adult, P2X3 protein is expressed in a number of sites outside of sensory ganglia in the embryonic mouse (Kidd et al., 1998; Boldogkoi et al., 2002). For example, P2X3 immuno-reactivity is evident between E9.5 and E14 in: the hindbrain, midbrain, marginal layer of the diencephalon and motor neuron precursors within the CNS; paravertabral sympathetic chain; testis; aorta. However, P2X3 protein is no longer detectable in the rodent CNS by P14 (Kidd et al., 1998). Similarly, P2X3 immuno-staining (and ATP gated channels with characteristics resembling P2X3) is widespread in perinatal rat SCG neurons, but largely disappears by P17 (Dunn et al., 2005). P2X3 immuno-reactivity is found in DRG and trigeminal ganglion neurons as early as E9.5. In contrast to the adult expression pattern, P2X3 protein is found within the majority of sensory neurons between E9.5-E14.5, rather than being predominantly restricted to small IB4-positive sensory neurons (Boldogkoi et al., 2002; Ruan et al., 2004). P2X3 expression progressively disappears from the majority of large myelinated neurons and CGRP-positive nociceptors during the first two post-natal weeks, so that by P14 the expression pattern of P2X3 in sensory ganglia resembles that of the adult (Ruan et al., 2004). The restriction of P2X3 expression to IB4-positive nociceptive sensory neurons in the adult suggests an important role for ATP and P2X3 in pain processing. This is discussed in more detail below.

Immuno-histochemistry has detected both P2X2 and P2X3 proteins in the same adult rat sensory neuron terminal arbors, suggesting that P2X3 subunits are able to form both homo-multimeric ion channels and hetero-multimeric ion channels, in combination with P2X2 subunits, in these neurons (Vulchanova et al., 1997). Heterologous expression of P2X subunits in cell lines has demonstrated that P2X3 homo-multimers have a high binding affinity for agonists and display rapid activation and rapid desensitisation kinetics following repeat agonist exposure (North et al., 1997; for a review see Ralevic and Burnstock, 1998). P2X2 homo-multimers, however, have a low agonist affinity combined with slow activation rates and slow, only partial, desensitisation following repeated agonist exposure (Evans et al., 1992; Collo et al., 1996). P2X3 subunit containing channels are also unique in that they respond to the synthetic agonist $\alpha\beta$ meATP. Hetero-multimeric P2X2/P2X3 channels have activation kinetics that combine properties of both subunits in that they display sensitivity to $\alpha\beta$ meATP, have high affinity, rapid agonist binding and slow,

incomplete, desensitisation (Ralevic and Burnstock, 1998). Experimental evidence suggests that heterologously expressed P2X2/P2X3 hetero-multimeric receptors contain one P2X2 subunit and two P2X3 subunits (Jiang et al., 2003).

Electrophysiological recordings of sensory neurons appear to confirm the existence of P2X3 homo- and P2X2/P2X3 hetero-multimeric channels, but not P2X2 homo-multimeric channels, in adult rat trigeminal and dorsal root ganglion neurons (Lewis et al., 1995; Roberson et al., 1996; Cook et al., 1997). However, homo-multimeric P2X2 channels appear to be present in adult nodose neurons (Zhong et al., 2001)

P2X3 and Pain

As mentioned above, ATP has been shown to elicit pain. For example, application of ATP, via iontophoresis, to the forearms of human volunteers produces a modest burning pain (Hamilton et al., 2000). Similarly, the injection of ATP into the trapezius of human volunteers produces a moderate sensation of pain (Mork et al., 2003). The injection of ATP into the footpad of rats produces nocifensive behaviour that includes paw licking and lifting. Such nocifensive behaviour is an indication of the animals suffering pain (Bland Ward et al., 1997; Hamilton et al., 1999). Furthermore, ATP injection into the footpads of rats suffering carrageenan induced inflammation, produces an enhanced pain response in comparison to injection into naive rats, suggesting enhanced sensitisation of P2X receptors by inflammation (Hamilton et al., 2001). Further studies have revealed that the enhanced sensitivity may be due to the release of the inflammatory mediators, SP and bradykinin. Downstream signalling following the binding of SP and bradykinin to their receptors is thought to potentiate the activation of P2X3 and P2X2/3 ion channels by phosphorylating them (Paukert et al., 2001). The observation that CFA-induced inflammation produces an increase in P2X3 protein expression in small-medium neurons of the DRG, further suggests that P2X3 plays a role in the generation of inflammatory hyperalgesia (Xu and Huang, 2002). Moreover, the intrathecal administration of antisense oligo-deoxynucleotides against P2X3 or subcutaneous injection of a specific P2X3 antagonist can reduce CFA (but not carrageenan) induced thermal and mechanical hyperalgesia in the rat (Honore et al., 2002; Barclay et al., 2002; Jarvis et al., 2002; Wu et al., 2004).

The role of P2X3 in the generation of inflammatory hyperalgesia suggests that this ion channel may also play a role in the aetiology of neuropathic pain following nerve trauma, a hypothesis that is supported by data showing that P2X3 is up-regulated in some experimental nerve injury paradigms. For example, the expression of P2X3 protein has been shown to be up-regulated in small-medium sized neurons of the DRG following chronic constriction injury of the rat sciatic nerve (Novakovic et al., 1999). An increase in the number of neurons showing P2X3 immunoreactivity was also observed in the trigeminal ganglion following either ligation/section or chronic constriction of the mandibular inferior alveolar nerve in adult rats (Eriksson et al., 1998). Both these injury models are partial injury models in that the ganglion from which the damaged nerve generates will contain ATF3-positive damaged neurons and ATF3-negative “spared” neurons (see above). A more detailed examination of P2X3 mRNA expression following two partial nerve injury models in the rat demonstrates that P2X3 mRNA expression only increases in undamaged or “spared” trigeminal and dorsal root ganglion neurons, whilst it actually decreases in damaged, ATF3-positive, neurons (Tsuzuki et al., 2001). In accordance with this observation, P2X3 protein expression is significantly reduced in L4 and L5 DRG when the rat sciatic nerve (arising from L4, 5 and 6 DRG) is completely transected (Bradbury et al., 1998). A similar decrease in P2X3 protein expression was observed in L5 and L6 DRG using a model of tight spinal nerve ligation of both L5 and L6 nerves, a model that effectively leaves no “spared” neurons in the corresponding DRG (Kage et al., 2002). Further analysis of activation kinetics suggested that the channels down-regulated were homomultimeric P2X3 channels without a P2X2 component. Interestingly, no alteration in P2X3 immunoreactivity was observed in the small subpopulation of A- β fibre large DRG neurons that express this ion channel. One can postulate that this decrease in P2X3 expression in damaged neurons is partly the consequence of the reduced ability of damaged neurons to retrogradely transport neurotrophic factor/neurotrophic factor receptor complexes from their central and/or peripheral terminals back to the cell body. Spared neurons may well have greater accessibility to target field-derived neurotrophic factors and this may be partly responsible for the increase in P2X3 expression observed. In contradiction to the other published data described above, an injury model that involves ligation of the rat L5 spinal nerve, failed to promote an

increase in P2X3 protein expression in “spared” L4 DRG neurons (Fukuoka et al., 2002). The reasons behind this unexpected observation are unclear.

The up-regulation of P2X3 mRNA and protein in spared neurons following nerve injury supports the notion that this ion channel could play a role in the generation of mechanical and thermal hyperalgesia that can occur following nerve injury. In support of this hypothesis, antisense oligo-deoxynucleotides or siRNA knockdown of P2X3 expression in DRG neurons (following intrathecal administration) reduces the degree of hyperalgesia that results from partial sciatic nerve and spinal nerve ligation in rats (Honore et al., 2002; Barclay et al., 2002; Dorn et al., 2004). Similarly, subcutaneous injection of a specific and selective P2X3 antagonist, blocks the generation of thermal hyperalgesia and mechanical allodynia following sciatic nerve chronic constriction injury in the rat (Jarvis et al., 2002). Moreover, sciatic nerve injury appears to enhance the cell surface expression of P2X3 containing channels by inducing an alteration in receptor trafficking, and this has been proposed to contribute to the generation of hyperalgesia and allodynia (Chen et al., 2005). After peripheral nerve injury there is extensive sprouting of post-ganglionic sympathetic nerves into the site of injury (neuroma) and DRG (McLachlan et al., 1993; Ramer et al., 1997). Since sympathetic neurons can release ATP as a neurotransmitter, P2X3 and P2X2/P2X3 channels may mediate part of the sympathetic maintenance/enhancement of neuropathic pain that results in complex regional pain syndromes (reviewed in Janig and Habler, 2000). A significant up-regulation of P2X3 protein expression in the terminal arbors of CGRP immuno-reactive nociceptive neurons, but not non-peptidergic nociceptive neurons, in a mouse bone cancer model has recently led to the suggestion that P2X3 is involved in the aetiology of bone cancer pain (Gilchrist et al., 2005).

Two different laboratories have generated P2X3^{-/-} mice (Cockayne et al., 2000; Souslova et al, 2000). In both strains of mice $\alpha\beta$ meATP fails to elicit currents in nodose or dorsal root ganglia neurons, demonstrating a lack of functional P2X3 homo-multimeric or P2X2/P2X3 hetero-multimeric receptors. P2X3^{-/-} mice also display a dramatically reduced pain and electrophysiological response to intraplantar injection of ATP, highlighting the importance of P2X3 and P2X2/P2X3 multimeric channels in mediating the response to ATP. Both strains of mice also display a

modest reduction in hindpaw licking and lifting after interplantar injection of formalin, confirming that P2X3 plays a role in the generation of inflammatory hyperalgesia. In contrast, P2X3^{-/-} mice show normal responses to acute noxious thermal and mechanical stimuli. The effect that P2X3 deletion has on the generation of hyperalgesia and allodynia following sciatic nerve lesion has not been tested to date. Interestingly, the reduced inflammatory pain response to formalin is also observed in P2X2^{-/-} and P2X2^{-/-}/P2X3^{-/-} double knock-out mice (Cockayne et al., 2005), suggesting a role for the P2X2/P2X3 heterodimeric receptors in inflammatory pain sensation. Unexpectedly, the development of thermal hyperalgesia in response to chronic inflammation induced by CFA, but not short term inflammation induced by carrageenan or capsaicin, is markedly potentiated in P2X3^{-/-} mice compared to wild type mice (Souslova et al., 2000). In addition, P2X3^{-/-} mice appear to have a deficit in response to non-noxious thermal stimuli in the absence of an inflammatory lesion. A more thorough investigation of the response of P2X3^{-/-} animals to noxious and innocuous heat stimuli reveals a blunted response of dorsal horn second order sensory neurons to innocuous heat and a paradoxical increased avoidance of noxious heat and cold by P2X3^{-/-} animals placed in a thermal gradient. In addition P2X3^{-/-} animals show significantly shorter latencies of withdrawal to noxious temperatures in the tail-flick test (Shimizu et al., 2005). Systemic application of specific P2X3 antagonists in wild type mice failed to reproduce the behavioural phenotype of P2X3^{-/-} mice, suggesting that the phenotype of the null-mutant mice is partially due to compensatory changes in the expression of other P2X / P2Y receptor subunits, and/or other ion channels, in the absence of P2X3.

Another striking phenotype of P2X3^{-/-} mice is bladder hypo-reflexia. Null mutant mice show an increased bladder capacity and decreased voiding frequency (Cockayne et al., 2000). Bladder distension has subsequently been shown to induce the release of ATP from bladder endothelial cells of mice, and application of ATP to the bladder endothelium, or bladder distension, rapidly induces activity of pelvic nerve afferents. These latter effects are absent in P2X3^{-/-} mice (Vlaskovska et al., 2001) and P2X2^{-/-} and P2X3^{-/-}/P2X2^{-/-} mice (Cockayne et al., 2005). These observations strongly suggest a role for P2X3 homo-multimeric and P2X2/P2X3 hetero-multimeric channels in regulating mechanosensory signal transduction in the rodent bladder. Evidence to suggest that these purine channels may also play an important role in

regulating sensory perception in the human bladder comes from the observations that an increase in the expression of P2X3 and P2X2 proteins is observed in the bladder urothelium of patients suffering from interstitial cystitis (Tempest et al., 2004). In addition, human bladder endothelial cells release ATP and increase expression of P2X3 when mechanically stretched (Sun and Chai, 2004). Antagonists of the P2X3 and P2X2 receptor could thus be of therapeutic potential in the treatment of overactive bladder and in relieving the pain of chronic interstitial cystitis.

Neurotrophic factor regulation of P2X3 expression

Since P2X3 expression is predominantly localised to the IB4-reactive subpopulation of small-diameter sensory neurons, it would seem likely that its expression will be regulated by members of the GDNF family of neurotrophic factors rather than the neurotrophins. Indeed, intrathecal administration of GDNF or artemin to rats following sciatic nerve lesion significantly reduces the injury induced decrease in P2X3 observed in damaged DRG neurons (Bradbury et al., 1998; Wang et al., 2003; Gardell et al., 2003). Intrathecal administration of GDNF to non-lesioned adult rats also increases the expression of P2X3 in cervical and lumbar DRG neurons that project to the inner region of lamina II in the dorsal horn of the spinal cord (Ramer et al., 2001). In contrast, whilst null deletion of the neurturin receptor GFR- α 2 reduces the size and degree of terminal arborization of IB4-positive c-fibre nociceptors, it does not decrease the number of DRG neurons expressing P2X3, suggesting that this GDNF family member does not regulate P2X3 expression in intact neurons (Lindfors et al., 2006). Perhaps surprisingly, intrathecal administration of NGF to non-lesioned rats appears to produce a small, but significant, increase in the number of DRG neurons expressing P2X3 protein (Ramer et al., 2001). The additional DRG neurons that express P2X3 are CGRP-positive and project to more superficial laminae of the dorsal horn than those of control animals, as well as to the ventro-medial afferent bundle that lies close to the central canal of the spinal cord. It is not clear whether NGF causes *de-novo* expression of P2X3 in some peptidergic nociceptors or merely increases expression of this ion channel to make it more readily detectable by immuno-histochemistry. In either case, the fact that NGF appears to increase the expression of P2X3 in peptidergic nociceptive neurons may partly explain why pre-

existing inflammatory conditions (that will increase NGF expression in the vicinity of the inflammation (Hefti et al., 2006)) sensitise the response of sensory neurons to ATP (Hamilton et al., 2001; Paukert et al., 2001; Xu and Huang, 2002).

4.1.4. Nav1.6 and 1.7

In chapter 2, I outlined the structure and function of the voltage-gated sodium channels with particular reference to the TTX-resistant sodium channel alpha subunits, Nav1.8, and Nav1.9. In this chapter, I investigate the transcriptional regulation of two TTX-sensitive (TTX-S) sodium channel alpha subunits, Nav1.6 and Nav1.7.

As discussed in chapter 2, sodium channels are responsible for the current flow required within sensory neurons to propagate an action potential. Electrophysiological recordings indicate that there are two general sodium currents in DRG neurons, one sensitive to TTX (TTX-S), and one unaffected by TTX (TTX-R) (Kostyuk et al., 1981; Caffrey et al., 1992; Roy and Narahashi, 1992). The TTX-S current is the predominant current in large proprioceptive and mechanoreceptive sensory neurons, whereas small nociceptive neurons contain both the TTX-S and TTX-R currents (Roy and Narahashi 1992; Rush et al., 1998; Cummins et al., 1998; Herzog et al., 2003).

In general, TTX-S channels tend to have a low threshold for activation (-55 to -40mV) and are rapidly activating and inactivating (reviewed in Lai et al., 2004). The application of TTX to distal axons, blocks nerve impulse conduction completely, providing evidence that TTX-S channels are required to mediate action potential generation in both myelinated and unmyelinated axons (Brock et al., 1998; Gold et al., 2003).

Nav1.6

Nav1.6 is encoded by the gene *Scn8a* and was initially isolated from the rat CNS and PNS (Schaller et al., 1995). Nav1.6 is broadly expressed throughout the nervous system, in both neurons and glia of the CNS, PNS and enteric nervous system

(Schaller et al., 1995; Black and Waxman, 1996; Felts et al., 1997; Black et al., 2002; Bartoo et al., 2005). Nav1.6 produces a strong, persistent sodium current with high firing rates (Smith et al., 1998) and has been shown to cluster at the nodes of Ranvier of mature myelinated sensory, motor and other CNS neuron axons. In addition, Nav1.6 is also localized to some neuronal dendrites and synapses within the CNS (Caldwell et al., 2000; Tzoumaka et al., 2000). Nav1.6 channels within myelinated sensory neurons display rapid repriming kinetics and the rapid development of closed-state inactivation. These two properties are significantly different to other TTX-sensitive sodium channels and account for the high repetitive firing rates, and the lack of response to slow depolarising stimuli, that are characteristic of myelinated sensory neurons (Cummins et al., 1998 and 2001; Herzog et al., 2003a). The lack of response to slow depolarising stimuli is important, since it ensures that transient nodal after currents, which can occur after action potential propagation through the nodes, do not trigger additional, spontaneous action potentials.

Two important studies have used the optic nerve as a model to address the mechanism that leads to the clustering of Nav1.6 at the nodes of Ranvier in mature myelinated neurons (Boiko et al., 2001; Kaplan et al., 2001; reviewed in Salzer, 2002). The optic nerve is populated by axons from retinal ganglion cells (RGCs). RGC axons display clearly demarcated myelinated regions, outside of the eye in the optic nerve, and unmyelinated regions, within the retina, making them an ideal system in which to study the role of myelination in regulating sodium channels clustering and expression. RGCs express two TTX-S sodium channels, Nav1.2 and Nav1.6. The expression of these two TTX-S sodium channels, and their axonal location, appear to be developmentally regulated in the mouse in accordance with the timing of myelination (Boiko et al., 2001; Kaplan et al., 2001). Nav1.2 is expressed prior to Nav1.6 during embryonic development, and it is initially localised at developing nodes of Ranvier. As myelination continues, Nav1.2 is gradually down-regulated at the maturing nodes and replaced by Nav1.6, so that by adulthood Nav1.2 is only present within the retina whilst Nav1.6 is localised at the nodes of Ranvier in myelinated axons (Boiko et al., 2001). The study of *shiverer* mice, a strain that displays severe hypomyelination within the CNS as a result of oligodendrocyte dysfunction, has confirmed that the myelination process regulates the expression and localization of Nav1.6 in RGC axons. In *shiverer* mice, the expression of Nav1.2 is

abundant in all regions of damaged, poorly myelinated RGC axons, whereas Nav1.6 is only detectable in the few nodes that retain functional integrity (Boiko et al., 2001). It appears that oligodendrocytes release a soluble factor that is responsible for regulating the initial clustering of Nav1.2 at immature RGC axonal nodes. However, a physical interaction between Nav1.6 and myelinating oligodendrocytes would appear to be required for clustering of this channel to mature RGC nodes of Ranvier (Kaplan et al., 2001).

Several proteins are known to interact with TTX-sensitive sodium channel alpha units and are thought to help direct their trafficking to nodes, as well as regulating their functional characteristics. These include sodium channel beta-subunits $\beta 1$ and $\beta 3$, neurofascin-186 and calmodulin (Ratcliffe et al., 2001; Herzog et al., 2003b). Nav1.6, in particular, has been shown to interact with ankyrin-G, βIV spectrin, neurofascin, NrCAM, calmodulin and FHF2 (Jenkins and Bennet, 2001; Herzog et al., 2003b; Wittmack et al., 2004). A further study has suggested that contactin-associated-protein regulates the formation of transverse bands in axonal paranodal regions, and these are important in regulating nodal formation. Indeed, the lack of contactin-associated-protein, and the subsequent loss of transverse bands, leads to aberrant Nav1.6 and Nav1.2 expression in myelinated CNS neurons (Rios et al., 2003).

To date, the mechanism that regulates the nodal clustering of Nav1.6 in myelinated peripheral sensory neurons has not been fully determined. However, of note is the observation that contactin-associated-protein deletion does not lead to aberrant expression of Nav1.6 or Nav1.2 in myelinated peripheral neurons in the same way as it does in myelinated CNS neurons (Rios et al., 2003). Furthermore, a recent publication has also suggested that, unlike the situation in the CNS, immature PNS nodes do not undergo a period of transient Nav1.2 expression prior to Nav1.6 channels populating the nodes, rather Nav1.6 is the major sodium channel expressed at immature nodes (Shafer et al., 2006). These two observations suggest that myelinating glial cells within the PNS use a different instructive mechanism, compared to those within the CNS, to regulate nodal Nav1.6 expression. This hypothesis is further supported by the observation that Nav1.6 is associated with fibroblast growth factor homologous factor 2 (FHF2) at sensory neuron nodes of Ranvier, but not motor neuron or optic nerve nodes (Wittmack et al., 2004). Ectopic

expression of the closely related factor, FHF2B, in the neuronal cell line ND7/23 increases the amplitude of the sodium current that is generated by Nav1.6, demonstrating that the association of FHF2 family members with Nav1.6 has functionally important consequences (Wittmack et al., 2004).

Whilst the current view is that Nav1.6 expression is largely restricted to myelinated neurons, and is indeed the main effector of saltatory action potential conduction within these neurons, evidence has also been presented to suggest that Nav1.6 plays a functional role in generating sodium currents in unmyelinated C-fibre nociceptive sensory neurons of rat and mouse (Black et al., 2002). Nav1.6 protein appears to be expressed throughout the entire length of peripherin-positive C-fibres innervating the epidermis (DRG derived) and the cornea (trigeminal ganglion derived). Expression is generally low and in a continuous pattern, not being restricted to nodal or other specific regions. A number of naturally occurring mouse Nav1.6 null-mutant strains have been identified. These mouse strains lack functional expression of Nav1.6 due either to the insertion of transposable elements into the *Scn8a* gene or spontaneous mutations in the gene. Animals in these *med* (*Motor End-Plate Disease*) mouse lines develop various motor disorders within the first two weeks of birth and die by three weeks (Duchen and Stefani, 1971; Burgess et al., 1995; Kohrman et al., 1996a and b; Garcia et al., 1998; Hamann et al., 2003). C-fibre nociceptors (and nodes of myelinated axons) do not stain for Nav 1.6 protein in postnatal *med* mice. The lack of functional Nav1.6 in *med* mice C-fibres does not lead to conduction block in these neurons, but it does lead to a significant reduction in compound action potential amplitude and conduction velocity, thus highlighting the functional significance of Nav1.6 sodium channels in these neurons (Black et al., 2002). The absence of conduction block in c-fibres from *med* mice reflects the fact that C-fibre nociceptors also express the TTX-sensitive sodium channel, Nav1.7 (see below). The observation that Nav1.6 is expressed in unmyelinated C-fibres of the sensory nervous system is not the only report of Nav1.6 being expressed in unmyelinated fibres. Nav1.6 channels also appear to be present in unmyelinated parallel fibres derived from granule cells in the molecular layer of the cerebellar cortex (Krzemien et al., 2000; Tzoumaka et al., 2000; Levin et al., 2006).

Nav1.6 appears to be responsible for the phenomenon of resurgent sodium currents in cerebellar Purkinje cells and large DRG neurons. In this phenomenon sodium channels open transiently during recovery from activation, thereby generating a “resurgent” sodium current that flows immediately after action potential propagation and displays very slow decay kinetics. Resurgent sodium currents are thought to be critical in determining the rapid firing pattern of Purkinje cells and are virtually eliminated in *med* mice and conditional knockout mice containing a Purkinje cell specific deletion of Nav1.6 (Raman and Bean, 1997; Raman et al., 1997; Levin et al., 2006). The role that resurgent currents play in regulating the excitability of myelinated sensory neurons is not clear, but such currents certainly exist in large myelinated DRG neurons and are dependent on Nav1.6 expression (Cummins et al., 2005).

The role of Nav1.6 in injury and inflammation

There is a scarcity of data investigating the role of Nav1.6 in the aetiology of neuropathic and inflammatory pain syndromes. This is likely due to the widespread assumption that Nav1.6 is only expressed in myelinated neurons (despite Black et al., 2002) and the even more widespread assumption that unmyelinated nociceptive C-fibre neurons are the only neurons involved in the aetiology of these pathological conditions. In a study examining a possible link between Nav1.6 and pathological pain conditions, the expression of Nav1.6 mRNA has been shown to decrease in ipsilateral L4 DRG (containing “spared” neurons) and L5 DRG (containing damaged neurons) following L5 tight spinal nerve ligation (Kim et al., 2002). This has been interpreted as an indication that Nav1.6 is not involved in the generation of the ectopic electrical discharges that are associated with the onset of hyperalgesia/allodynia following sciatic nerve lesion. However, this study measured Nav1.6 mRNA levels by an RNase protection assay on total RNA extracted from L4 and L5 DRG. This approach can only measure a global change in Nav1.6 mRNA expression within all neurons and cannot determine whether certain neuronal subpopulations show increased or decreased expression of Nav1.6 mRNA. In addition, it is not clear whether Nav1.6 protein levels alter following sciatic nerve lesion. In any case, a decrease in Nav1.6 mRNA/protein expression following sciatic nerve injury could be causative in the aetiology of neuropathic pain/hyperalgesia as its expression may be replaced by that of other TTX-S

sodium channels like Nav1.3 and Nav1.6 (e.g. Kim et al., 2001 and 2002). This in turn may alter the response of myelinated sensory neurons to slow, sub-threshold depolarisations and lead to ectopic firing (see above). Therefore, to date, it is not entirely clear whether altered Nav1.6 sodium channel expression, or modulation of its functional properties, contributes to the generation of lesion induced spontaneous ectopic sodium currents, and hence neuropathic pain. Likewise, no data has emerged, to date, that directly addresses whether altered Nav1.6 expression or function contributes to the generation of inflammatory hyperalgesia. However, it has recently been demonstrated that p38 MAP kinase can phosphorylate Nav1.6, thereby leading to a reduced sodium current density. The reduced current density has been postulated to be the result of the phosphorylation event targeting Nav1.6 channels for ubiquitination and degradation (Wittmack et al., 2005). P38 MAP kinase has been shown to be activated in lumbar DRG neurons following sciatic nerve lesion and NGF driven inflammation, and is causally related to the generation of hyperalgesia/allodynia (Jin et al., 2002; Ji et al., 2002; Obata et al., 2004). These observations further raise the possibility that aberrant Nav1.6 expression/modulation is involved in the generation of neuropathic and/or inflammatory pain. Clearly this hypothesis needs to be investigated further.

Despite no obvious function in the generation of pathological pain, following nerve injury or inflammation, aberrant Nav1.6 expression and function has been implicated in the onset of other neurological disorders.

Nav1.6 and Multiple Sclerosis

The regulation of Nav1.6 expression, and its subsequent nodal localization, by myelination raises the possibility that this sodium channel may play a role in the aetiology of pathological disease symptoms in demyelinating disorders. Multiple Sclerosis (MS) is an inflammatory, demyelinating disease of the CNS. Originally it was thought that myelinating oligodendrocytes were the cell type most affected by the disease. However, more recently it has become evident that axonal and neuronal degeneration occurs in both MS and experimental autoimmune encephalomyelitis (EAE), a mouse model showing many features of MS (Reviewed in Bechtold et al., 2005).

Initial studies, on the optic nerve, established that voltage-gated sodium channels can participate in the production of calcium-mediated axonal degeneration, following anoxia, by providing a route for persistent sodium influx that drives reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange through the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, NCX (Stys et al., 1992). Later studies in spinal cord dorsal columns provided further evidence for the involvement of sodium channels and NCX in axonal degeneration following exposure to anoxia (Imaizumi et al., 1998) and high levels of nitric oxide, a characteristic of MS (Kapoor et al., 2003). In accordance with these observations, non-specific blocking of sodium channels by flecainide or phenytoin in mice with EAE prevents axonal degeneration in the spinal cord and attenuates the reduction in compound axonal potential that is characteristic of the disease (Lo et al., 2003; Bechtold et al., 2004). However, until recently the identity of the sodium channels involved in triggering calcium-mediated axonal degeneration has remained a mystery. It has now been established that mice with EAE show abnormal retinal ganglion cell (RGC) and optic nerve expression of Nav1.2 and Nav1.6 (Craner et al., 2003). In particular, mice with EAE show widespread expression of Nav1.2 in RGCs and degenerating optic nerve nodes with significantly reduced expression of Nav1.6, a situation that is characteristic of immature RGCs and optic nerve. Further in the progression of the disease, mice with EAE display random diffuse expression of both TTX-S sodium channels along the entire optic nerve. Furthermore, mice with EAE also display diffuse expression of Nav1.2 and Nav1.6 protein in demyelinated spinal cord axons, and the expression of Nav1.6, in particular, coincides with that of the beta amyloid precursor protein, a marker of axonal injury, and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) (Craner et al., 2004 a). This data strongly supports the notion that aberrant Nav1.6 (and Nav1.2) expression, as a result of defective myelination and/or demyelination, contributes to the axonal and neuronal degeneration that is characteristic of EAE. Nav1.6 protein has also been shown to be expressed in a diffuse pattern, overlapping that of NCX, within demyelinated axons residing in areas of acute MS plaques in human optic nerve and spinal cord post-mortem tissue, suggesting that the aetiology of EAE and MS are the same (Craner et al., 2004b). A recent publication has also implicated Nav1.6 as having a causative role in the activation of microglia and macrophages that is concomitant with the onset of inflammation in the early stages of MS and EAE (Craner et al., 2004). Sodium channel blockade may, therefore, not only reduce neuronal degeneration in the latter

stages of MS/EAE, but may also ameliorate the inflammatory insult that contributes to the onset of these pathological conditions.

Nav1.6 and other neurological disorders

The analysis of *med* mice has suggested that aberrant Nav1.6 expression and function may play a role in the aetiology of a number of neurological disorders. As mentioned above, the various strains of *med* mice develop a number of progressively worsening motor disorders within the first two weeks after birth (a time period coinciding with the replacement of Nav1.2 with Nav1.6 at developing nodes of Ranvier) and neonates of most strains die by three weeks. Neonatal *med* mice display certain symptoms of several human diseases including; dystonia, motor endplate disease and inherited cerebellar ataxia (Burgess et al., 1995; Garcia et al., 1998; Hamann et al., 2003; Levine et al., 2006).

Motor end plate disease has been postulated to arise in *med* mice because changes in the conduction velocity and amplitude of motor neuron action potentials, together with aberrant sprouting of motor neuron terminal arbors, prevent current penetration through the neuromuscular junction (Duchen, 1970; Duchen and Stefani, 1971; Kearney et al., 2002). A recent publication has demonstrated robust Nav1.6 expression in the Schwann cells that wrap around motor neuron terminal arbors at the neuromuscular junction. These Schwann cells are absent in *med* mice, and it has been postulated that their absence (probably due to the lack of Nav1.6 expression) contributes to the aberrant sprouting of motor neuron terminal arbors (Musarella et al., 2006).

Other aspects of *med* mouse motor disorders are likely to arise from inappropriate signalling through brain regions that regulate coordination, such as the cerebellum and basal ganglia. For example, TTX-sensitive resurgent and steady-state sodium currents decrease disproportionately relative to transient currents in Purkinje cells of juvenile *med* mice, thereby changing the kinetics of total sodium currents in these cells. The result of changed sodium channel kinetics is that the action potential activation

threshold of Purkinje cells is shifted by +10mv and the rate of spontaneous, repetitive firing of action potentials within these cells is reduced (Harris et al., 1992; Raman and Bean, 1997; Raman et al., 1997; Smith et al., 1998). Because *med* mice die within three weeks of birth and lack Nav1.6 expression in all cell types that normally express this sodium channel, it has not previously been possible to determine the extent to which the specific lack of Nav1.6 expression in Purkinje cells, or other cells within the cerebellum, directly contributes to the behavioural phenotype of *med* mice. Neither has it been possible to determine whether Nav1.6 deletion in adult cells of the cerebellum leads to adult mice with a similar behavioural phenotype to that of juvenile *med* mice. A recent study, using conditional deletion of Nav1.6 in Purkinje cells, granule cells or both, has attempted to address these questions (Levin et al., 2006). Whilst adult mice with a granule cell specific deletion of Nav1.6 only exhibit minor behavioural abnormalities, adult mice lacking Nav1.6 specifically in Purkinje cells display ataxia, tremor and impaired coordination that correspond to a significant drop in both the ratio of resurgent-to-transient currents and spontaneous firing rates in Purkinje cells. This data clearly demonstrates that the lack of Nav1.6 expression in Purkinje cells is responsible for many of the motor related behavioural abnormalities of *med* mice.

To date, no data has emerged regarding the transcriptional or translational regulation of Nav1.6 expression by neurotrophic factors.

Nav1.7

The existence of a sodium channel with the expression pattern and functional characteristics of Nav1.7 was initially suggested by a study on PC12 cells that revealed a TTX-sensitive sodium current that was distinct from that generated by Nav1.2, a sodium channel that was known to be expressed in this cell line, and that was rapidly up-regulated by NGF- induced PC12 differentiation (D'Arcangelo et al., 1993; Toledo-Aral, 1995). Northern blotting of PC12 cell RNA, using a probe against a highly conserved region of all known sodium channels, revealed a novel 11 kb transcript that did not correspond in size to any known sodium channels and was also present in RNA extracted from rat DRGs, but not rat brain. This transcript was shown

to be rapidly up-regulated by NGF-induced PC12 cell differentiation, in accordance with the NGF induced increase in sodium currents (Toledo-Aral, 1995). The first cDNA corresponding to Nav1.7 was initially isolated from the human medullary carcinoma cell line (hMTC) and termed human neuroendocrine sodium channel (hNE-Na), (Klugbauer et al., 1995). A rabbit homologue was cloned in the same year from a rabbit Schwann cell library (Belcher et al., 1995) and the cloned cDNA was used to identify the location of the corresponding gene, Scn9a, within the mouse genome (Beckers et al., 1996). The rat homologue of Nav1.7, also known as PN1, was isolated soon afterwards from PC12 cell and rat DRG cDNA libraries (Toledo-Aral et al., 1997; Sangameswaran et al., 1997).

An initial screen of rat tissues, using Western Blotting, RNase protection assay and Northern Blotting, demonstrated that Nav1.7 mRNA and protein are principally expressed in sensory and sympathetic ganglia of the PNS and are virtually undetectable in the brain, spinal cord and non-neuronal tissues (Toledo-Aral et al., 1997). A more sensitive RT-PCR assay has revealed low-level expression of Nav1.7 mRNA in adult rat brain and heart, but not skeletal muscle (Sangameswaran et al., 1997). Indeed, a recent publication has demonstrated expression of Nav1.7 mRNA in the atrio-ventricular node of the mouse heart (Marionneau et al., 2005). Functional Nav1.7 also appears to be expressed in mouse smooth muscle myocytes and mouse pancreatic B cells (Saleh et al., 2005; Vignali et al., 2006)

In situ hybridization and immunohistochemistry have localised Nav1.7 mRNA and protein expression specifically to neurons within embryonic and adult rat DRG, with no apparent expression in either satellite cells or Schwann cells (Toledo-Aral et al., 1997; Sangameswaran et al., 1997). Interestingly, Nav1.7 protein expression is highest at the terminal arbors of cultured embryonic rat DRG neurons, with only low level, diffuse expression along neurites and within cell bodies (Toledo-Aral et al., 1997). The majority of adult and embryonic rat DRG neurons appear to express Nav1.7 mRNA, although with some variation in the levels of expression between neurons (Felts et al., 1997; Black et al., 1996; Toledo-Aral et al., 1997). In accordance with this observation, embryonic DRG also display widespread neuronal expression of Nav1.7 protein (Toledo-Aral et al., 1997). In contrast, immunohistochemistry has revealed that Nav1.7 protein is predominantly expressed in small, presumptive

nociceptive, DRG neurons in the adult rat and guinea pig (Porreca et al., 1999, Gould et al., 2000; Djouhri et al., 2003). An electrophysiological analysis of adult guinea pig DRG neurons expressing high levels of Nav1.7 protein has demonstrated that, whilst nociceptive neurons predominantly express this sodium channel, some low-threshold mechanoreceptors also express Nav1.7 at high levels and Nav1.7 is more closely correlated to action potential conduction velocity than cell size (Djouhri et al., 2003). A recent publication has investigated alternative splicing of Nav1.7 mRNA in both human and rat DRG (Raymond et al., 2004). Four alternative splice variants of Nav1.7 exist in human and rat DRG, encompassing changes in the splicing of exon 5 and exon 11 of the Scn9a gene by the use of alternative splice donor sites. Whilst all splice variants are reduced in rat lumbar DRG following L5 spinal nerve ligation, two of the transcripts are significantly enriched in comparison to the others, suggesting that alternative splicing of Nav1.7 may play a role in the generation of neuropathic pain associated with spinal nerve ligation (Raymond et al., 2004). The differences in the functional properties of the Nav1.7 channels encoded by each splice variant have not yet been determined.

Over-expression of mouse Nav1.7 in mouse DRG neurons has revealed that this sodium channel produces a fast activating and inactivating TTX-sensitive current that is characterised by having very slow rates of closed state inactivation and repriming (Herzog et al., 2003a). The properties of ectopically expressed Nav1.7 are similar to the properties of native TTX-sensitive currents in nociceptive A-delta and C-fibre neurons, further suggesting that Nav1.7 is largely responsible for generating the TTX-sensitive currents in nociceptive neurons (Herzog et al., 2003a). The ectopic expression of human Nav1.7, in the non-neuronal cell lines CHO and HEK293, has revealed that the human homologue of rat Nav1.7 has similar electrophysiological properties to its rodent counterpart (Cummins et al., 1998; Akiba et al., 2003).

Nav1.7 and Pain

The observation that Nav1.7 is generally expressed at higher levels in nociceptive sensory neurons of the DRG, compared to proprioceptive neurons and the majority of low-threshold mechanoreceptive neurons, suggests that this sodium channel may have

a role in the response of sensory neurons to noxious stimuli and may be involved in pain signalling. The expression of Nav1.7 predominantly in nociceptive neurons also raises the possibility that it is involved in the aetiology of inflammatory and/or neuropathic pain.

Conditional transgenic mice, using cre-recombinase mediated deletion of loxP flanked Nav1.7 exons that encode the voltage sensor of the channel, have been used to investigate the role of Nav1.7 in normal nociception and in the generation of pathological pain states (Nassar et al., 2004). Globally deleting floxed Nav1.7 exons during the embryonic period confers neonatal lethality, apparently because of a failure of neonates to feed, precluding an investigation of the role Nav1.7 in normal nociception. To circumvent this problem, a Nav1.8 promoter driven cre-recombinase was used, by the same authors, to delete Nav1.7 specifically in nociceptive sensory neurons of the mouse PNS. Surprisingly, isolated Nav1.7 deficient DRG neurons only show a small reduction in peak TTX-sensitive current, probably reflecting the continued expression, or perhaps increased expression, of Nav1.1 and Nav1.6 in Nav1.7 deficient nociceptive neurons. Similarly, deleting Nav1.7 in afferent nociceptive neurons does not significantly alter the evoked electrophysiological response of spinal cord dorsal horn neurons following peripheral stimulation with innocuous or noxious thermal (hot or cold) stimuli. However, Nav1.7^{-/-} mice show a small, but significant, reduction in their sensitivity to noxious heat in the Hargreaves test (Nassar et al., 2004). The evoked electrophysiological response of dorsal horn neurons to peripheral noxious mechanical stimulation, but not low threshold mechanical stimuli, is significantly reduced in the absence of nociceptor Nav1.7. In accordance with this observation, mice with a nociceptive neuron specific deletion of Nav1.7, show a pronounced analgesia to noxious, but not low-threshold, mechanical stimuli in behavioural tests.

The most dramatic phenotype of mice containing a nociceptive neuron specific deletion of Nav1.7 is a striking deficit in the development of inflammatory hyperalgesia. Nav1.7 deficient mice show significantly less nocifensive behaviour in the first and second phase responses to intraplantar injection of formalin (Nassar et al., 2004, 2005). In addition, the marked thermal and mechanical hyperalgesia that arises following intraplantar CFA injection is virtually absent in mice lacking Nav1.7

expression in nociceptive neurons. Thermal hyperalgesia is also significantly reduced in Nav1.7^{-/-} mice following NGF and carrageenan induced inflammation (Nassar et al., 2004).

The role that Nav1.7 plays in the generation of neuropathic pain has been investigated in mice containing a nociceptive neuron specific deletion of Nav1.7, and also in mice with a nociceptor specific double-deletion of both Nav1.7 and the TTX-resistant sodium channel, Nav1.8 (Nassar et al., 2005). Both mutant mouse strains display a normal development of mechanical allodynia following L5 spinal nerve ligation, suggesting that neither sodium channel plays a role in the aetiology of allodynia following nerve damage. This is, perhaps, a surprising result in light of the role that Nav1.7 plays in determining normal sensory thresholds to noxious mechanical stimuli and the significant change in the ratios of Nav1.7 splice variants that is observed in the same lesion model (Nassar et al., 2004; Raymond et al., 2004). However, in accordance with the null mutant data, Nav1.7 mRNA expression within DRG neurons appears to fall significantly following spinal nerve ligation (Kim et al., 2002). Furthermore, Nav1.7 protein levels decrease significantly in DRG from human patients suffering from traumatic central or peripheral axotomy (Coward et al., 2001).

The data from both conditional Nav1.7 null mice and conditional Nav1.7/Nav1.8 double-null mice demonstrates that Nav1.7 plays a role in determining the response to high-threshold painful thermal and, in particular, mechanical stimuli. The data also demonstrates that Nav1.7 plays an important role in the generation of inflammatory thermal and mechanical hyperalgesia (Nassar et al., 2004, 2005). Other experimental evidence supports this latter conclusion. For example carrageenan-induced inflammation has been shown to increase Nav1.7 protein expression within DRG neurons in parallel with an increase in TTX-sensitive sodium currents and the development of hyperalgesia (Black et al., 2004). Moreover, Herpes virus vector mediated antisense knockdown of Nav1.7 has been shown to ameliorate CFA induced, inflammatory thermal hyperalgesia in mice (Yeomans et al., 2005). In addition, the anti-inflammatory cyclooxygenase (COX) inhibitors ibuprofen and NS-398 can prevent the CFA-induced up-regulation in Nav1.7 protein levels observed in adult rat DRG when they are applied prior to CFA administration (Gould et al., 2004).

Importantly, the COX inhibitor associated amelioration of CFA induced Nav1.7 up-regulation corresponds with a reduction in inflammation-induced hyperalgesia.

The important role that Nav1.7 plays in regulating normal nociceptive thresholds to noxious stimuli has been highlighted by the recent discovery that mutation of this sodium channel is associated with the painful condition, primary erythralgia (also called erythromelalgia). This neuropathy is characterised by burning pain and redness of the skin of the extremities, in response to warm stimuli or moderate exercise, and can either arise spontaneously or be inherited (Layzer et al., 2001). The symptoms of this condition are probably due to a combination of peripheral sensory neuron hyper-excitability and an alteration of sympathetic vasomotor activity (Rush et al., 2006). A number of research groups have now identified at least 10 point mutations in the *Scn9a* gene that are associated with either familial or spontaneous (sporadic) erythralgia (Kanadia et al., 2003; Yang et al., 2004; Drenth et al., 2005; Michiels et al., 2005; Dib-Hajj et al., 2005; Han et al., 2006). The effects that two of these point mutations have on the functional properties of Nav1.7 have been investigated by ectopically expressing mutated Nav1.7 in a kidney cell line (Cummins et al., 2004). Both mutations shift the activation threshold of Nav1.7 by 15 mV in a hyperpolarizing direction, as well as significantly slowing the rate of channel closing. In addition, mutant channels show a much larger response to slow, small depolarising stimuli. The net result of these changes *in-vivo* is likely to be hyper-excitability of primary sensory neurons. The transfection of cDNAs encoding mutant channels into cultured DRG neurons has been used to investigate the electrophysiological properties of Nav1.7 channels carrying a different erythralgia-associated point mutation to those investigated by Cummins et al., (Dib-Hajj et al., 2005). Once again, the mutation confers a gain of function phenotype on mutated channels that is characterised by a lowered activation threshold and delayed channel closure. Interestingly, one of the first mutations of Nav1.7 to be characterised as being involved in erythralgia, L858H, (Yan et al., 2004) produces functional hyper-excitability when over-expressed in sensory neurons but hypo-excitability when over-expressed in sympathetic neurons, clearly demonstrating that the physiological functional effects of any one ion channel is partly dependant on the cellular context in which it is expressed (Rush et al., 2006). Despite the growing data implicating aberrant Nav1.7 function in the aetiology of

erythralgia, one group has documented cases of the disease that are not associated with mutation of Nav1.7 (Burns et al., 2005).

Regulation of Nav1.7 expression by neurotrophic factors

Initial studies on the regulation of Nav1.7 mRNA expression were carried out using Northern Blotting analysis of RNA extracted from PC12 cells (D'Arcangelo et al., 1993; Toledo-Aral et al., 1995). A brief 1 minute exposure of PC12 cells to NGF has been shown to rapidly up-regulate the expression of the 11Kb Nav1.7 message. Nav1.7 mRNA expression peaks at 3 hours post-NGF exposure and returns to basal levels by 24 hours. Patch clamping experiments have revealed that the increase in Nav1.7 mRNA expression is accompanied by a dramatic increase in sodium current that is first apparent at eight hours after NGF application and persists for at least 24 hours (Toledo-Aral et al., 1995). Interestingly, the up-regulation of Nav1.7 mRNA appears to be dependent on new protein synthesis, suggesting that NGF-induced immediate early genes mediate the increase in sodium channels expression. A brief exposure to interferon- γ , epidermal growth factor or basic fibroblast growth factor also increases the expression of Nav1.7 mRNA in PC12 cells (Toledo-Aral et al., 1995).

In contrast to PC12 cells of the sympatho-adrenal lineage, NGF does not appear to regulate Nav1.7 expression in sensory neurons. Transgenic mice that over-express NGF specifically in skin do not show increased expression of Nav1.7 mRNA in DRG neurons (Fjell et al., 1999a). Moreover, Schwann cells or Schwann cell conditioned media, that are a source of NGF, do not increase neuronal expression of Nav1.7 mRNA when added to cultured adult DRG neurons, although they increase the neuronal expression of other sodium channels that have previously been shown to be regulated by NGF (Hinson et al., 1997). In agreement with this, TTX-sensitive currents do not decrease in TrkA expressing nociceptive neurons (in which Nav1.7 is the main TTX-S sodium channel) in animals that have reduced levels of target field NGF, as a result of immunization-induced anti-NGF antibody production. TTX-resistant sodium currents, however, are reduced in these animals in accordance with

the known ability of NGF to regulate both TTX-resistant sodium channel alpha-subunits, thus demonstrating the effectiveness of anti-NGF antibody production in NGF-immunized mice (Fjell et al., 1999b). It can be hypothesised that the reduction in the expression of Nav1.7 mRNA and protein within DRG neurons that has previously been observed following peripheral nerve lesion (Coward et al., 2001; Kim et al., 2002) occurs as a result of the loss of accessibility of DRG neurons to target field-derived factors. This would suggest that additional target-field derived factors, other than NGF, normally regulate the expression of Nav1.7 in adult DRG neurons.

In this chapter I will determine whether the other target field-derived factor/s that regulate Nav1.7 mRNA expression in sensory neurons could be artemin or MSP.

4.1.5. LIF

Leukaemia inhibitory factor (LIF) is a neuropoietic cytokine (also known as cholinergic differentiation factor, or CDF) that has pleiotropic activity in several adult and embryonic systems. In the haematopoietic system, LIF induces the proliferation of haematopoietic stem cells (Fletcher et al., 1990; Leary et al., 1990) as well as the differentiation of leukaemia cells (Tomida et al., 1984) and megakaryocytic progenitor cells (Metcalf et al., 1990). LIF affects bone resorption (Abe et al., 1986) and also inhibits adipogenesis by negative regulation of lipoprotein lipase (Mori et al., 1989). Furthermore, LIF inhibits kidney epithelial cell and embryonic stem cell differentiation (Tomida et al., 1990; Smith et al., 1988; Williams et al., 1988). In the latter case, LIF acts to maintain the multipotent developmental potential of embryonic stem cells. Within the nervous system, LIF displays neurotrophic factor like activity with effects on both neuronal differentiation and survival within the CNS and PNS. LIF can also modulate astrocyte and oligodendrocyte differentiation (Yamamori et al., 1989; Murphy et al., 1991; Murphy et al., 1993; Murphy et al., 1997; Horton et al., 1998; Thier et al., 1999; Arce et al., 1999).

LIF belongs to the family of gp130 cytokines, (also known as the interleukin-6 family of cytokines). Family members include interleukin-6 (IL-6), IL-11, ciliary derived

neurotrophic factor (CNTF), Oncostatin M, cardiotrophin-1 (CT-1), and cardiotrophin-like cytokine (CLC) (reviewed in Taga, 1996; Murphy et al., 1997) All the members of this cytokine family utilise a common β receptor signalling subunit, gp130. gp130 can form hetero- or homodimers with additional α and/or β receptor subunits. IL-11 and IL-6 signal by inducing homodimerisation of gp130, whereas CNTF, LIF and CT-1 induce heterodimerisation of gp130 and the LIF β receptor subunit (LIFR). OSM induces heterodimerisation of gp130 and the OSM β -receptor subunit (Gearing et al., 1991, Taga et al., 1996; Tanaka et al., 1999). IL-6, IL-11 and CNTF initially bind to ligand specific α subunits (IL6R α , IL-11 α , CNTFR α) before inducing β subunit dimerisation and signal transduction.

Neither gp130 nor LIFR contain intrinsic tyrosine kinase activity, so in order for the receptor complex to become active, membrane bound janus kinases (JAKs) must phosphorylate tyrosine residues in the cytoplasmic region of gp130. Signal transducers and activators of transcription (STAT) family members can then bind via their SH2 domain to these phosphorylated residues. Phosphorylated STAT dimers are then translocated to the nucleus where they initiate transcription of target genes (reviewed in Heinrich et al., 1998).

LIF and its role in the peripheral sensory nervous system

LIF has neurotrophic properties on developing peripheral sensory neurons. For example, LIF promotes the differentiation of sensory neurons from proliferating progenitors in neural crest cells cultures. LIF also promotes the survival of late embryonic sensory neurons with an efficacy comparable to NGF (Murphy et al., 1991; Murphy et al., 1993; Horton et al., 1998; Horton et al., 1996) LIF is retrogradely transported and accumulates in specific DRG neurons following intra-neural injection into the sciatic nerve of adult rats. The neurons that express functional LIF receptors and retrogradely transport this cytokine are predominantly small in diameter, with the majority (~81%) co-labelling for CGRP. Interestingly, 62% of LIF containing neurons are immuno-positive for TrkA and 34% are immuno-reactive for IB4 (Thompson et al., 1997), suggesting that LIF can exert neurotrophic effects on a

sub-population of nociceptive adult sensory neurons that are partly peptidergic and partly IB4-reactive.

LIF appears to play an important role in the response to nerve injury and, in particular, may enhance axonal regeneration. LIF is not normally detectable in the adult nervous system (Yammamori, 1991), however, an increase in LIF mRNA and protein is observed following sciatic nerve ligation, transection or crush. This increase occurs at the site of injury within dedifferentiating Schwann cells (Banner and Paterson, 1994; Bolin et al., 1995; Sun and Zigmond, 1996; Hirota et al., 1996; Kurek et al., 1996; Ito et al., 1998; Thompson and Majithia, 1998). LIF expression is not enhanced within DRG following sciatic nerve lesion. In contrast, LIF is induced in both the lesioned nerve and in sympathetic ganglia following post-ganglionic sympathetic nerve lesion (Sun and Zigmond 1996 a and b). LIF can initiate enhanced neurite outgrowth in freshly plated, CGRP-positive, cultured DRG neurons, but only in the presence of added NGF (Cafferty et al., 2001). LIF also enhances the elongation of established neurites in more mature DRG cultures in the absence of NGF. A conditioning lesion, prior to a second lesion, has been shown to enhance the regenerative capacity of sciatic nerves lesioned for a second time (Sjoberg and Kanje, 1990). The conditioning lesion appears to change the intrinsic growth characteristics of injured nerves, from branched growth to more elongated growth, and the effect appears to require novel gene transcription (Smith and Skene, 1997). The regeneration enhancing effects of a conditioning lesion are lost in LIF^{-/-} mice, suggesting that the primary lesion induces Schwann cells to produce LIF, and that Schwann cell-derived LIF is responsible for enhanced regeneration following a second lesion (Cafferty et al., 2001). Surprisingly, *in-vivo* peripheral nerve regeneration appears to be unaffected in LIF^{-/-} mice in the absence of a conditioning lesion, despite the fact that an *in-vitro* assay demonstrates that damaged nerves from wild type mice, but not LIF^{-/-} mice, can enhance neurite outgrowth from cultured DRG neurons (Ekstrom et al., 2000). However, this unexpected result may be as a result of the extended time points at which regeneration was assessed in this study if LIF, as has been suggested (Cafferty et al., 2001) only accelerates the rate of initial nerve regeneration.

Since LIF is up-regulated following nerve lesion, there is a possibility that it plays a role in the generation of inflammatory and neuropathic pain. Experimental evidence is

now building to support this hypothesis. For example, intraplantar injection of LIF into juvenile rats induces mechanical allodynia, but not thermal hyperalgesia, that can be prevented by a LIF blocking antibody (Thompson et al., 1996). In addition, intrathecal administration of LIF, via a mini osmotic pump, can induce the sprouting of sympathetic nerves into adjacent DRG and their peripheral nerves (Thompson and Majithia, 1997). Furthermore, the sprouting of sympathetic neurons into DRG that occurs following spinal nerve ligation can be significantly reduced by a gp130 blocking antibody and increased by exogenous LIF. Sympathetic innervation of DRG and damaged nerves has been implicated in the aetiology of neuropathic pain, and in particular in sympathetically maintained complex regional pain syndromes (reviewed in Janig and Habler, 2000). LIF null-mutant mice show a deficit in the number of macrophages, neutrophils, mast cells and T lymphocytes that infiltrate the lesion site in the first few days following sciatic nerve injury, and this is concomitant with a reduced inflammatory response (Sugiura et al., 2000). This would suggest that LIF might mediate some aspects of inflammatory hyperalgesia, although nociceptive thresholds were not measured in this study. In contrast, LIF knockout mice display an increased inflammatory response to CFA injection, a treatment that appears to raise the endogenous levels of LIF within the skin of wild type mice (Banner et al., 1998). Moreover, exogenous LIF ameliorates CFA induced thermal and mechanical hyperalgesia in rats but does not change nociceptive thresholds in non-CFA treated animals, suggesting that endogenous LIF normally acts in an anti-inflammatory manner. In agreement with this hypothesis, LIF mRNA levels within damaged nerves are negatively correlated with the number of macrophages infiltrating the nerve in post-mortem samples from human patients with varying peripheral neuropathies (Ito et al., 2001). However, LIF mRNA levels are dramatically increased in damaged human nerves, posing the question of whether LIF up-regulation is either protective or causative in human cases of peripheral painful neuropathy. The demonstration that exogenous LIF can reverse deficits in sensory neuron conduction velocity and tail flick latency in mice with cisplatin induced sensory neuropathy, mainly by beneficial effects on Schwann cells, suggests that the former scenario is true (Ozturk et al., 2005).

A number of studies have examined the role of LIF in modulating the widespread changes in neuronal gene expression that occur within DRG following nerve trauma.

For example, it is now well established that placing adult DRG neurons in culture leads to a time-dependent increase in the expression of galanin mRNA and protein that mimics the *in-vivo* changes in galanin expression following sciatic nerve lesion (Hokfelt et al., 1987; Villar et al., 1989; Wiesenfeld-Hallin., 1992; Kerekes et al., 1997, Zhang et al., 1998; Ozturk and Tonge, 2001; Holmes et al., 2005). The addition of LIF to adult and embryonic rodent DRG cultures significantly increases the number of galanin immuno-reactive neurons after 3 days, but only in the absence of exogenous NGF (Corness et al., 1998; Ozturk and Tonge., 2001). Furthermore, significantly fewer DRG neurons from LIF^{-/-} mice express galanin message associated peptide (GMAP) and galanin mRNA after 72 hours in culture compared to cultured DRG neurons from wild-type mice, demonstrating the importance of Schwann cell derived LIF in regulating galanin expression following nerve injury (Kerekes et al., 1999). The addition of exogenous LIF to DRG cultures from LIF^{-/-} mice restores the number of galanin mRNA and GMAP-positive neurons to that seen in DRG cultures from wild type mice. In accordance with the above in-vitro data, DRG from LIF^{-/-} mice contain dramatically fewer galanin and GMAP immuno-reactive neurons following sciatic nerve transection compared to DRG from wild type mice (Corness et al., 1996, Sun and Zigmond, 1996).

LIF also appears to play a role in modulating the up-regulation of DINE mRNA expression within small lumbar DRG neurons following sciatic nerve injury (Kato et al., 2002). Intra-sciatic nerve injection of a neutralizing anti-gp130 antibody ameliorates the increase in DINE mRNA expression following sciatic nerve transection in adult rats. Interestingly, intra-sciatic nerve injection of LIF or an anti-NGF antibody can induce DINE mRNA expression in lumbar DRG neurons of non-lesioned rats, and the effects of LIF and anti-NGF are additive, suggesting that DINE expression is induced in sensory neurons by a combination of increased Schwann cell-derived LIF and a reduction in NGF availability following nerve lesion.

Reg-2 is a secreted protein that is not normally expressed in the adult nervous system. Reg-2 is rapidly induced in a subset of small DRG neurons following sciatic nerve crush or transection and appears to enhance axonal regeneration by acting as a Schwann cell mitogen (Livesey et al., 1997; Averill et al., 2002). By 7 days post sciatic nerve lesion, Reg-2 is no longer expressed in a subset of small, damaged DRG

neurons, rather its expression is prominent in a sub-population of medium- to large-sized damaged DRG neurons. Reg-2 is normally expressed in embryonic sensory neurons, however, it is not expressed in embryonic sensory neurons from LIF receptor knockout mice, suggesting that LIF controls its developmental expression in sensory neurons (Livesey et al., 1997; Averill et al., 2002). This observation raises the possibility that Schwann cell-derived LIF induces Reg-2 expression in sensory neurons following sciatic nerve lesion. Since, galanin, DINE and Reg-2 have been postulated to have a neuroprotective role following nerve trauma (Holmes et al., 2000; Holmes et al., 2005; Kiryu-Seo et al., 2000; Livesey et al., 1997; Averill et al., 2002), the above data re-affirms the potentially important role that LIF plays in promoting neuronal regeneration following injury.

Whilst the available experimental data suggests that Schwann cell derived LIF may, at least partly, drive the changes in DINE, galanin and Reg-2 expression that occur within sensory neurons following nerve lesion, this does not appear to be the case for two neuropeptides, SP and CGRP, that also displays dramatic changes in expression following nerve trauma. The down-regulation of SP peptide following sciatic nerve transection is of the same magnitude in LIF^{-/-} mice as it is in wild type mice (Sun and Zigmond, 1996a). The application of LIF to the proximal transected sciatic nerve stump can reduce the axotomy-induced down-regulation of SP mRNA in lumbar DRG neurons (Zhang et al., 1995). The apparent up-regulation of substance P mRNA in axotomized DRG neurons by LIF mirrors the effects of LIF in up-regulating the expression of SP mRNA and peptide in sympathetic ganglia following sympathetic nerve transection, and the ability of LIF to increase the expression of SP mRNA in cultured sympathetic neurons (Ludlam et al., 1995; Sun and Zigmond 1996b). Exogenous LIF increases the expression of CGRP peptide in explanted mouse lumbar DRGs compared to explants cultured in the absence of growth factors (Ozturk et al., 2002). This data is a little difficult to interpret however, since explanted lumbar DRGs, unlike dissociated lumbar DRG neuron cultures, do not appear to be a good model of sciatic nerve lesion. In the absence of added growth factors the number of CGRP immuno-positive neurons within lumbar DRG explants hardly changes over time, and NGF does not appear to enhance CGRP expression (Ozturk et al., 2002). In contrast, lumbar DRG neurons display a dramatic reduction in CGRP staining both *in-*

vivo, following sciatic nerve lesion, and *in-vitro* in dissociated cultures. In both these latter cases, exogenous NGF significantly prevents the fall in CGRP expression (Noguchi et al., 1990; Mulder et al., 1997; Sterne et al., 1998; Shi et al., 2001; Shadiack et al., 2001; Lindsay et al., 1989; Verge et al., 1995; Jiang and Smith, 1995; Price et al., 2005). There is no data documenting the effects of exogenous LIF on the expression of CGRP in dissociated sensory neuron cultures.

To date, there is no published data addressing the question of whether LIF plays a role in regulating the transcriptional expression of the sodium channels Nav1.6, 1.7, 1.8 or 1.9, either in culture or following nerve lesion. Similarly, it is not known whether LIF can regulate the expression of ATF3, P2X3, VR1 or PACAP in sensory neurons. In this chapter, I will report the results of experiments to investigate whether LIF can regulate the transcription of these mRNAs, as well as the transcription of galanin, SP and α - and β -CGRP mRNAs, in dissociated cultures of adult mouse DRG neurons.

4.2 Materials and Methods

4.2.1 Neuronal Cultures

Neuronal cultures of adult DRG were set up to identify the effects of NGF, Artemin and additionally MSP on the expression of various mRNAs in sensory neurons. DRG was the most suitable of the sensory ganglia to culture at this age. Practically, it is the easiest to dissect and culture, and, as each mouse contains a large number of DRG, it facilitates setting up a large number of dense cultures from a small number of mice, thus allowing many different experimental conditions to be included in each experiment. In addition, the majority of other studies on gene regulation and nerve injury and regeneration in adult sensory neurons, as well as models of neuropathic pain and sensory neuropathy, have used rodent DRG.

Adult CD-1 Mice (approximately P60) were killed with CO₂ followed by cervical dislocation. All subsequent dissections and preparations were carried out in a laminar flow hood using standard sterile techniques. Dissections were performed under a stereomicroscope using a fibre optic light to effectively illuminate samples, but prevent overheating of the specimen. Ganglia were initially dissected using forceps and scissors that had previously been sterilised by flaming in alcohol. Tungsten needles were required to complete the dissection by removing any adherent connective tissue. Dissections were carried out in filter sterilised L15 medium, pH 7.3, supplemented with penicillin and streptomycin as previously.

Dorsal Root Ganglia (DRG):

The DRG dissection in the adult is essentially the same as that for the postnatal mouse (see 3.2), but requiring tougher instruments to cut through bone. Several ganglia are present per mouse and run the length of the spinal cord. Dissection from the adult firstly involved the removal of the skin from the back. The whole spinal column was then removed and cut into 2 or 3 pieces to facilitate the next step of the dissection. As for the postnatal and embryonic mouse, cuts were then made by inserting one scissor

blade inside the spinal column and cutting the full length of the column on both sides. The ventral section of the spinal column was now removed and the spinal cord taken out. The DRG were visible on either side of the dorsal half of the column, spread out uniformly along the length of the column. The membrane covering the surface of the spinal column was removed, followed by each DRG. At this age there is an abundance of nerves and connective tissue, and so time had to be spent using tungsten needles to remove as much adherent material as possible. Ganglia were transferred to 15ml falcon tubes using a flame-sterilised, siliconised glass Pasteur pipette. Approximately 50 ganglia per culture were dissected, and the ganglia were divided into approximately 10 ganglia per tube to allow thorough enzymatic degradation of collagen and connective tissue within the ganglia, prior to dissociation of the ganglia into a single cell suspension.

Because of the increasing amounts of collagenous connective tissue in older ganglia, the use of collagenase was required initially to breakdown this tissue, prior to trypsinization. Once all ganglia were dissected 980µl Hanks Balanced Salt Solution (+ Calcium(0.097mg/ml) and Magnesium (0.0185mg/ml)) and 20µl collagenase (100mg/ml) (Sigma) was added to each tube. Ganglia were initially incubated on ice for 30mins followed by incubation at 37°C for 25mins. Ganglia were then washed twice in CMF-HBSS and further enzymatically treated by incubation with 0.05% trypsin, for 30 minutes, at 37°C. To arrest trypsin action, ganglia were washed in F12 + HIHS. Ganglia could then be mechanically dissociated by gentle trituration using a fire-polished, narrowed, siliconized Pasteur pipette to produce a single cell suspension. This trituration was done in F14 medium supplemented with albumax I and glutamine. The trituration was monitored at various stages by examining droplets of the dissociated cell suspension under an inverted phase-contrast microscope.

Once a single cell suspension was obtained, cells were made to up to the required volume in F14 + Albumax and plated onto 35mm culture dishes (Greiner). As these cultures were to be used for RT-PCR they were plated at high density (approximately 1000-2000 cells per dish). Dishes had been previously prepared by coating with substratum of poly-DL-ornithine (0.5mg/ml in 0.15M borate buffer, pH 8.4, overnight at room temperature) and then laminin (20µg/ml, Sigma in F14 medium, 4 hours at

37°C). Prior to plating, dishes were washed twice with F12+HIHS to remove laminin and then 1ml F14 supplemented with Albumax I and 2mM glutamine was added to each dish. Next, 1ml of cell suspension was added per dish and cells were grown with or without neurotrophic factors in a humidified 5.5% CO₂ incubator, at 37°C for 48 or 96hours.

In the adult, sensory neurons do not require neurotrophic support for survival so caspase inhibitors were not required. Cells were, therefore, either grown without factors or in the presence of NGF (10ng/ml), Artemin (10ng/ml) or MSP (50ng/ml). LIF was also used in some cultures at a concentration of 50ng/ml.

Following incubation cells were collected for RNA extraction in 350µl RLT buffer (+ 1% β mercaptoethanol) (Qiagen) ready for RNA extraction.

To ensure any observed changes in mRNA expression were not due to differential survival of neurons in the presence of neurotrophic factors, survival counts were done at 3, 48 and 96 hours after plating using a 1cm grid. Survival at 48 hrs and 96hrs was expressed as a percentage of the 3 hour count.

4.2.2. RNA extraction

RNA was extracted using the Qiagen RNeasy kit (described in 2.2.5), and Stratascript reverse transcriptase was used for reverse transcription (described previously in 2.2.5).

4.2.3. Real-time PCR-Stratagene MX3000P

Real Time PCR was used to identify changes in gene expression in adult sensory neurons cultured for 96 hours in the presence or absence of NGF, Artemin or MSP.

Reverse Transcription of RNA samples was done as in chapter 2 (see 2.2.5.1) and PCRs set up as in 2.2.5.2. All genes mentioned in chapters 2 and 3 were investigated, and this study was also extended further to examine other potentially interesting

genes. Newer genes included many that are up-regulated following axotomy or nerve damage, and also in in-vitro cultures. Experiments were performed ostensibly to see if NGF, ART and MSP could reverse axotomy-induced changes in gene expression.

Additional genes investigated included: the purine receptor, P2X3; the sodium channels Nav1.3, Nav1.6 and Nav1.7; Damage Induced Neuronal Endopeptidase (DINE); Activating Transcription Factor 3 (ATF3). The latter two mRNAs are expressed at very low levels *in vivo* under normal circumstances but are massively up-regulated following axotomy or nerve damage (Tsujino et al., 2000, Kiryu-Seo et al., 2000).

All genes outlined in 3.2.5 were investigated alongside several others. The primers used and reaction conditions for new genes are outlined below.

Primers:

GENE	PRIMER SEQUENCE	MGCL CONCENTRATION (MM)	ANNEALING TEMPERATURE (°C)
P2X3	Forward: 5-GTG CTT CCC GCT AAG ACC TG-3' Reverse: 5'-TTT GGA AAT GGA TGG ATG CTT GG-3'	3	52
Nav1.6	Forward: 5'-GAC ACA CAG AGC AAG CAG ATG- 3' Reverse: 5'-GGA GAG AAT GAC CAC CAC AAA G-3'	3	52

Nav1.7	Forward: 5'-GCT ACA TCA TCA TAT CCT TCC TG-3' Reverse: 5'-GAA CAT CTC AAA GTC GTC CTC-3'	3	49
Damage Induced Neuronal Endopeptidase (DINE)	Forward: 5'-GTA TTG GCA CCA TCA TTG-3' Reverse: 5'-GTG AAG TTG TCA TAG AGG-3'	3	52
Activating Transcription Factor 3	Forward: 5'-GAG AGT GTG AAT GCT GAG-3' Reverse: 5'-TCT GTT GGA TAA AGA GGT TC-3'	3	51

Table 4.1. Primer sequences, annealing temperatures and Mg concentration used for detection of new genes.

4.3. Results

4.3.1. Developmental expression patterns of the new mRNAs assayed in chapter 4

The expression of DINE, ATF3, P2X3, Nav1.6 and Nav1.7 mRNAs were quantified by real time QPCR using RNA that had been extracted from E16, P0, P5 and adult trigeminal, nodose and dorsal root ganglia. Results are shown in figures 4.1 and 4.2. In this chapter, results were statistically compared by the use of an ANOVA (analysis of variance) test. This test is useful as, although similar to the t-test, it can be used to compare several sets of data. This was thus useful in this chapter, when several variables were apparent.

As anticipated from the published literature (see above), the levels of DINE and ATF3 mRNAs were extremely low in all ganglia studied, at all ages (figure 4.1 B and C).

The developmental expression pattern of P2X3 mRNA within sensory ganglia is shown in figure 4.1A. All three sensory ganglia displayed similar patterns of P2X3 mRNA expression and the amounts of P2X3 mRNA expressed were of the same order of magnitude within each of the three ganglia. In general, fairly constant levels of P2X3 mRNA were expressed between E16 and P5 in all three ganglia and this is followed by a marked drop in expression between P5 and adulthood. The drop in P2X3 mRNA levels between P5 and the adult was most marked in the trigeminal ganglion.

This chapter also explores the expression of two TTX-sensitive sodium channel alpha-subunits, Nav1.6 and Nav1.7. The developmental expression pattern of Nav1.6 mRNA is very similar in both trigeminal and dorsal root ganglia and is markedly different to the expression patterns of the TTX-resistant sodium channels, Nav1.8 and Nav1.9 (see chapter 3). In both neural crest-derived sensory ganglia, Nav1.6 mRNA

levels increased approximately 3-fold from E16 to P5, followed by a modest drop in the levels of expression between P5 and adulthood (figure 4.2 A). In both ganglia the levels of Nav1.6 mRNA expressed in the adult were higher than those expressed at E16. Nav1.6 mRNA showed a slightly different developmental pattern of expression in nodose ganglia compared to trigeminal and dorsal root ganglia. In this placode-derived ganglion the increase in expression between E16 and neonatal ages was modest and the peak of expression was at P0. However, once again, as in the case of the neural crest-derived sensory ganglia, there was a small drop in the level of Nav1.6 mRNA between P5 and adulthood, so that in the adult the levels of Nav1.6 mRNA were slightly lower than those at E16 (figure 4.2A).

The developmental expression pattern of Nav1.7 mRNA more closely resembles that of the TTX-resistant channels, Nav1.8 and Nav1.9, than Nav1.6 mRNA does. In all ganglia, significantly less Nav1.7 mRNA was expressed in the adult compared to E16 (figure 4.2B). In the case of the nodose ganglion, Nav1.7 mRNA expression levels peak at P0, whereas in trigeminal and dorsal root ganglia, peak expression of Nav1.6 mRNA occurs at E16.

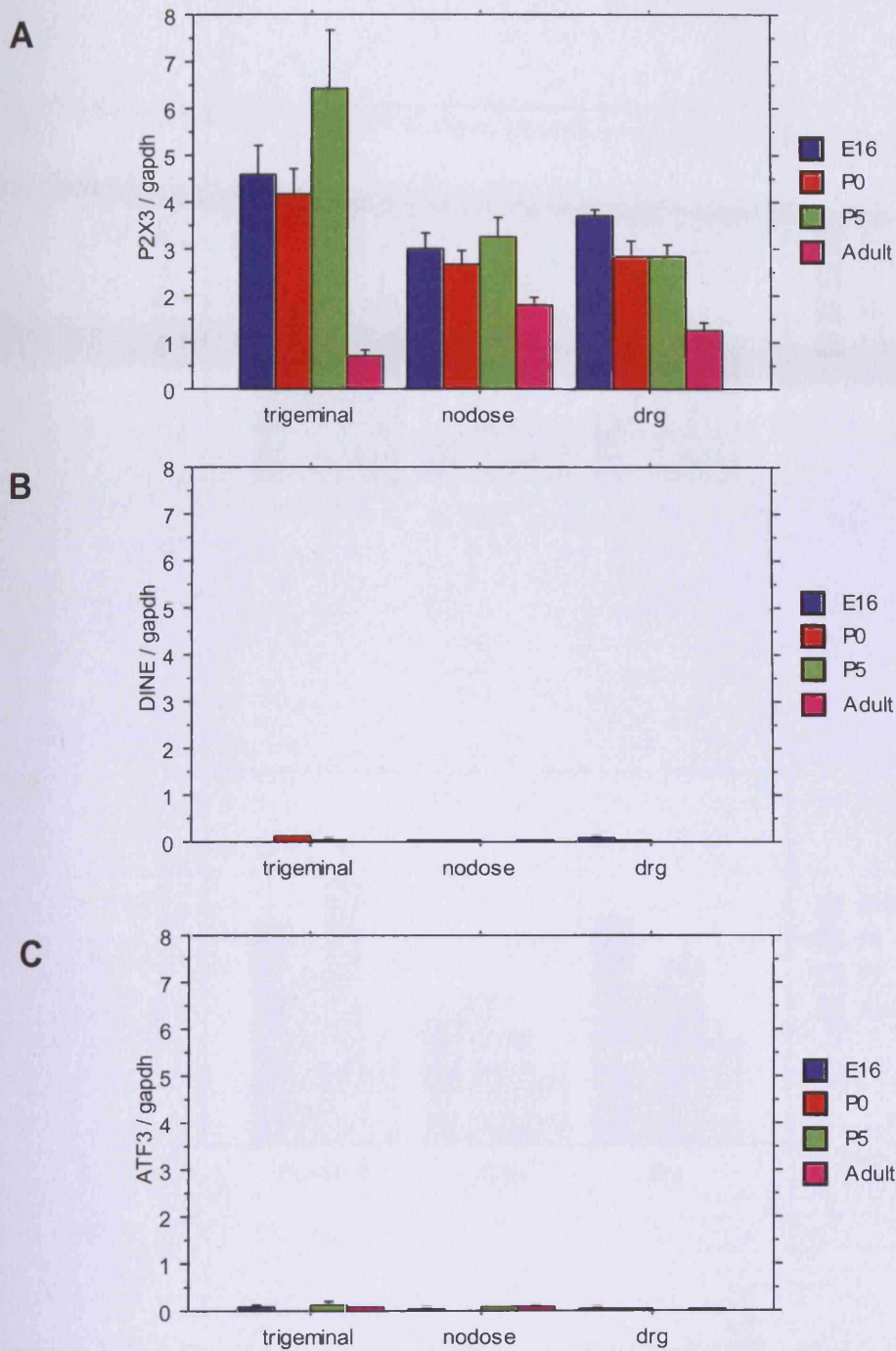


Figure 4.1. Developmental expression of *P2X3*, *DINE* and *ATF3* mRNAs in sensory neurons

Trigeminal ganglia, nodose ganglia and DRG were collected from E16, P0, P5 and adult mice and RNA extracted. Expression of *P2X3*, *DINE* and *ATF3* mRNAs were quantified via real-time QPCR.

Error bars = +/- standard error, n = 6

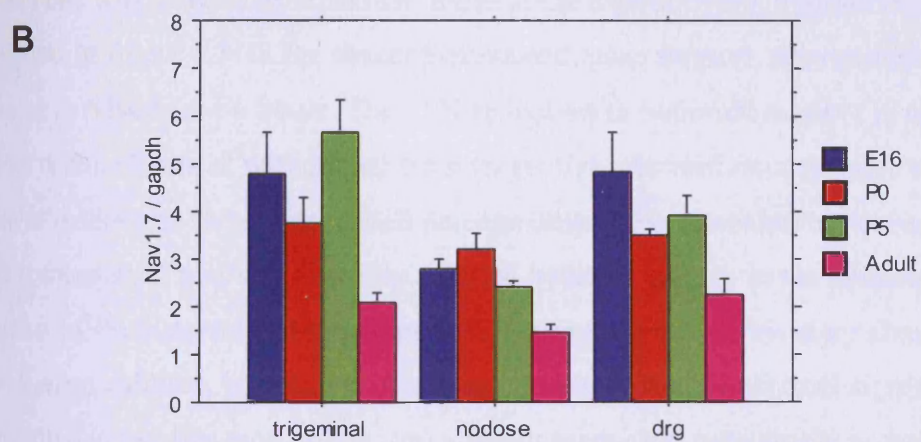
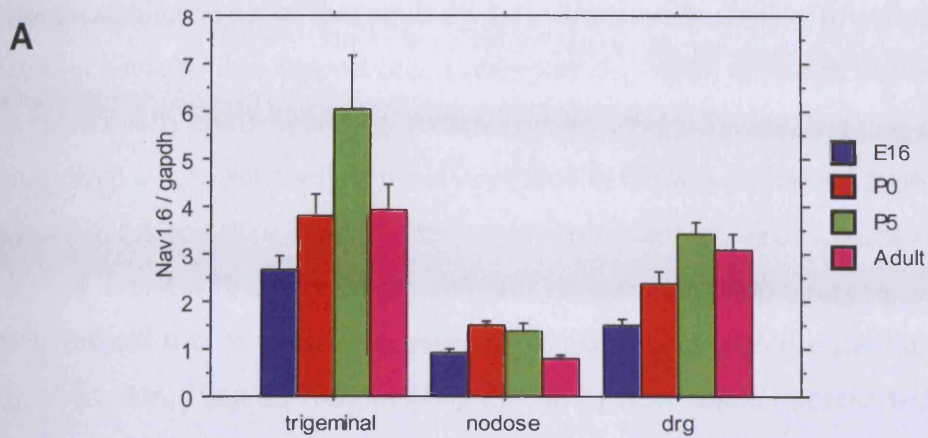


Figure 4.2. Developmental expression of *Nav1.6* and *Nav1.7* mRNAs in sensory neurons

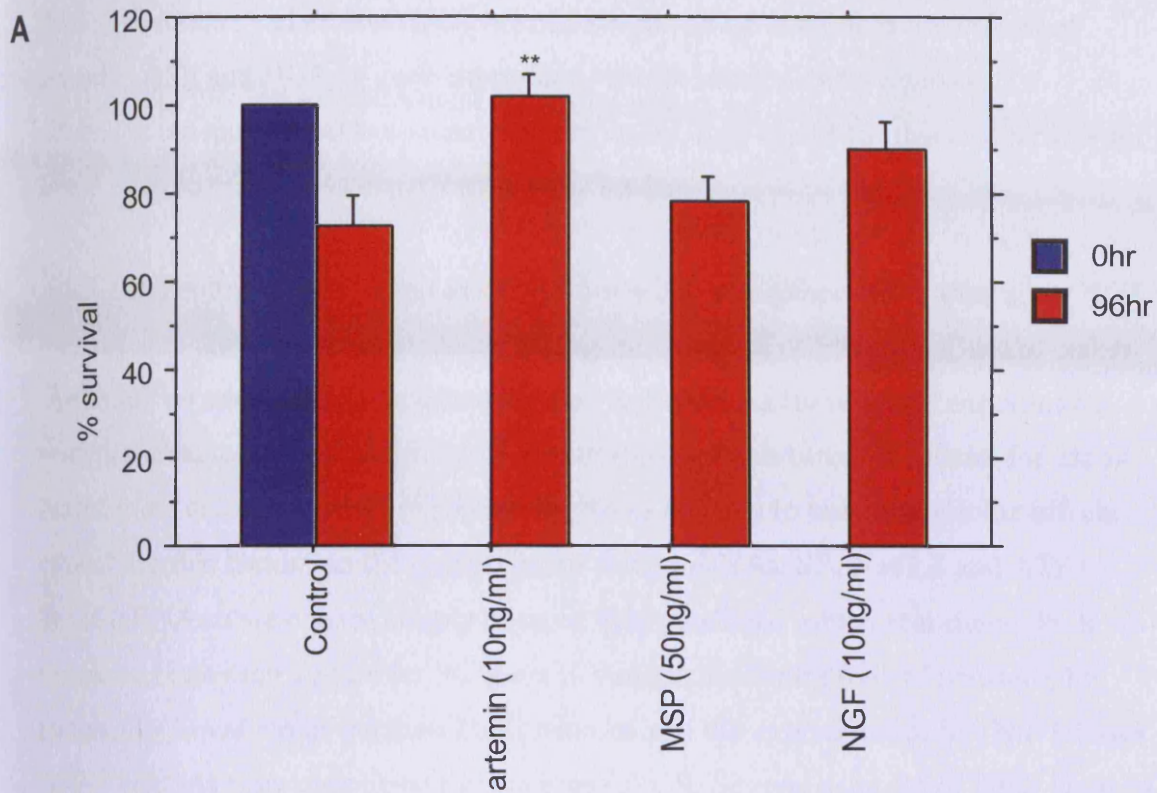
Trigeminal ganglia, nodose ganglia and DRG were collected from E16, P0, P5 and adult mice and RNA extracted. Expression of *Nav1.6* and *Nav1.7* mRNAs were quantified via real-time QPCR.

Error bars = +/- standard error, n = 6

4.3.2. Survival of adult DRG neurons in culture

It is well-established belief that adult sensory neurons can survive in culture in the absence of neurotrophic support (e.g. Lindsay et al., 1988), although this view has been occasionally challenged (e.g. Acheson et al., 1995). To confirm that DRG neuron survival was not compromised over time in culture, or indeed further enhanced in the presence of additional neurotrophic factors, experiments were performed to assess the survival of adult DRG neurons over 96 hours in culture, either without the addition of exogenous neurotrophic factors or with the addition of 10ng/ml artemin, 10ng/ml NGF or 50ng/ml MSP (these concentrations were determined in a series of pilot experiments and were used for all future experiments - see 4.3.3 below). Neuronal numbers were initially determined 3 hours after plating to allow sufficient time for the neurons to adhere to the laminin substratum. A second neuron count was made 96 hours after plating and the percentage of healthy, phase bright cells was calculated relative to those at the 3-hour count. The survival data is presented in figure 4.3. In the absence of neurotrophic support, approximately 75 % of neurons survived for 96 hours. The 25% reduction in neuronal number is unlikely to be due to the effects of withdrawal from target field-derived neurotrophic support, rather it is likely to reflect lethal cell damage during the dissociation procedure. A similar number of neurons were lost after 96 hours of culture in the presence of 50ng/ml MSP. 10ng/ml NGF appears to increase neuronal survival by about 10 % over control cultures, however this increase does not reach statistical significance. Interestingly, the presence of 10ng/ml artemin seemed to completely prevent neuronal loss at 96 hours. This increase in survival was statistically significant compared to control cultures and MSP supplemented cultures. The apparent survival promoting effect of artemin, and to a lesser extent NGF, may have more to do with both factors having a hypertrophic effect on neuronal morphology, thus making small neurons more obvious and easier to count at 96 hrs, rather than direct effects on neuronal survival. This is discussed more fully below. Since neuronal survival was not significantly compromised by the lack of neurotrophic factor support, all future

culture experiments analysing the effects of NGF, MSP and artemin on adult sensory neuron gene expression were carried out in the absence of caspase inhibitors.



B

	P value	Significance
Control, artemin	0.0069	**
Control, MSP	0.4923	
Control, NGF	0.0638	
artemin, MSP	0.0202	*
artemin, NGF	0.1829	
MSP, NGF	0.1905	

Figure 4.3. Survival of cultured adult DRG neurons

Adult DRG neurons were cultured in the presence or absence of artemin (10ng/ml), NGF (10ng/ml) or MSP (50ng/ml) for 96 hours. The number of phase bright healthy neurons were counted 3 hours after plating (0hrs) and then a further 96 hours later. Survival was calculated as a percentage of this '0hr' count. Error bars are +/- standard error, where n = 4, * = p<0.05, ** = p<0.01

4.3.3. Pilot neurotrophic factor dose responses

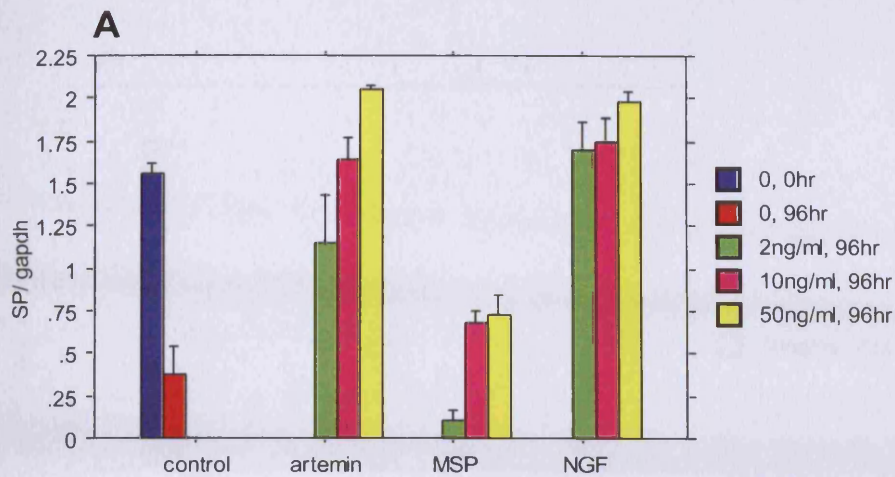
Throughout this chapter several experiments were set up to explore the effects of artemin, MSP and NGF on gene expression within cultured DRG neurons. To determine the most suitable concentration of each factor to use for these experiments, initial pilot dose response experiments were set up.

Adult DRG cultures were set up as outlined in 4.2.1. and either MSP, artemin or NGF was added to dishes at concentrations of 2ng/ml, 10ng/ml or 50ng/ml. Control dishes containing no neurotrophic factors were also included. As these initial experiments were designed to determine suitable concentrations of neurotrophic factors for use in future, more comprehensive, experiments, it was decided to only analyse the effects of neurotrophic factors on the expression of three mRNAs, SP, Nav1.8 and ATF3. These mRNAs were chosen simply because they produced robust real time-QPCR reactions. Following culture for 96 hours in various concentrations of neurotrophic factors, RNA was extracted from DRG neurons and the expression of SP, Nav1.8 and ATF-3 mRNAs were quantified by real time-QPCR. Several samples of DRG neurons were lysed immediately following dissociation to provide RNA for a time 0 time-point that could be used to evaluate the levels of mRNA expression within DRG neurons *in-vivo*. The data from the pilot dose response experiments is presented in figures 4.4 to 4.6. Statistical analyses were performed using ANOVA tests to compare data groups (n = 4). Statistical comparisons between the different neurotrophic factor concentrations and controls are presented in the tables underneath each graph.

In chapters 2 and 3, t-tests were sufficient to analyse the statistical significance between data sets, so data was analysed and graphs were presented in an Excel spreadsheet format. However, in this chapter several data sets are being compared against each other, thus requiring a statistical package that could carry out more in-depth analyses. For this reason, results in this chapter were calculated in StatView and hence the graphs appear in a different format.

The expression of SP mRNA in control cultures and cultures supplemented with different concentration of either artemin, MSP or NGF is shown in figure 4.4. The levels of SP mRNA expressed by adult DRG neurons grown in the absence of neurotrophic factor support decreased markedly over time in culture. This is in accordance with the neonatal sensory neuron culture data presented in chapter three of this thesis and previously published data from sciatic nerve lesion experiments (Nielsch et al., 1987; Zhang et al., 1995; Sterne et al., 1998). The decrease in SP mRNA was significantly attenuated by the addition of NGF at all concentrations and artemin at 10ng/ml and 50ng/ml. The expression enhancing effects of NGF were not significantly different between 2ng/ml and 50ng/ml. Likewise, 50ng/ml artemin was not significantly more effective in enhancing SP mRNA expression than 10ng/ml artemin. MSP could also partially prevent the time-dependent decrease in SP mRNA expression that occurred in cultured DRG neurons, although not at a concentration of 2ng/ml.

The amount of Nav1.8 mRNA expressed by cultured adult DRG neurons also decreased over time in culture in the absence of neurotrophic factor support (figure 4.5). Once again, this is in agreement with the neonatal culture data presented in chapter 3 of this thesis and the published literature documenting the expression of this sodium channel following sciatic nerve lesion (Okuse et al., 1997; Cummins et al., 1997; Tate et al., 1998; Dib-hajj et al., 1998; Novakovic et al., 1998; Sleeper et al., 2000; Decosterd et al., 2002). The addition of 2, 10 or 50ng/ml of NGF or artemin significantly attenuated the decrease in Nav1.8 mRNA expression. Once again, as in the case of SP mRNA, there was no statistically significant difference between the regulatory effects of 2ng/ml NGF and 50ng/ml NGF. In the case of Nav1.8 mRNA, 50ng/ml artemin was significantly more effective in ameliorating the decrease in mRNA expression than 2ng/ml or 10ng/ml artemin. Whilst all concentrations of MSP were capable of enhancing Nav1.8 mRNA expression, only 50ng/ml had a statistically significant effect on Nav1.8 mRNA expression.



B

artemin	P value	Significance
2ng/ml, 10ng/ml	0.1101	
2ng/ml, 50ng/ml	0.0251	*
2ng/ml, control	0.4010	
10ng/ml, 50ng/ml	0.1572	
10ng/ml, control	0.0028	**
50ng/ml, control	0.0011	**

C

MSP	P value	Significance
2ng/ml, 10ng/ml	0.0053	**
2ng/ml, 50ng/ml	0.0026	**
2ng/ml, control	0.4849	
10ng/ml, 50ng/ml	0.7805	
10ng/ml, control	0.8717	
50ng/ml, control	0.1407	

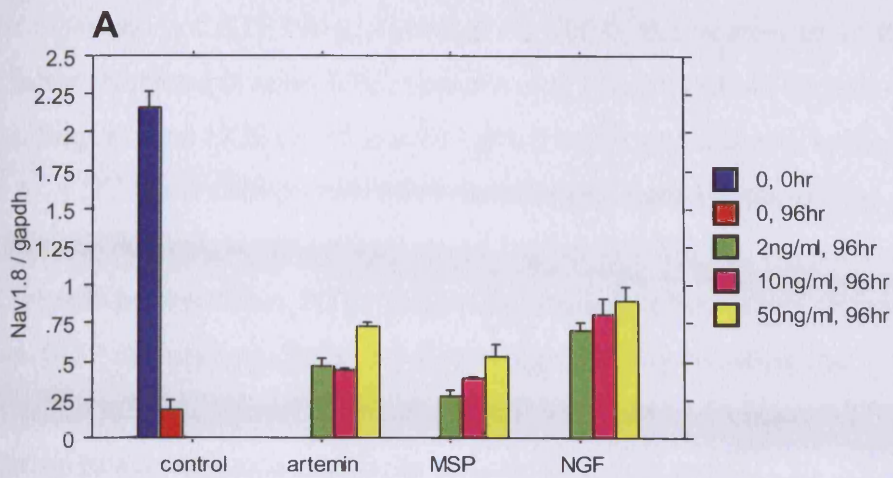
D

NGF	P value	Significance
2ng/ml, 10ng/ml	0.8359	
2ng/ml, 50ng/ml	0.1698	
2ng/ml, control	<0.0001	**
10ng/ml, 50ng/ml	0.2276	
10ng/ml, control	<0.0001	**
50ng/ml, control	<0.0001	**

Figure 4.4. Effect of increasing concentrations of artemin, MSP and NGF on the expression of SP mRNA in cultured adult mouse DRG neurons

Adult mouse DRG neurons were cultured for 96 hours in the presence and absence of concentrations of Artemin MSP and NGF ranging from 2ng/ml to 50ng/ml. Expression of SP mRNA was quantified using real-time QPCR and all results were normalised against GAPDH mRNA levels. Results of ANOVA analyses are outlined in B-D.

Error bars = +/- standard error, n = 3 - 4 * = <0.05 , ** = <0.01



B

artemin	P value	Significance
2ng/ml, 10ng/ml	0.5597	
2ng/ml, 50ng/ml	0.0034	**
2ng/ml, control	0.0069	**
10ng/ml, 50ng/ml	0.0012	**
10ng/ml, control	0.0040	**
50ng/ml, control	0.0001	**

C

MSP	P value	Significance
2ng/ml, 10ng/ml	0.2733	
2ng/ml, 50ng/ml	0.0219	*
2ng/ml, control	0.5298	
10ng/ml, 50ng/ml	0.1402	
10ng/ml, control	0.1115	
50ng/ml, control	0.0074	**

D

NGF	P value	Significance
2ng/ml, 10ng/ml	0.4138	
2ng/ml, 50ng/ml	0.1696	
2ng/ml, control	0.0011	**
10ng/ml, 50ng/ml	0.5202	
10ng/ml, control	0.0003	**
50ng/ml, control	0.0001	**

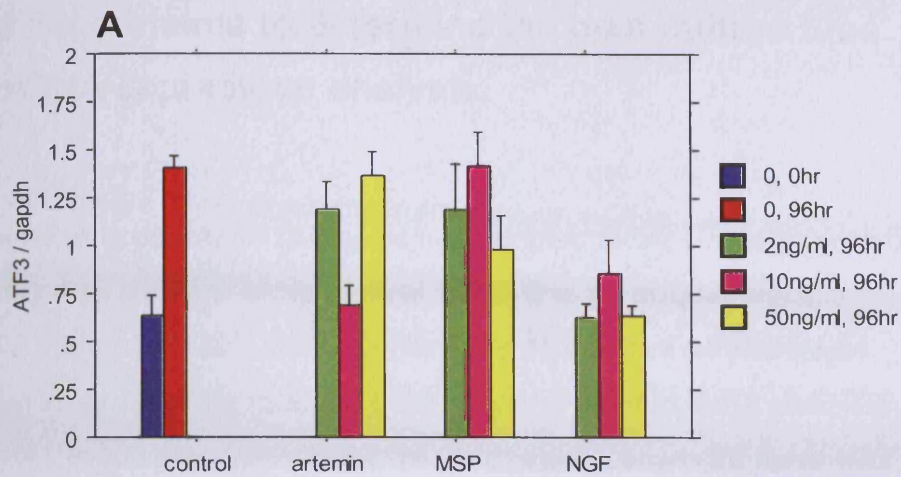
Figure 4.5. Effect of increasing concentrations of artemin, MSP and NGF on the expression of Nav1.8 mRNA in cultured adult mouse DRG neurons

Adult mouse DRG neurons were cultured for 96 hours in the presence and absence of concentrations of Artemin MSP and NGF ranging from 2ng/ml to 50ng/ml. Expression of Nav1.8 mRNA was quantified using real-time QPCR and all results were normalised against GAPDH mRNA levels. Results of ANOVA analyses are outlined in B-D.

Error bars = +/- standard error, n = 3 - 4 * = <0.05 , ** = <0.01

As anticipated from previously published data documenting the effects of nerve damage on the expression of ATF3 (e.g. Averill et al., 2004), the expression of this transcription factor increased in adult DRG neurons over time in culture (figure 4.6), Both artemin (10ng/ml) and NGF (2, 10 and 50 ng/ml) could significantly reduce the up-regulation of ATF3. Interestingly, artemin was not significantly effective in reducing ATF3 mRNA levels at concentrations of 2ng/ml or 50ng/ml, suggesting that the effects of artemin on regulating ATF3 mRNA expression follow a bell shape dose response curve. MSP did not seem to be capable of significantly reducing the expression of ATF3 mRNA. However, 50ng/ml MSP was able to decrease ATF3 mRNA expression to a level that was close to statistical significance.

In light of the data presented above, I decided to use 10ng/ml artemin and NGF and 50ng/ml MSP in all subsequent experiments designed to investigate the modulation of DRG neuron gene expression by these neurotrophic factors.



B

artemin	P value	Significance
2ng/ml, 10ng/ml	0.0240	**
2ng/ml, 50ng/ml	0.3544	
2ng/ml, control	0.1880	
10ng/ml, 50ng/ml	0.0036	**
10ng/ml, control	0.0005	**
50ng/ml, control	0.7531	

C

MSP	P value	Significance
2ng/ml, 10ng/ml	0.4500	
2ng/ml, 50ng/ml	0.4787	
2ng/ml, control	0.4849	
10ng/ml, 50ng/ml	0.1529	
10ng/ml, control	0.8717	
50ng/ml, control	0.7531	

D

NGF	P value	Significance
2ng/ml, 10ng/ml	0.2120	
2ng/ml, 50ng/ml	0.9519	
2ng/ml, control	0.0002	**
10ng/ml, 50ng/ml	0.2307	
10ng/ml, control	0.0023	**
50ng/ml, control	0.0003	**

Figure 4.6. Effect of increasing concentrations of artemin, MSP and NGF on the expression of ATF3 mRNA in cultured adult mouse DRG neurons

Adult mouse DRG neurons were cultured for 96 hours in the presence and absence of concentrations of Artemin MSP and NGF ranging from 2ng/ml to 50ng/ml. Expression of ATF3 mRNA was quantified using real-time QPCR and all results were normalised against GAPDH mRNA levels. Results of ANOVA analyses are outlined in B-D.

Error bars = +/- standard error, n = 3 - 4 * = <0.05 , ** = <0.01

4.3.4. Pilot experiments to determine the best culture time point for mRNA expression analysis.

Pilot experiments were performed (using the neurotrophic factor concentrations that were empirically determined as being optimal in 4.3.3) to investigate whether extracting RNA from adult DRG cultures after 48 or 96 hours of culture would provide the best assay range for measuring the effects of artemin, MSP and NGF on regulating mRNA expression. Once again, the “test genes” chosen for these pilot experiments were SP, Nav1.8 and ATF3. Adult DRG cultures, supplemented with either 10ng/ml NGF, 50ng/ml MSP or 10ng/ml artemin, were set up and incubated for either 48 or 96 hours prior to RNA extraction and purification. Control dishes containing no neurotrophic factors were also set up in parallel. Once again, samples of DRG neurons were lysed immediately following dissociation to provide RNA for a time 0 time-point for evaluating the levels of mRNA within DRG neurons *in-vivo*. The results of these pilot experiments are shown in figures 4.7. Significant differences in mRNA expression levels, compared to control cultures, at 96 hours are indicated by * ($p<0.05$) or ** ($p<0.01$) directly above bars as determined by anova statistical analysis.

Once again, these cultures demonstrate a marked decrease in the expression of SP and Nav1.8 mRNAs over time in culture, whilst the expression of ATF3 mRNA increased. SP mRNA expression levels dropped 8-fold by 48 hours and this drop was increased to 15-fold by 96 hrs. At 48 hours, 10ng/ml artemin was able to increase the expression of SP mRNA by 3-fold in comparison to the levels in control cultures. By 96 hours, artemin increased the levels of SP mRNA more than 10-fold compared to control cultures. Similarly, 10ng/ml NGF doubled SP mRNA expression compared to control cultures at 48 hours and this difference between NGF supplemented cultures and control cultures was increased to 5-fold by 96 hours. At 48 hours, 50ng/ml MSP increased the expression of SP mRNA, although this increase was not statistically significant. However, by 96 hours 50ng/ml MSP was as effective as 10ng/ml NGF in ameliorating the decline in SP mRNA expression (figure 4.7A).

Nav1.8 mRNA levels within control adult DRG cultures dropped by 6-fold compared to *in-vivo* levels over the first 48 hours of culture, and this decrease reached 10-fold by 96 hours (figure 4.7B). Artemin appeared to be ineffective in attenuating the decrease in Nav1.8 mRNA expression at 48 hours, whereas 10ng/ml NGF and 50ng/ml MSP were able to slightly, but significantly, increase the expression of this TTX-resistant sodium channel mRNA. At 96 hours, mainly due to the continued decreasing expression of Nav1.8 mRNA in the absence of neurotrophic factor support, all three neurotrophic factors could significantly increase the levels of Nav1.8 mRNA compared to those in control cultures. However, it is clear that these three neurotrophic factors were less able to rescue Nav1.8 mRNA expression at 96 hours than SP mRNA expression.

The mRNA levels of the injury induced factor, ATF3, increased more than 6-fold after 48 hours in control cultures compared to the presumptive *in-vivo*, or time 0, level (figure 4.7C). It should be noted that in the case of ATF3 and DINE mRNAs, but not the other mRNAs assayed in this chapter, the time 0 mRNA expression levels are significantly higher than those found *in-vivo*, since the dissection and dissociation procedures appear to stimulate immediate-early gene type expression of these mRNAs (S Wyatt personal communication - data not shown.). At 48 hours, all three neurotrophic factors tested appeared to be able to reduce the expression of ATF3 mRNA by around 25% compared to control cultures, although the reduction was only statistically significant in the case of NGF and artemin. After 96 hours in culture, ATF3 mRNA levels in DRG neurons cultured in the absence of neurotrophic factor support dropped 25% compared to 48 hour control culture levels. However, all three neurotrophic factors could reduce the expression of this transcription factor mRNA in a statistically significant manner at this time-point.

It is clear from the data presented above that determining mRNA expression levels in DRG neurons after 96 hours of culture, rather than 48 hours of culture, provides an assay with the best dynamic range for investigating the effects of neurotrophic factors on regulating gene expression. Therefore, all subsequent real time-QPCR analysis of gene expression was performed on RNA extracted from adult DRG neurons that had been in culture for 96 hours.

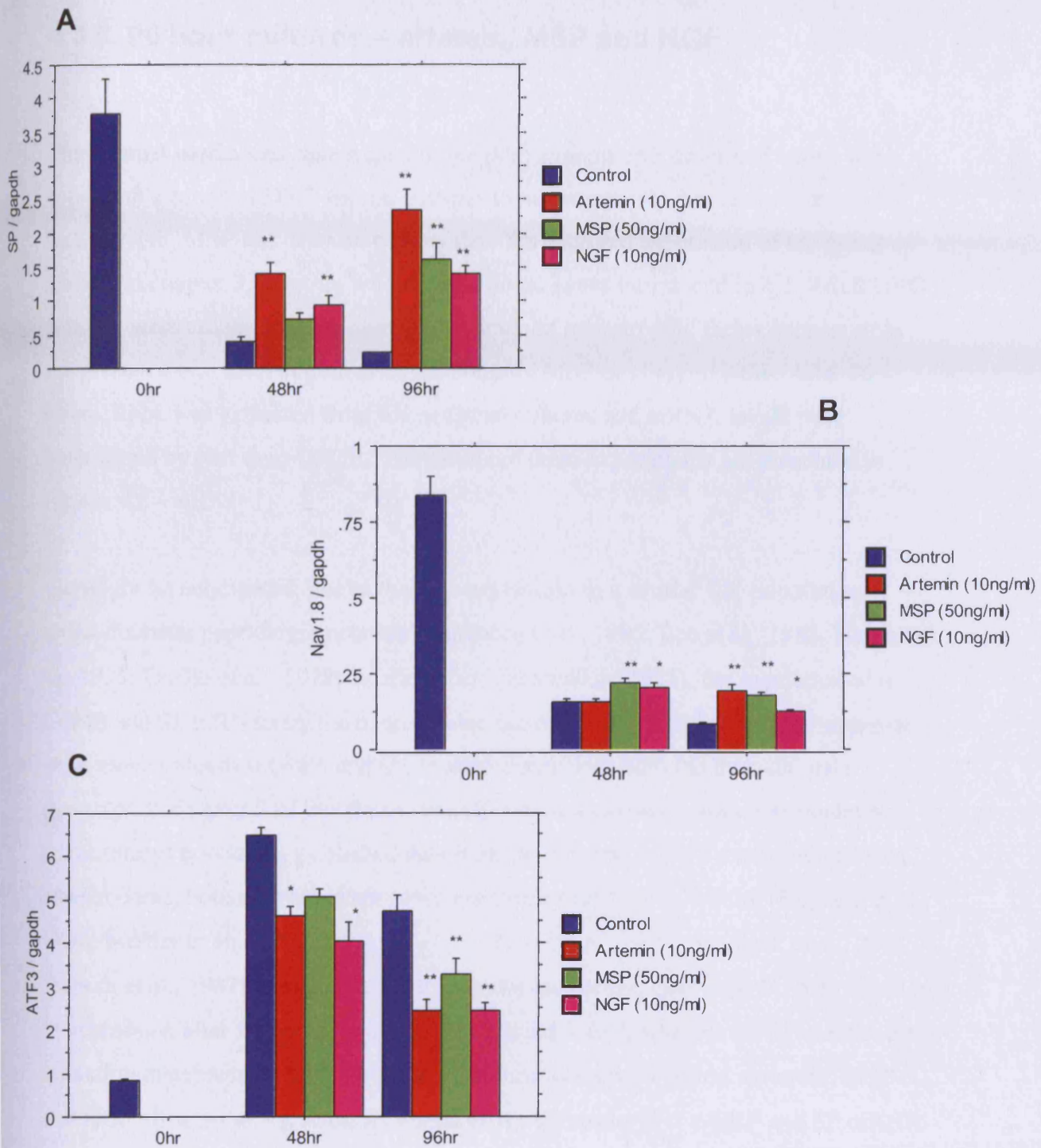


Figure 4.7. Effect of artemin, MSP and NGF on the expression of SP, Nav1.8 and ATF3 mRNAs in adult mouse DRG neurons cultured for 48 or 96 hours

Adult mouse DRG neurons were cultured in the presence or absence of artemin (10ng/ml), MSP (50ng/ml) or NGF (10ng/ml) for 48 or 96 hours. SP (A) Nav1.8 (B) and ATF3 (C) mRNA levels were quantified using real-time QPCR. * = $p < 0.05$, ** = $p < 0.01$ as determined by two-tailed t-test, in comparison to control samples at that time-point.

Error bars = +/- standard error, $n = 4$

4.3.5. 96 hour cultures – artemin, MSP and NGF

The optimal parameters determined in the pilot experiments described above were applied in a series of DRG neuron cultures to determine whether the neurotrophic factors NGF, MSP and artemin can regulate the neuronal expression of the genes studied in chapter 3, together with the additional genes introduced in 4.1. Adult DRG neurons were cultured for 96 hours either without neurotrophic factor support or in the presence of either 10ng/ml artemin, 50ng/ml MSP or 10ng/ml NGF. After 96 hours, RNA was extracted from the neuronal cultures and mRNA levels were determined by real time-QPCR. The results of these experiments are presented in figures 4.8 – 4.13.

As might be anticipated, due to their co-expression in a similar sub-population of small-diameter peptidergic neurons (Lundberg et al., 1985; Lee et al., 1985; Hokfelt et al., 1975; Cuello et al., 1978; Skofitsch and Jacobowitz, 1985), the regulation of α -CGRP and SP mRNAs by the neurotrophic factors NGF, artemin and MSP appeared to be almost identical (4.8A and C). In accordance with both the neonatal data presented in chapter 3 of this thesis, and (if one views *in-vitro* culture as model of nerve injury) previously published data from *in-vitro* and *in-vivo* experiments using adult rodents, both mRNAs were down-regulated over time in culture (Noguchi et al., 1990; Mulder et al., 1997; Sterne et al., 1998; Shi et al., 2001; Shadiack et al., 2001; Nielsch et al., 1987; Zhang et al., 1995). In the case of α -CGRP mRNA, the reduction in expression after 96 hours in culture was around 3-fold, whereas for SP mRNA the reduction in expression after 96 hours in culture was almost 8-fold. Artemin, MSP and NGF all acted to significantly attenuate the decreases in α -CGRP and SP mRNA expression in cultured adult DRG neurons. All three neurotrophic factors appeared to have a similar efficacy in promoting the transcriptional expression of α -CGRP, effectively doubling the levels of α -CGRP mRNA expressed by adult DRG neurons after 96 hours in culture compared to control cultures. Artemin appeared to be the most effective neurotrophic factor at regulating SP mRNA expression. 10ng/ml artemin could increase the levels of SP mRNA expressed by DRG neurons that have

been in culture for 96 hours by 6-fold compared to the levels of this neuropeptide mRNA in control cultures. 10ng/ml NGF and 50ng/ml MSP were only able to increase SP mRNA levels by 3-fold compared to control cultures.

The levels of β -CGRP mRNA expressed by adult DRG neurons also decreased over time in culture, although the decrease, only being 2-fold, is not as dramatic as that of α -CGRP mRNA (figure 4.8B). NGF, at 10ng/ml, entirely prevented the decrease in β -CGRP mRNA levels. Whilst MSP and artemin had a tendency to increase the levels of this neuropeptide mRNA compared to controls after 96 hours in culture, the increases in β -CGRP mRNA levels were not statistically significant. However, in a second series of experiments, carried out to produce the “additive” data that is presented below, both MSP and artemin were able to significantly enhance the levels of β -CGRP mRNA in DRG cultures compared to control cultures (figure 4.14 B).

The two TTX-R sodium channel mRNAs showed a dramatic 10-fold decrease in neuronal expression following 96 hours in culture in the absence of neurotrophic factor support (figure 4.9 A and B). This decrease in expression levels reflects the down-regulation previously observed within small-diameter neurons of the DRG following peripheral nerve damage (Okuse et al., 1997; Cummins et al., 1997; Tate et al., 1998; Dib-hajj et al., 1998; novakovic et al., 1998 Sleeper et al., 2000; Decosterd et al., 2002). The addition of artemin, MSP or NGF significantly increased the expression of both mRNAs compared to control cultures. However, these increases in the expression of both mRNAs, compared to control cultures, was only of the order of 2-fold for all the neurotrophic factors. This would suggest that other neurotrophic factors are involved, in addition to artemin, MSP and NGF, in regulating the expression of these TTX-resistant sodium channels within adult sensory neurons. This is discussed more fully below.

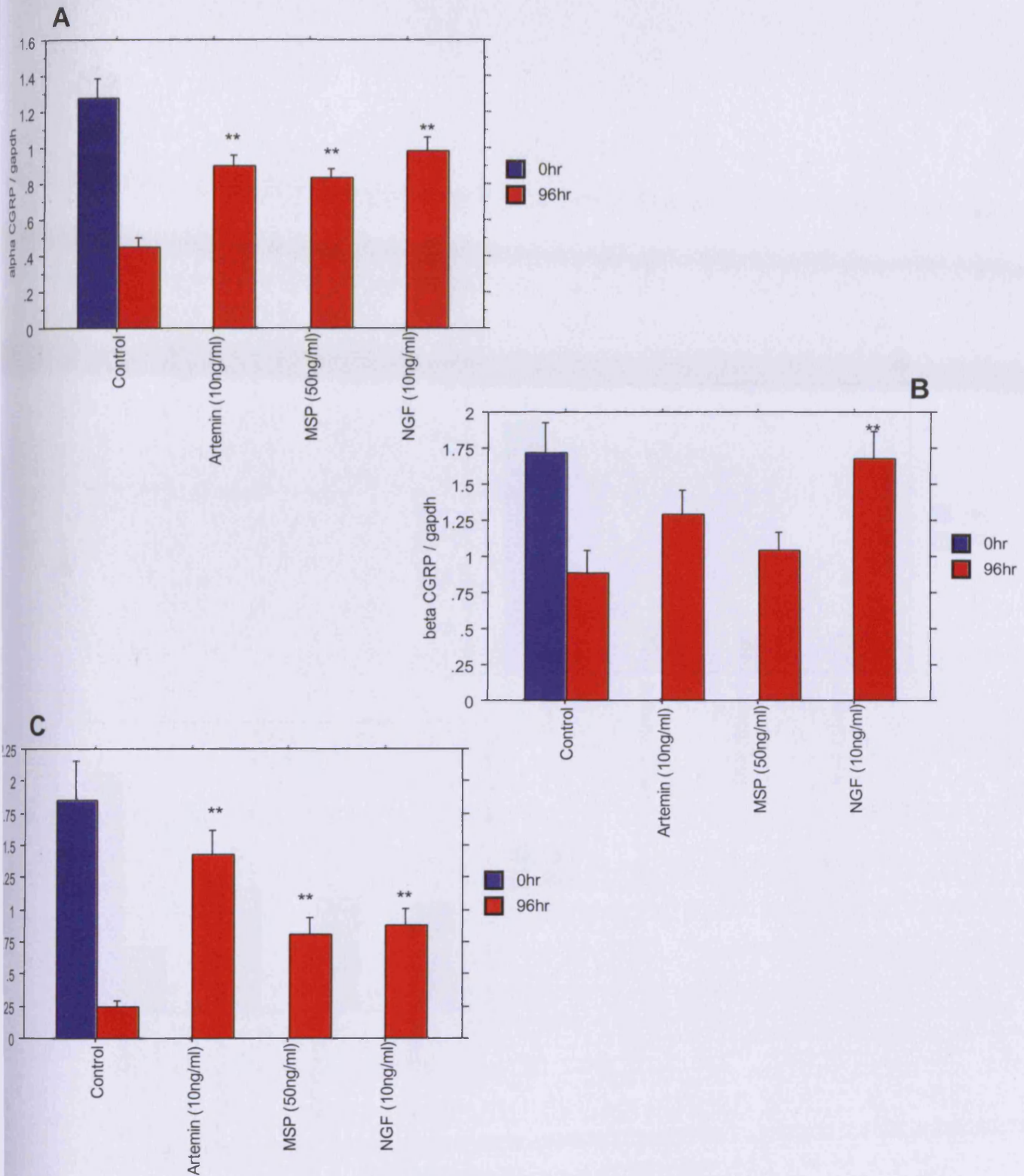


Figure 4.8 Expression of alpha CGRP, beta-CGRP and SP mRNAs in adult DRG cultured in the presence of artemin, MSP or NGF

Adult mouse DRG were cultured for 96 hours in the presence of artemin (10ng/ml), MSP (50ng/ml) or NGF (10ng/ml). Cultures with no factors were included as a control for comparison. Expression of alpha-CGRP(A), beta-CGRP (B) and SP (C) mRNAs were quantified using real-time QPCR. Error bars = +/- standard error, n = 15-20. Significant differences to control 96 hours are illustrated. * = $p < 0.05$, ** = $p < 0.01$ as determined by two tailed t-test.

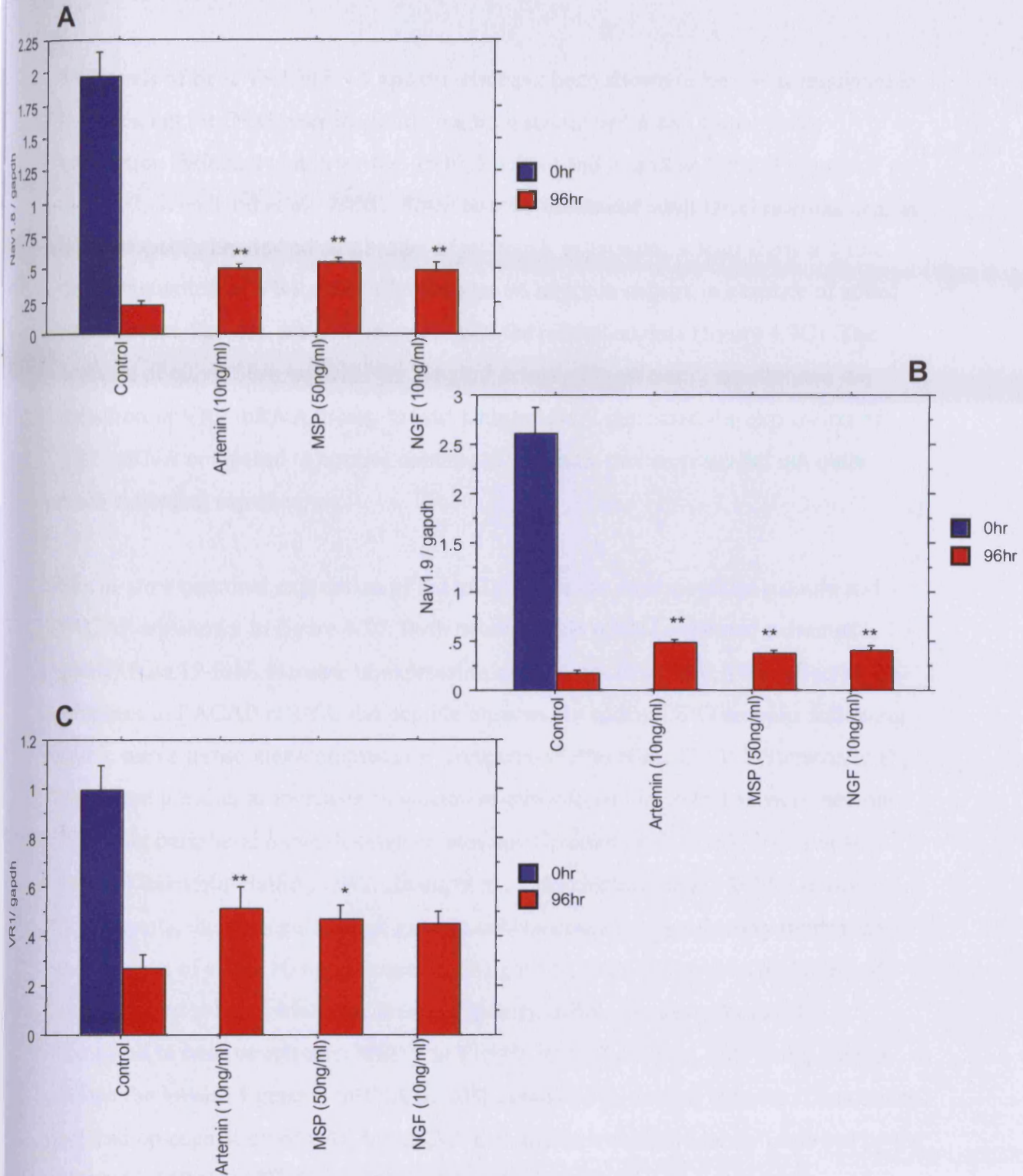


Figure 4.9. Expression of *Nav1.8*, *Nav1.9* and *VR1* mRNAs in adult DRG cultured in the presence of artemin, MSP or NGF

Adult mouse DRG were cultured for 96 hours in the presence of artemin (10ng/ml), MSP (50ng/ml) or NGF (10ng/ml). Cultures with no factors were included as a control for comparison. Expression of *Nav1.8* (A), *Nav1.9* (B) and *VR1* (C) mRNAs were quantified using real-time QPCR. Error bars = +/- standard error, n = 15-20. Significant differences to control 96 hours are illustrated. * = $p < 0.05$, ** = $p < 0.01$ as determined by two tailed t-test.

The levels of both VR1 mRNA and protein have been shown to be down-regulated in injured adult rat DRG neurons following both sciatic nerve and spinal nerve transection (Michael and Priestley, 1999; Michael and Priestley, 2002; Fukuoka T et al., 2002; Wendland et al., 2003). Since *in-vitro* culture of adult DRG neurons can, in many respects, be viewed as a model of axotomy, my results, which show a 4-fold down-regulation of VR1 mRNA levels over 96 hours in culture in absence of added neurotrophic factors, are in agreement with the published data (figure 4.9C). The addition of either 50ng/ml MSP or 10ng/ml artemin significantly ameliorated the reduction in VR1 mRNA levels. Whilst 10ng/ml NGF increased the expression of VR1 mRNA compared to control cultures at 96 hours, this increase did not quite reach statistical significance.

The *in-vitro* neuronal expression of the mRNAs for the neuropeptides galanin and PACAP are shown in figure 4.10. Both neuropeptide mRNAs showed a dramatic, greater than 15-fold, increase in expression over 96 hrs in culture. This reflected the increases in PACAP mRNA and peptide observed in rodent DRG neurons following sciatic nerve transection/compression (Jongsma-Wallin et al., 2001; Pettersson et al., 2004) and the similar increases in galanin expression within rodent sensory neurons following peripheral nerve damage or axotomy (Hokfelt et al., 1987; Villar et al., 1989; Wiesenfeld-Hallin., 1992; Zhang et al., 1998; Holmes et al., 2005). In my experiments, the up-regulation of galanin mRNA could be significantly inhibited by the addition of either 10 ng/ml artemin, 50 ng/ml MSP or 10ng/ml NGF. NGF and artemin were able to reduce the levels of galanin mRNA by as much as 60% compared to control cultures. MSP was slightly less efficacious, only being able to reduce the levels of galanin mRNA by 40% compared to control cultures. The culture-induced up-regulation of PACAP mRNA expression was significantly inhibited by the addition of 10ng/ml NGF to cultures. The addition of either 50 ng/ml MSP or 10 ng/ml artemin to DRG cultures also ameliorated some of the increase in PACAP mRNA levels observed in control cultures, although the effect did not reach statistical significance.

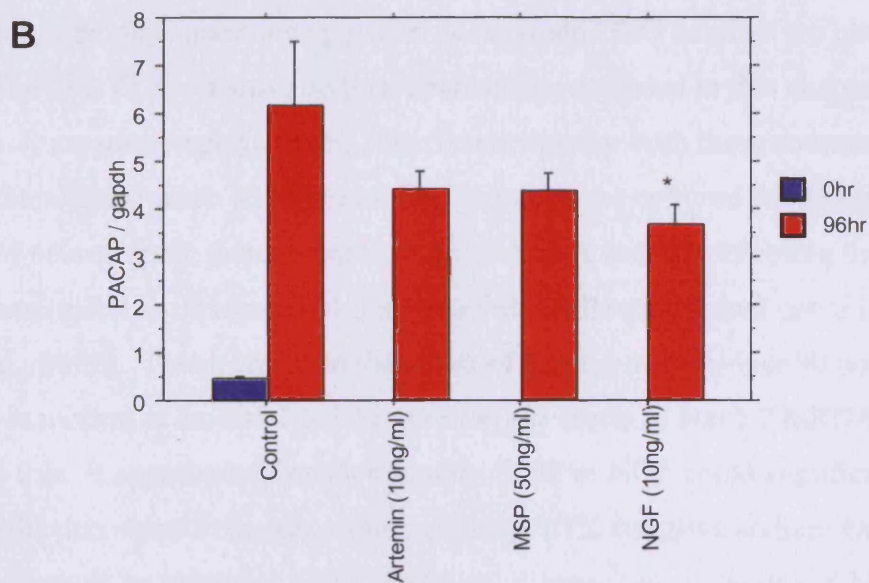
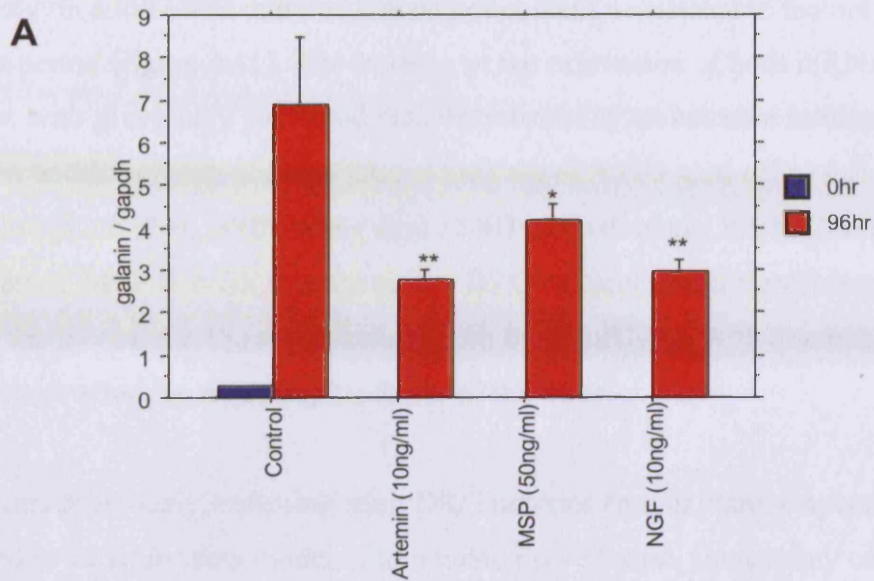


Figure 4.10. Expression of galanin and PACAP mRNAs in adult DRG cultured in the presence of artemin, MSP or NGF

Adult mouse DRG were cultured for 96 hours in the presence of artemin (10ng/ml), MSP (50ng/ml) or NGF (10ng/ml). Cultures with no factors were included as a control for comparison. Expression of galanin (A) and PACAP (B) mRNAs were quantified using real-time QPCR. Error bars = +/- standard error, n = 15-20. Significant differences to control 96 hours are illustrated. * = $p < 0.05$, ** = $p < 0.01$ as determined by two tailed t-test.

ATF3 and DINE mRNAs were also significantly up-regulated, by 3- and 50-fold, respectively, in adult DRG cultures that do not contain neurotrophic factors over a 96 hour time period (figure 4.11). The increase in the expression of both mRNAs is in agreement with previously published data demonstrating an increase in their expression within sensory neurons (and in the case of ATF3 glial cells) following nerve lesion (Kato et al, 2002; Wang et al., 2003; Averill et al., 2004). The addition of either artemin, MSP or NGF to adult mouse DRG cultures could significantly attenuate the increase in the expression of both these mRNAs, with artemin appearing to be the most effective neurotrophic factor of the three.

As discussed previously, culturing adult DRG neurons can, in many respects, be considered to be an *in-vitro* model of peripheral nerve lesion, since many of the changes in neuronal gene expression that occur following sciatic or spinal nerve constriction/ligation/transection appear to occur when DRG neurons are placed in culture. The two TTX-sensitive sodium channels investigated in this chapter, Nav1.6 and Nav1.7, are no exception to this rule. Transcripts for both these sodium channels decreased markedly when adult mouse DRG neurons are cultured for 96 hours in the absence of neurotrophic factor support (figures 4.12 A and B), reflecting the decrease in both these mRNAs observed in rat lumbar DRG following spinal nerve ligation (Kim et al., 2002). The decrease in the levels of Nav1.6 mRNA over 96 hours in culture was modest at around 3.5-fold, whereas the levels of Nav1.7 mRNA decreased nearly 10-fold. It appears as if neither artemin, MSP or NGF could significantly attenuate the decreases in the expression of either TTX-sensitive sodium channel mRNA, although, as observed in the “additive” cultures below, NGF and MSP appeared to have a tendency to slightly increase the expression of both mRNAs.

Perhaps surprisingly, P2X3 mRNA expression did not appear to significantly change when adult DRG neurons are placed in culture for 96 hours (figure 4.12 C) This data contradicts the previously published observation that P2X3 mRNA levels decrease significantly in damaged, ATF 3-positive, sensory neurons following nerve transection (Tsuzuki et al., 2001). Neither MSP nor NGF significantly altered the expression levels of P2X3 mRNA over the 96 hour culture period. Whilst the addition

of 10ng/ml artemin appeared to significantly decrease the expression of P2X3 mRNA in adult DRG neuron cultures, the decrease was small and inconsistent, not being apparent in later tissue culture experiments (see 4.3.6 and 4.3.7).

Figure 4.13 shows the expression of GAPDH mRNA within adult DRG cultures after 96 hours. In accordance with the low amount of neuronal death observed in adult mouse DRG cultures (figure 4.3), GAPDH mRNA levels did not markedly change in control cultures compared to those at time 0. The addition of either 10ng/ml artemin, 10ng/ml NGF or 50ng/ml MSP to cultures did not significantly alter the expression of GAPDH mRNA compared to control cultures.

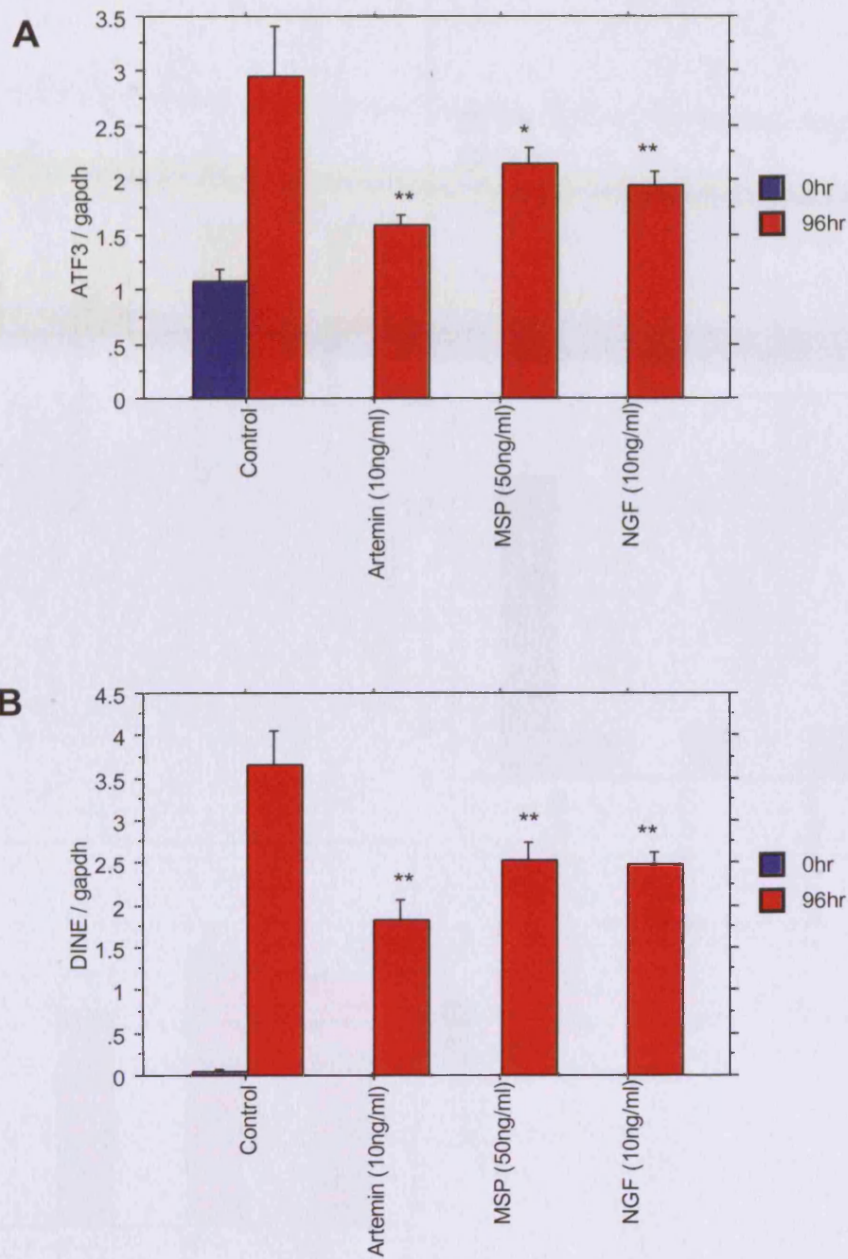


Figure 4.11. Expression of *ATF3* and *DINE* mRNAs in adult DRG cultured in the presence of artemin, MSP or NGF

Adult mouse DRG were cultured for 96 hours in the presence of artemin (10ng/ml), MSP (50ng/ml) or NGF (10ng/ml). Cultures with no factors were included as a control for comparison. Expression of *ATF3* (A) and *DINE* (B) mRNAs were quantified using real-time QPCR. Error bars = +/- standard error, n = 15-20.

Significant differences to control 96 hours are illustrated. * = $p < 0.05$, ** = $p < 0.01$ as determined by two tailed t-test.

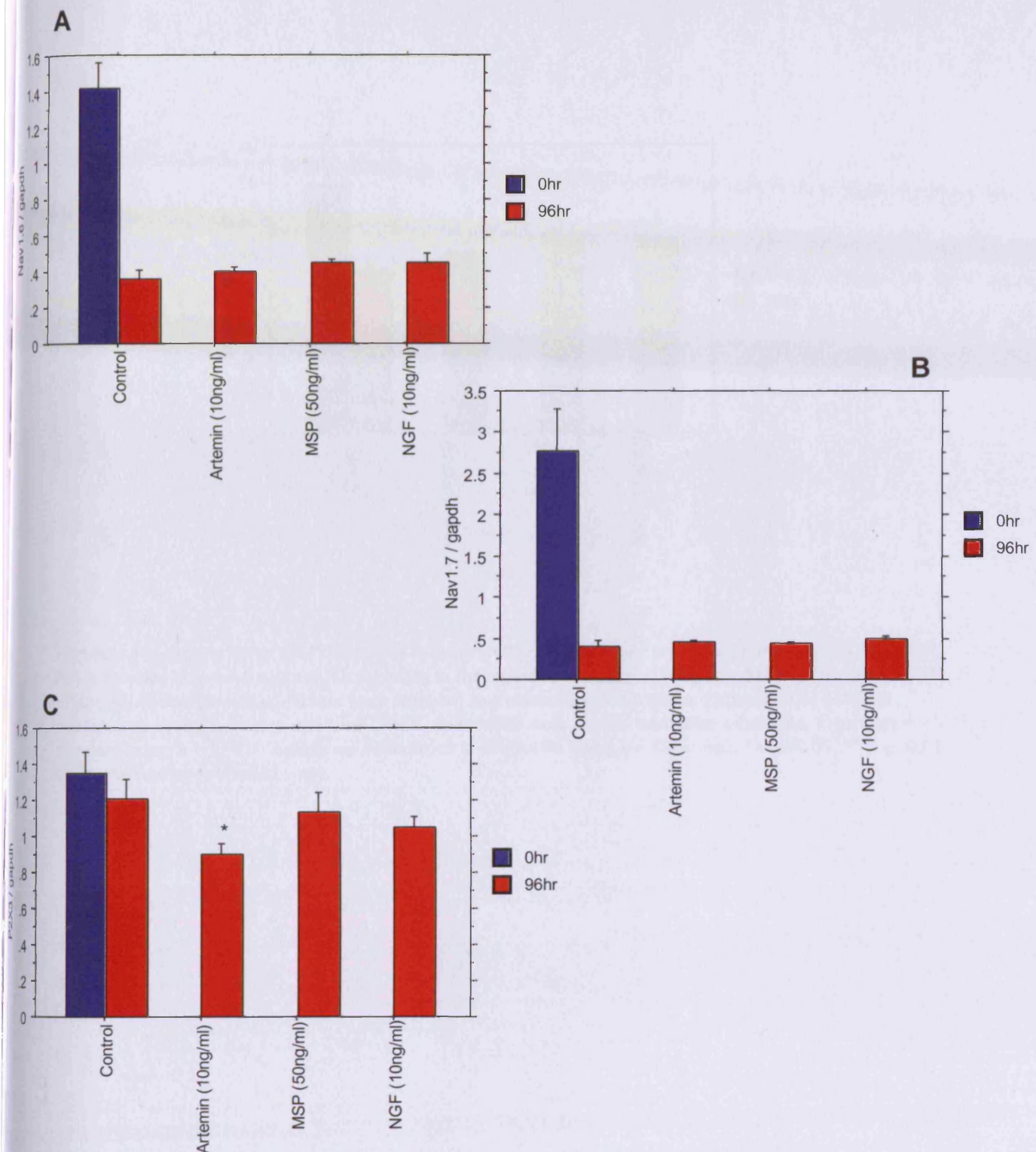


Figure 4.12 Expression of Nav1.6, Nav1.7 and P2X3 mRNAs in adult DRG cultured in the presence of artemin, MSP or NGF

Adult mouse DRG were cultured for 96 hours in the presence of artemin (10ng/ml), MSP (50ng/ml) or NGF (10ng/ml). Cultures with no factors were included as a control for comparison. Expression of Nav1.6 (A), Nav1.7 (B) and P2X3 (C) mRNAs were quantified using real-time QPCR. Error bars = +/- standard error, n = 15-20. Significant differences to control 96 hours are illustrated. * = $p < 0.05$, ** = $p < 0.01$ as determined by two tailed t-test.

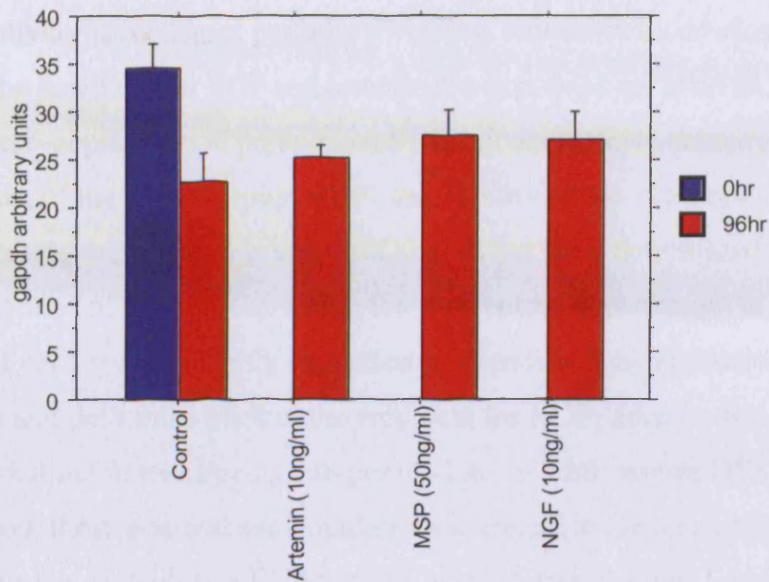


Figure 4.13. Expression of GAPDH mRNA in adult DRG cultured in the presence of artemin, MSP or NGF. Adult mouse DRG were cultured for 96 hours in the presence of artemin (10ng/ml), MSP (50ng/ml) or NGF (10ng/ml). Cultures with no factors were included as a control for comparison. Expression of GAPDH mRNA was quantified using real-time QPCR, and results were used to normalise other data. Error bars = +/- standard error, n = 15-20. Significant differences to control 96 hours are illustrated. * = $p < 0.05$, ** = $p < 0.01$ as determined by two tailed t-test.

4.3.6. Additive experiments

In section 4.3.5. I have demonstrated that saturating levels of NGF, artemin and MSP can modulate the expression of a number of mRNAs in cultured adult mouse DRG neurons, effectively reversing, or partially reversing, culture-induced changes in gene expression. The receptors for NGF and artemin are expressed on specific, partially overlapping, sub-populations of predominantly small nociceptive sensory neurons *in-vivo*. In the case of the MSP receptor, RON, the identity of the sub-population of neurons expressing this receptor in the adult has not yet been determined. Although the sub-populations of neurons expressing these receptors may change in culture, it is still likely that each receptor is only expressed in a specific sub-population of neurons. In order to try and determine whether the receptors for NGF, artemin and MSP are expressed on distinct or overlapping sub-populations of adult mouse DRG neurons in culture, and how these neuronal sub-populations correlate to the sub-populations of neurons expressing each of the mRNAs investigated in this chapter, I carried out a series of “additive” tissue culture experiments where neurons were cultured either without neurotrophic factors, with a single neurotrophic factor or with combinations of two or three neurotrophic factors. The rationale behind this is that one may expect the effects of two neurotrophic factors on the expression of any one mRNA to be partially additive if the sub-populations of neurons expressing receptors for these neurotrophic factors, in addition to the mRNA in question, are not entirely overlapping. Conversely, if two neurotrophic factors do not produce an additive effect on regulating mRNA expression, it is likely that the majority of neurons responding to any one neurotrophic factor, and also expressing the mRNA of interest, contains receptors for the other neurotrophic factor i.e. the two sub-populations that express the mRNA of interest and respond to each neurotrophic factor are virtually entirely overlapping. This rationale is based on two assumptions. First, that each neurotrophic factor is being used at a saturating concentration for effects on modulating the transcriptional expression of the analysed genes. Second, that the intracellular signal transduction pathways that lead to an increase in transcriptional expression of the analysed genes are similar for each neurotrophic factor. Therefore, if signalling pathways are maximally activated with saturating concentrations of one neurotrophic

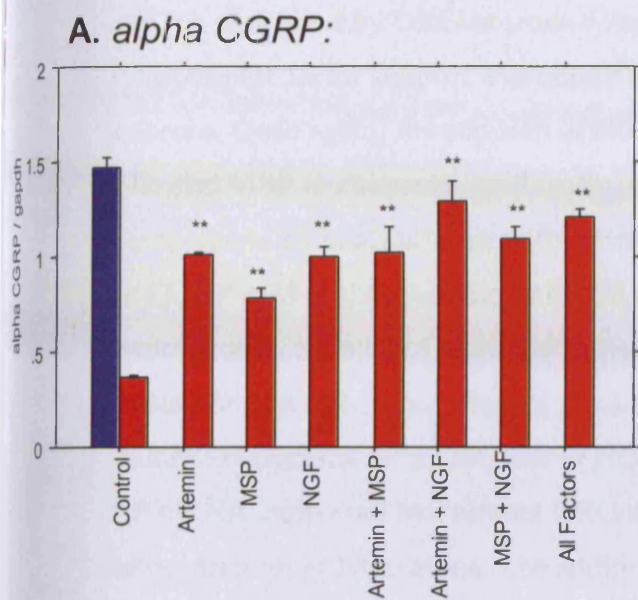
factor within any one cell, the addition of a second trophic factor should not increase transcription of the gene in question within the same cell.

The results of the “additive” experiments are presented in figures 4.14 - 4.21.

Determining the statistical significance of the data is quite complex due to the number of conditions under test, thus requiring ANOVA statistical analysis of data sets.

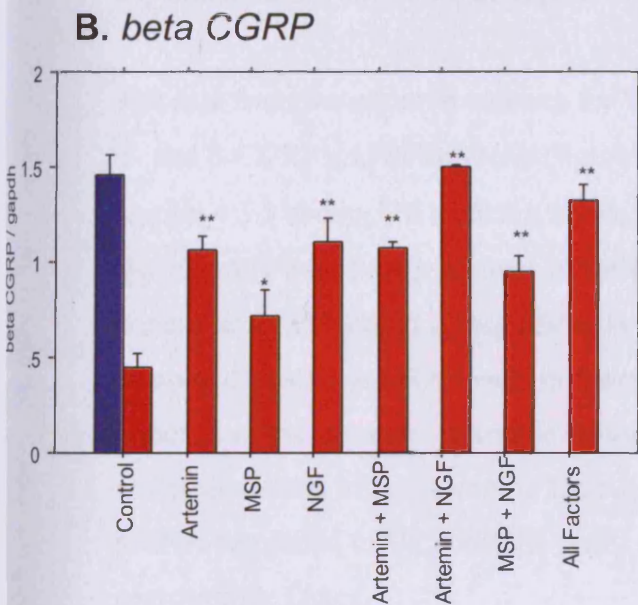
Significant differences in the expression of each mRNA in comparison to ‘control cultures’ at 96hours are highlighted by * ($p < 0.05$) or ** ($p < 0.01$) above the relevant bar. To prevent graphs from becoming crowded and over-complicated, other relevant comparisons are shown in tables C and D of each figure.

The expression of α -CGRP and β -CGRP mRNAs for the series of cultures that provided data for the “additive” experiments is shown in figure 4.14A and B. The data for both CGRP mRNAs are very similar. A 3- to 4-fold down-regulation of CGRP mRNAs was observed over time in control cultures, which was significantly reversed by the addition of either artemin, MSP or NGF. The addition of both 10ng/ml artemin and 10ng/ml NGF to cultures produced significantly higher levels of α -CGRP and β -CGRP mRNAs than the addition of either artemin or NGF alone, restoring both mRNAs to levels found *in-vivo*. The partially additive effects of NGF and artemin on promoting α - and β -CGRP mRNA expression suggests that these two factors act on two partially overlapping sub-populations of peptidergic DRG neurons. This in turn suggests that a sub-population of DRG neurons exist, at least in culture, that are peptidergic but do not express TrkA, rather they express the artemin receptor, GFR- $\alpha 3$. The addition of 50ng/ml MSP to cultures containing either 10ng/ml artemin or 10ng/ml NGF did not significantly increase α - or β -CGRP mRNA levels above those found in cultures containing either NGF alone or artemin alone, suggesting that few sensory neurons express the MSP receptor RON in the absence of either TrkA or GFR- $\alpha 3$. Similarly, the addition of MSP to cultures containing both artemin and NGF did not increase α - or β -CGRP mRNA levels above those in cultures containing just NGF and artemin.



C. alpha CGRP:

	P value	Significance
Art, art + MSP	0.8734	
Art, art + NGF	0.0165	*
Art, all factors	0.0802	
MSP, art + MSP	0.0255	*
MSP, MSP + NGF	0.0053	**
MSP, all factors	0.0003	**
NGF, art + NGF	0.0095	**
NGF, MSP + NGF	0.3765	
NGF, all factors	0.0563	



D. beta CGRP:

	P value	Significance
Art, art + MSP	0.9317	
Art, art + NGF	0.0018	**
Art, all factors	0.0459	*
MSP, art + MSP	0.0164	*
MSP, MSP + NGF	0.0879	
MSP, all factors	<0.0001	**
NGF, art + NGF	0.0045	**
NGF, MSP + NGF	0.3084	
NGF, all factors	0.0957	

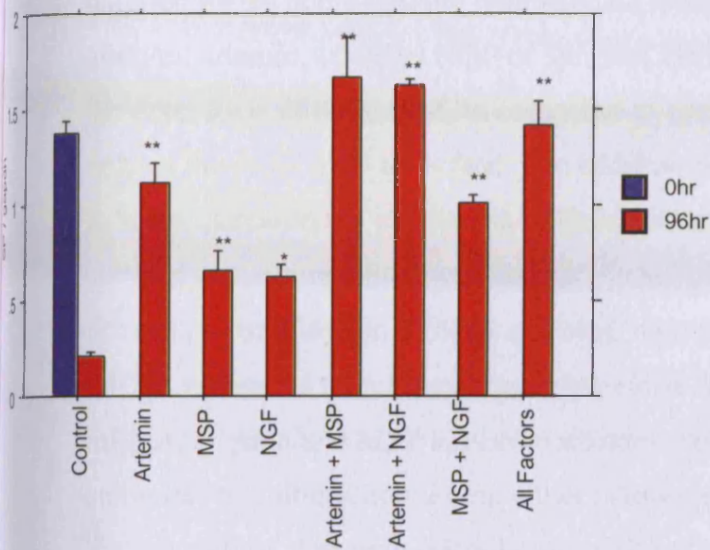
Figure 4.14. Additive effects of artemin, MSP and NGF on expression of alpha CGRP and beta CGRP mRNAs in adult mouse cultured DRG

Adult mouse DRG were cultured in the presence or absence of artemin (10ng/ml), NGF (10ng/ml), MSP (50ng/ml) or a combination of factors, for 96 hours. Expression of alpha CGRP (A) and beta CGRP (B) mRNAs were quantified using real-time Q-PCR. ANOVA statistical analyses were carried out. Significant differences to expression at 'control 96 hours' are indicated directly above bars, other relevant comparisons are outlined in C (alpha CGRP) and D (Beta CGRP). * = $p < 0.05$ ** = $p < 0.01$. Error bars = +/- standard error, n = 4

The “additive” experimental data for SP mRNA is shown in figure 4.15 A. Once again, in accordance with the data presented in section 4.3.5 above, the level of SP mRNA expressed by DRG neurons cultured for 96 hours in the absence of neurotrophic factor support was dramatically lower than in freshly dissociated neurons. Once again, the addition of either 10ng/ml artemin, 10ng/ml NGF or 50ng/ml MSP to cultures significantly increased the expression of SP mRNA compared to control cultures, with artemin having the greatest efficacy. As in the case of CGRP mRNAs, the addition of NGF and artemin to cultures increased the expression of SP mRNA to levels above that found in cultures with either factor alone. Interestingly, in contrast to the data for CGRP mRNAs, the addition of MSP to cultures containing either artemin or NGF alone significantly increased the levels of SP mRNA expressed by cultured DRG neurons to levels above those found with either artemin or NGF alone. The addition of all three neurotrophic factors to DRG cultures did not increase the expression of SP mRNA compared to cultures containing any combination of two neurotrophic factors.

The data from the additive cultures for VR1 mRNA is markedly different to that for α - and β -CGRP and SP mRNAs (figure 4.15 B). In agreement with the data from section 4.3.5 above, VR1 mRNA levels within adult mouse DRG neurons decreased significantly over time in culture in the absence of added neurotrophic factors. NGF, artemin and MSP could all significantly enhance the expression of VR1 mRNA compared to VR1 mRNA levels in control cultures, with NGF being the least effective of the three neurotrophic factors. However, unlike SP and CGRP mRNAs, no combinations of neurotrophic factors significantly increased the expression of VR1 mRNA compared to VR1 mRNA levels observed in cultures containing a single neurotrophic factor.

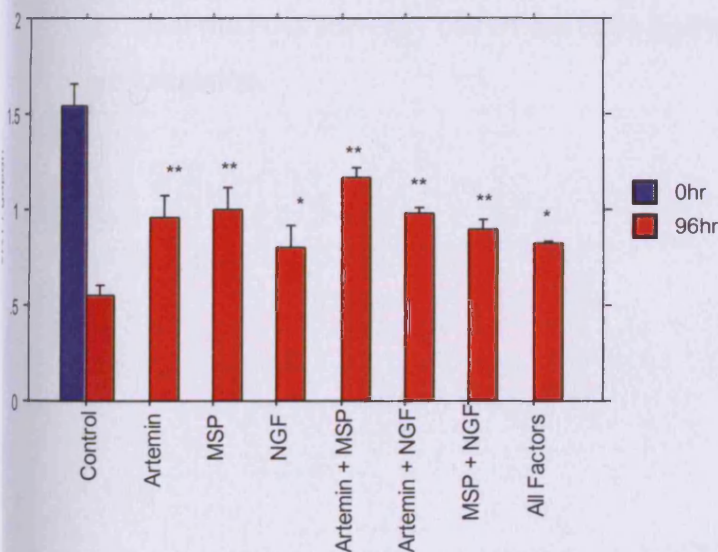
A. SP:



C. SP:

	P value	Significance
Art, art + MSP	0.0020	**
Art, art + NGF	0.0057	**
Art, all factors	0.0638	
MSP, art + MSP	<0.0001	**
MSP, MSP + NGF	0.0526	
MSP, all factors	<0.0001	**
NGF, art + NGF	<0.0001	**
NGF, MSP + NGF	0.0356	*
NGF, all factors	<0.0001	**

B. VR1:



D. VR1:

	P value	Significance
Art, art + MSP	0.0789	
Art, art + NGF	0.8559	
Art, all factors	0.2192	
MSP, art + MSP	0.1613	
MSP, MSP + NGF	0.3122	
MSP, all factors	0.1116	
NGF, art + NGF	0.1116	
NGF, MSP + NGF	0.4170	
NGF, all factors	0.8382	

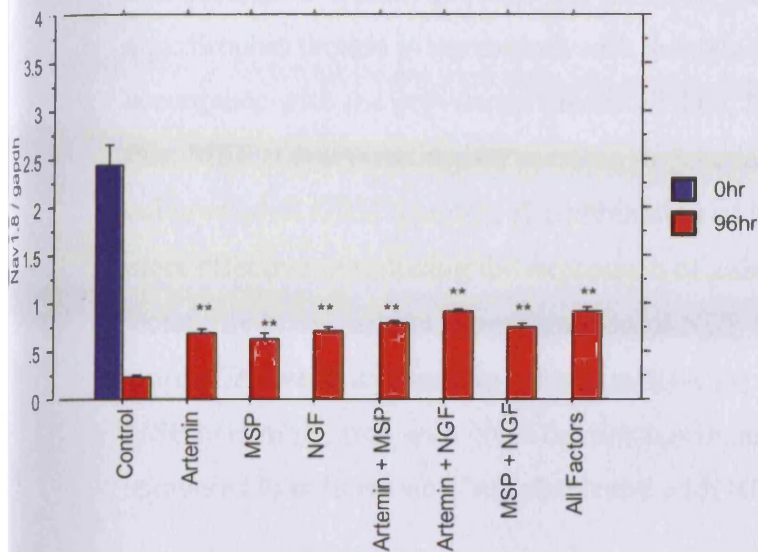
Figure 4.15. Additive effects of artemin, MSP and NGF on expression of SP and VR1 mRNAs in adult mouse cultured DRG

Adult mouse DRG were cultured in the presence or absence of artemin (10ng/ml), NGF (10ng/ml), MSP (50ng/ml) or a combination of factors, for 96 hours. Expression of SP (A) and VR1 (B) mRNAs were quantified using real-time Q-PCR. ANOVA statistical analyses were carried out. Significant differences to expression at 'control 96 hours' are indicated directly above bars, other relevant comparisons are outlined in C (SP) and D (VR1). * = $p < 0.05$ ** = $p < 0.01$.

Error bars = +/- standard error, n = 4

The “additive” culture data for the two TTX-resistant sodium channels Nav1.8 and Nav1.9 are similar, but not identical (figure 4.16 A and B). A greater than 10-fold decrease in the expression of both mRNAs was apparent in DRG neurons that were cultured for 96 hours without neurotrophic factor support. The addition of either 10ng/ml artemin, 10ng/ml NGF or 50ng/ml MSP to cultures significantly increased the expression of both mRNAs compared to control cultures, although increases are only of the order of 2- to 3- fold. The addition of both NGF and artemin to control cultures increased the expression of both mRNAs to higher levels than those found in cultures containing either neurotrophic factor alone in a partially additive way. In contrast, a combination of MSP and NGF did not increase the expression of either mRNA compared to cultures containing either factor alone. In the case of Nav1.9 mRNA, artemin and MSP appear to enhance expression in a partially additive way compared to cultures containing either neurotrophic factor. This is not the case for Nav1.8 mRNA. For both mRNAs, the addition of all three neurotrophic factors to cultures did not increase the levels of mRNA expressed compared to cultures containing combinations of any two of the neurotrophic factors, suggesting that very few neurons exist in adult mouse DRG cultures that express TTX-resistant sodium channel mRNAs and only one of the three neurotrophic factor receptors under investigation.

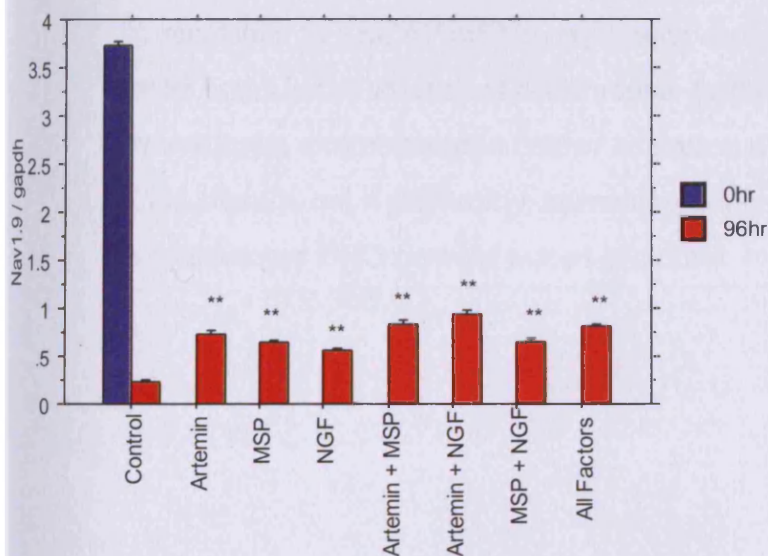
A. Nav1.8:



C. Nav1.8:

Treatment	P value	Significance
Art, art + MSP	0.1322	
Art, art + NGF	0.0014	**
Art, all factors	0.0015	**
MSP, art + MSP	0.0153	*
MSP, MSP + NGF	0.0683	
MSP, all factors	0.0001	**
NGF, art + NGF	0.0032	**
NGF, MSP + NGF	0.6171	
NGF, all factors	0.0035	**

B. Nav1.9:



D. Nav1.9:

Treatment	P value	Significance
Art, art + MSP	0.0225	*
Art, art + NGF	0.0001	**
Art, all factors	0.0863	
MSP, art + MSP	0.0003	**
MSP, MSP + NGF	0.9572	
MSP, all factors	0.0015	**
NGF, art + NGF	<0.0001	**
NGF, MSP + NGF	0.0628	
NGF, all factors	<0.0001	**

Figure 4.16. Additive effects of artemin, MSP and NGF on expression of Nav1.8 and Nav1.9 mRNAs in adult mouse cultured DRG

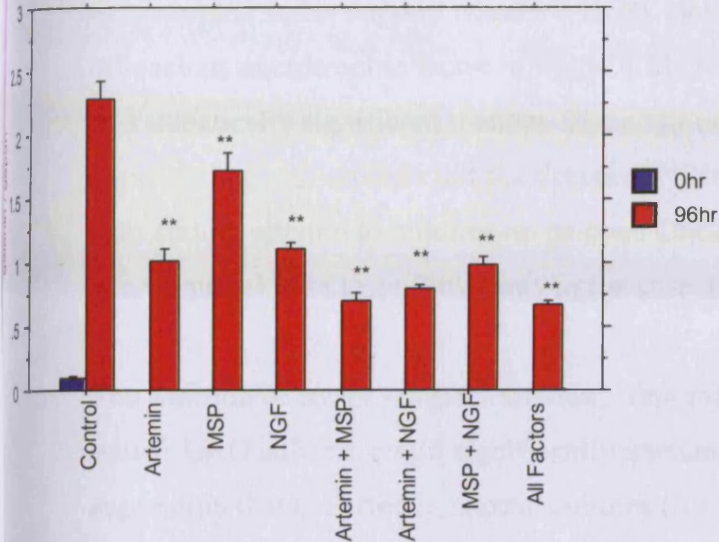
Adult mouse DRG were cultured in the presence or absence of artemin (10ng/ml), NGF (10ng/ml), MSP (50ng/ml) or a combination of these factors, for 96 hours. Expression of Nav1.8 (A) and Nav1.9 (B) mRNAs were quantified using real-time Q-PCR. ANOVA statistical analyses were carried out. Significant differences to expression at 'control 96 hours' are indicated directly above bars, other relevant comparisons are outlined in C (Scn10a) and D (Scn11a). * = $p < 0.05$ ** = $p < 0.01$.

Error bars = +/- standard error, n = 4

The regulation of galanin and PACAP mRNA expression in cultured adult mouse DRG neurons is shown in figure 4.17. The 10-fold up-regulation of galanin mRNA that occurred in control cultures was partially, but significantly, inhibited by all neurotrophic factors in agreement with the data presented in figure 4.10A. In accordance with the previously presented data, NGF and artemin were more effective than MSP at ameliorating the increase in galanin mRNA expression that occurred in cultured adult DRG neurons. A combination of MSP and artemin was significantly more effective in reducing the expression of galanin mRNA than either neurotrophic factor alone. In contrast, a combination of NGF and artemin was not significantly more effective at attenuating galanin mRNA expression than artemin alone. Similarly MSP in combination with NGF did not significantly reduce galanin mRNA levels compared to cultures only supplemented with NGF.

Neither NGF, MSP nor artemin could significantly affect the approximately 5-fold up-regulation in PACAP mRNA expression observed in adult DRG neurons cultured for 96 hours in the absence of neurotrophic factor support (figure 4.17B). Interestingly, a combination of either artemin and MSP or NGF together with MSP could slightly, but significantly, attenuate the increase in PACAP mRNA that occurs in adult mouse DRG neurons placed in culture.

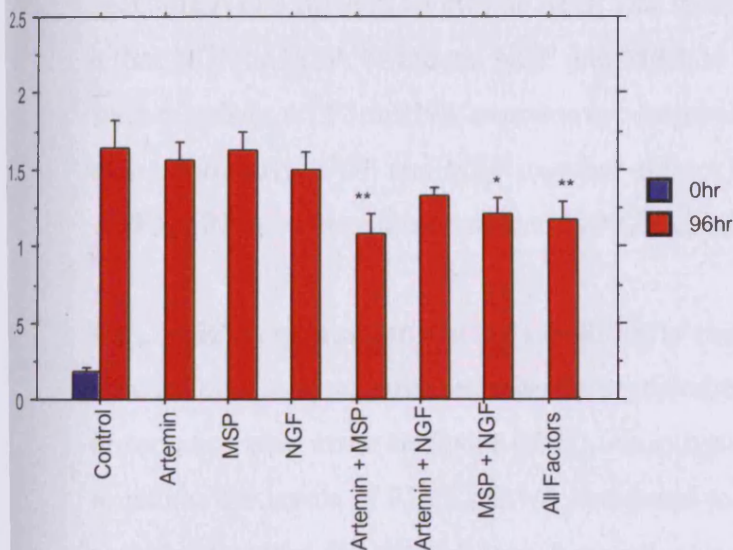
A. galanin:



C. galanin:

	P value	Significance
Art, art + MSP	0.0199	*
Art, art + NGF	0.0910	
Art, all factors	0.0105	*
MSP, art + MSP	<0.0001	**
MSP, MSP + NGF	<0.0001	**
MSP, all factors	<0.0001	**
NGF, art + NGF	0.0166	*
NGF, MSP + NGF	0.2970	
NGF, all factors	0.0015	**

B. PACAP:



D. PACAP:

	P value	Significance
Art, art + MSP	0.0084	**
Art, art + NGF	0.1707	
Art, all factors	0.0286	*
MSP, art + MSP	0.0037	**
MSP, MSP + NGF	0.0237	*
MSP, all factors	0.0134	*
NGF, art + NGF	0.3535	
NGF, MSP + NGF	0.1317	
NGF, all factors	0.0851	

Figure 4.17. Additive effects of artemin, MSP and NGF on expression of galanin and PACAP mRNAs in adult mouse cultured DRG

Adult mouse DRG were cultured in the presence or absence of artemin (10ng/ml), NGF (10ng/ml), MSP (50ng/ml) or a combination of these factors, for 96 hours. Expression of galanin (A) and PACAP (B) mRNAs were quantified using real-time Q-PCR. ANOVA statistical analyses were carried out. Significant differences to expression at 'control 96 hours' are indicated directly above bars, other relevant comparisons are outlined in C (galanin) and D (PACAP). * = $p < 0.05$ ** = $p < 0.01$.

Error bars = +/- standard error, n = 4

In accordance with the data presented in figure 4.11, all three neurotrophic factors could partially ameliorate the 50-fold increase in DINE mRNA expression that occurred in adult mouse DRG neurons cultured for 96 hours in the absence of neurotrophic factor support (figure 4.18 B). However, only artemin, the most efficacious neurotrophic factor in figure 4.11, could reduce DINE mRNA expression in a statistically significant manner. The addition of either MSP or NGF to cultures in combination with artemin did not decrease DINE mRNA levels to a greater extent than adding artemin to cultures on its own. Once again, MSP and NGF did not effect gene expression in an additive way in the case of DINE mRNA.

The addition of either 10ng/ml artemin, 10ng/ml NGF or 50ng/ml MSP to adult mouse DRG cultures could significantly attenuate the up-regulation in ATF3 mRNA expression that occurred in control cultures (figure 4.18A). In agreement with the data presented in figure 4.11A, artemin was the most effective neurotrophic factor in reducing ATF3 mRNA levels and MSP was the least effective. Additionally adding either NGF or MSP, or indeed NGF and MSP, to cultures containing artemin did not further reduce ATF3 mRNA expression compared to cultures containing artemin alone. Similarly, NGF and MSP together did not have an additive effect in reducing ATF3 mRNA expression compared to NGF alone.

P2X3 mRNA expression did not significantly change over time in culture in adult mouse DRG neurons cultured without neurotrophic factor support (figure 4.19A), an observation also made in figure 4.12C. As in figure 4.12C 10ng/ml artemin appeared to reduce the levels of P2X3 mRNA compared to control cultures, although in this case the decrease did not quite reach statistical significance. In fact, no neurotrophic factor or combination of neurotrophic factors significantly altered the expression of P2X3 mRNA in cultured mouse adult DRG neurons

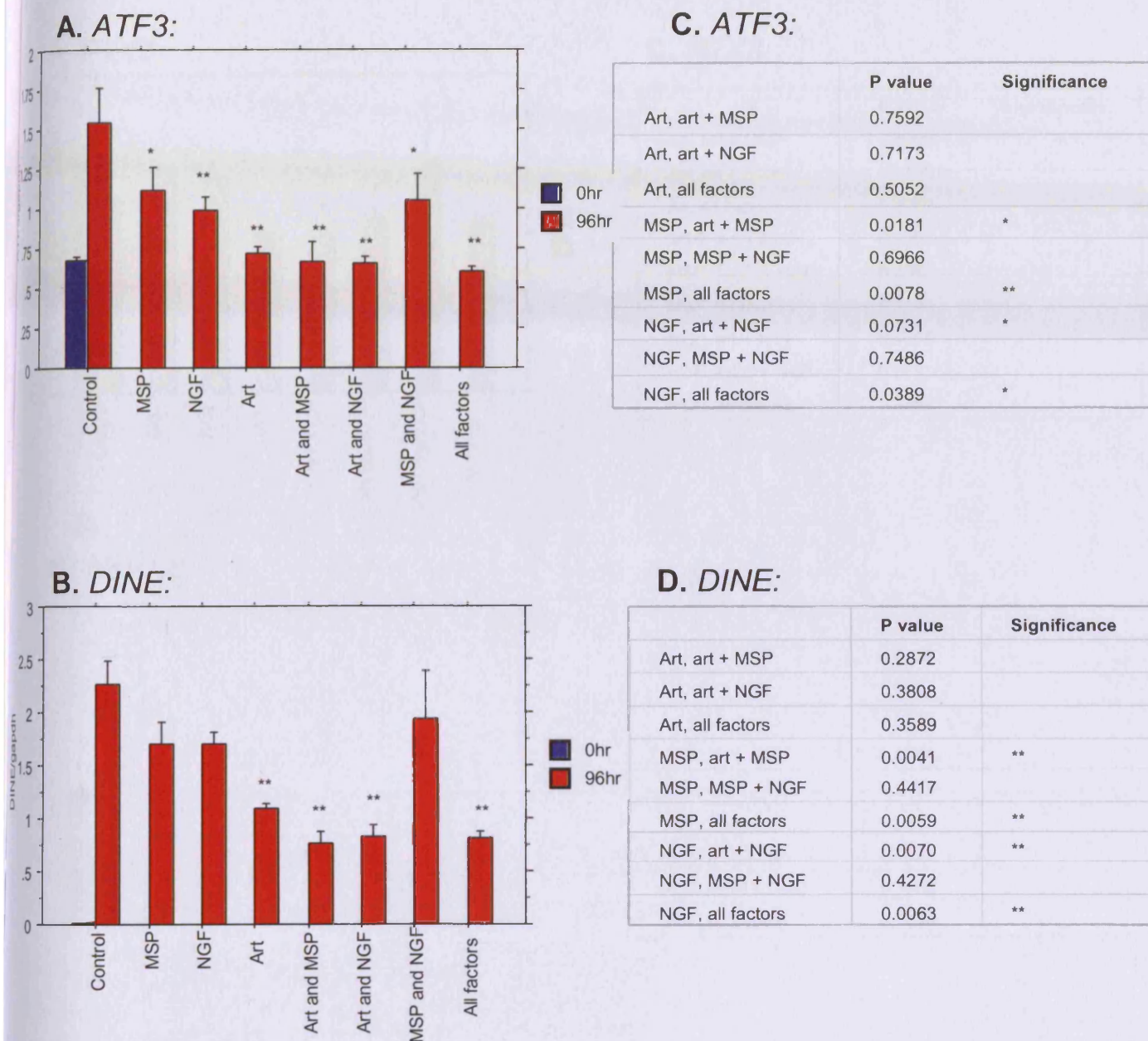
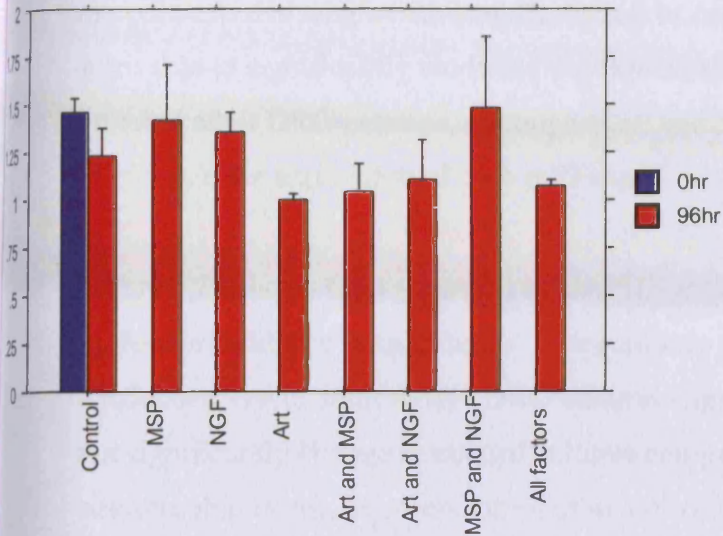


Figure 4. 18 Additive effects of artemin, MSP and NGF on expression of mRNA of genes of interest in adult mouse cultured DRG

Adult mouse DRG were cultured in the presence or absence of artemin (10ng/ml), NGF (10ng/ml), MSP (50ng/ml) or a combination of these factors, for 96 hours. Expression of ATF3 (A), DINE (B) mRNAs were quantified using real-time Q-PCR. ANOVA statistical analyses were carried out. Significant differences to expression at 'control 96 hours' are highlighted directly above bars, other relevant comparisons are outlined in C (ATF3) and D (DINE). * = $p < 0.05$ ** = $p < 0.01$.

Error bars = +/- standard error, n = 4

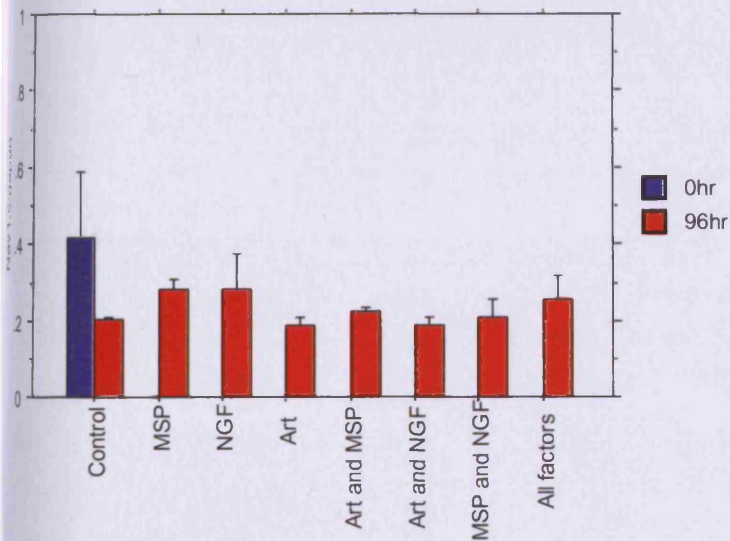
A. P2X3:



C. P2X3:

	P value	Significance
Art, art + MSP	0.8902	
Art, art + NGF	0.7175	
Art, all factors	0.8092	
MSP, art + MSP	0.2066	
MSP, MSP + NGF	0.8360	
MSP, all factors	0.2443	
NGF, art + NGF	0.4015	
NGF, MSP + NGF	0.6731	
NGF, all factors	0.3388	

B. Nav1.6:



D. Nav1.6:

	P value	Significance
Art, art + MSP	0.6238	
Art, art + NGF	0.9704	
Art, all factors	0.3480	
MSP, art + MSP	0.4698	
MSP, MSP + NGF	0.3423	
MSP, all factors	0.7647	
NGF, art + NGF	0.1921	
NGF, MSP + NGF	0.3060	
NGF, all factors	0.7490	

Figure 4.19. Additive effects of artemin, MSP and NGF on expression of P2X3 and Nav1.6 mRNAs in adult mouse cultured DRG

Adult mouse DRG were cultured in the presence or absence of artemin (10ng/ml), NGF (10ng/ml), MSP (50ng/ml) or a combination of these factors, for 96 hours. Expression of P2X3 (A), Nav1.6 (B) mRNAs were quantified using real-time Q-PCR. ANOVA statistical analyses were carried out. Significant differences to expression at 'control 96 hours' are indicated directly above bars, other relevant comparisons are outlined in C (P2X3) and D (Nav1.6).

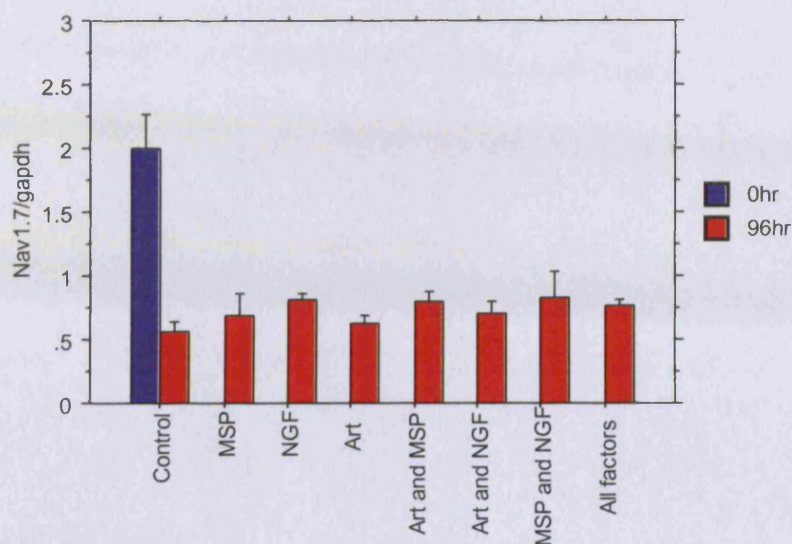
* = $p < 0.05$ ** = $p < 0.01$.

Error bars = +/- standard error, n = 4

The expression of levels of Nav1.6 and Nav1.7 mRNAs (figures 4.19B and 4.20) both decreased over time in control cultures as was observed in figure 4.12A and B. However, in the cultures set up for the “additive” data presented in figures 4.19B and 4.20, the drop in the expression of both mRNAs was attenuated compared to previous experiments. No single neurotrophic factor, or combination of neurotrophic factors, were able to significantly modulate the expression of either sodium channel mRNA in cultured adult DRG neurons, although MSP and NGF did appear to have a tendency to increase the expression of both mRNAs.

Figure 4.21 shows the expression of GAPDH mRNA within the neuronal cultures set up for the “additive” experiments. In accordance with the low amount of neuronal death observed in adult mouse DRG cultures (figure 4.3), GAPDH mRNA levels did not significantly change in control cultures compared to time 0. No single neurotrophic factor, or combination of neurotrophic factors, significantly altered the expression of GAPDH mRNA compared to control cultures.

A. Nav1.7:



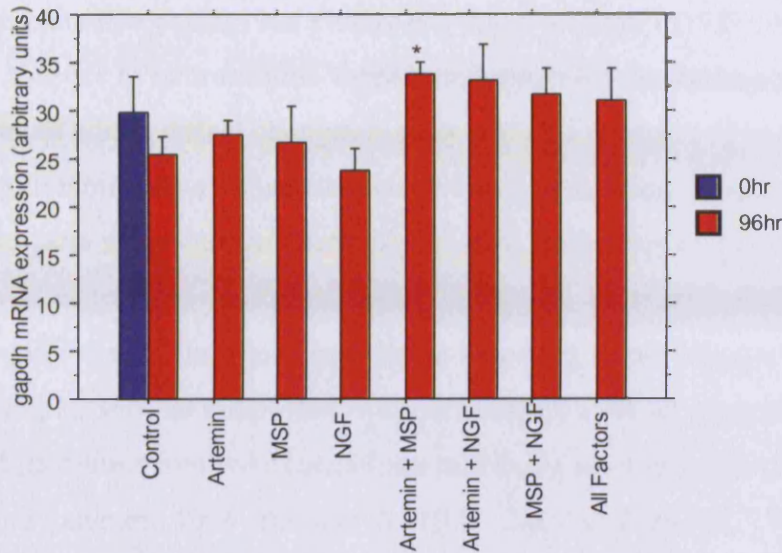
B. Nav1.7:

	P value	Significance
Art, art + MSP	0.3099	
Art, art + NGF	0.6275	
Art, all factors	0.3896	
MSP, art + MSP	0.5130	
MSP, MSP + NGF	0.3589	
MSP, all factors	0.6197	
NGF, art + NGF	0.5021	
NGF, MSP + NGF	0.8932	
NGF, all factors	0.7691	

Figure 4.20. Additive effects of artemin, MSP and NGF on expression of Nav1.7 mRNA in adult mouse cultured DRG

Adult mouse DRG were cultured in the presence or absence of artemin (10ng/ml), NGF (10ng/ml), MSP (50ng/ml) or a combination of these factors, for 96 hours. Expression of Nav1.7 (A) mRNA was quantified using real-time Q-PCR. ANOVA statistical analyses were carried out. Significant differences to expression at 'control 96 hours' are indicated directly above bars, other relevant comparisons are outlined in B (Nav1.7). * = $p < 0.05$ ** = $p < 0.01$. Error bars = +/- standard error, n = 4

A. GAPDH:



B. GAPDH:

	P value	Significance
Art, art + MSP	0.1099	
Art, art + NGF	0.1557	
Art, all factors	0.3448	
MSP, art + MSP	0.0724	
MSP, MSP + NGF	0.2053	
MSP, all factors	0.2485	
NGF, art + NGF	0.0232	*
NGF, MSP + NGF	0.0524	
NGF, all factors	0.0665	

Figure 4.21. Additive effects of artemin, MSP and NGF on expression of GAPDH mRNA in adult mouse cultured DRG

Adult mouse DRG were cultured in the presence or absence of artemin (10ng/ml), NGF (10ng/ml), MSP (50ng/ml) or a combination of these factors, for 96 hours. Expression of GAPDH mRNA was quantified using real-time Q-PCR. Significant differences to expression at 'control 96 hours' are indicated directly above bars, other relevant comparisons are outlined in B (GAPDH). * = $p < 0.05$ ** = $p < 0.01$. Error bars = +/- standard error, $n = 4$

4.3.7. The effects of LIF on sensory neuron gene expression

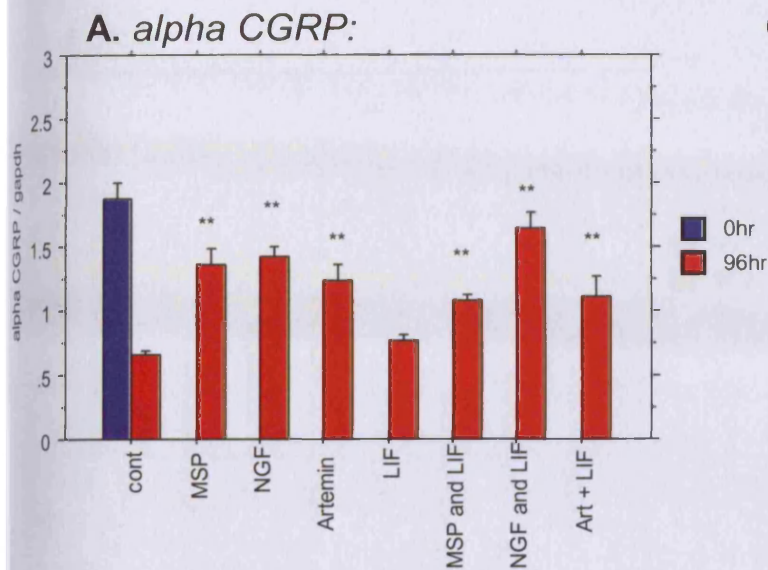
The data presented in this chapter has clearly displayed that adult DRG neurons cultured in the absence of neurotrophic support undergo many alterations in gene expression, most of which reflect changes in gene expression observed in damaged DRG neurons following nerve transection, constriction or ligation. Whilst many of these changes in gene expression are probably, at least partly, due to an alteration in the availability of target field-derived neurotrophic factors, alternative mechanisms may come into play to modulate gene expression following nerve damage or *in-vitro* culture. For example, another consequence of nerve injury is an up-regulation in LIF expression, and its release into the extracellular milieu, by de-differentiating Schwann cells (Banner and Paterson, 1994; Bolin et al., 1995; Sun and Zigmund, 1996; Hirota et al., 1996; Kurek et al., 1996; Ito et al., 1998; Thomson and Majithia, 1997). This up-regulation has been suggested to play a role in some axotomy related changes in gene expression, as discussed in the introduction to this chapter. The possibility arises that Schwann cells within dissociated DRG cultures release LIF into the culture medium and this may be partly responsible for orchestrating some of the changes in neuronal gene expression observed in adult DRG cultures. As a first step in determining the role of LIF in regulating the *in-vitro* expression of the mRNAs investigated in this chapter, neurons were cultured in the presence or absence of LIF, artemin, MSP or NGF and also with LIF in combination with artemin, MSP or NGF for 96 hours. To further determine whether the release of LIF from Schwann cells within DRG neuron cultures contributes to the changes in neuronal gene expression that occur in culture, I had planned to culture DRG neurons in the presence and absence of an antibody that functionally blocks LIF from binding to the LIF receptor component, gp130. Unfortunately, time constraints precluded the completion of this work.

The results of culture experiments that included LIF are shown in figures 4.22 - 4.29. ANOVA statistical analyses were carried out to compare results. Any significant difference to 'control' samples at 96 hours is shown directly above the relevant bar by the use of * ($p < 0.05$) or ** ($p < 0.01$). Details of other comparisons are shown in the table to the right of the appropriate graph.

Data showing the expression of CGRP mRNAs within adult DRG neurons in the “LIF culture” series of experiments is presented in figure 4.22. As previously demonstrated, α -CGRP mRNA showed a down-regulation over time in culture in the absence of neurotrophic factors, which was significantly attenuated by supplementing cultures with either NGF, MSP or artemin. The addition of 10ng/ml LIF to DRG cultures did not alter the expression of α -CGRP mRNA compared to control cultures. Similarly, the addition of LIF to cultures containing either artemin, NGF or MSP did not change the expression of α -CGRP mRNA compared to cultures containing the three neurotrophic factors alone (figure 4.22A).

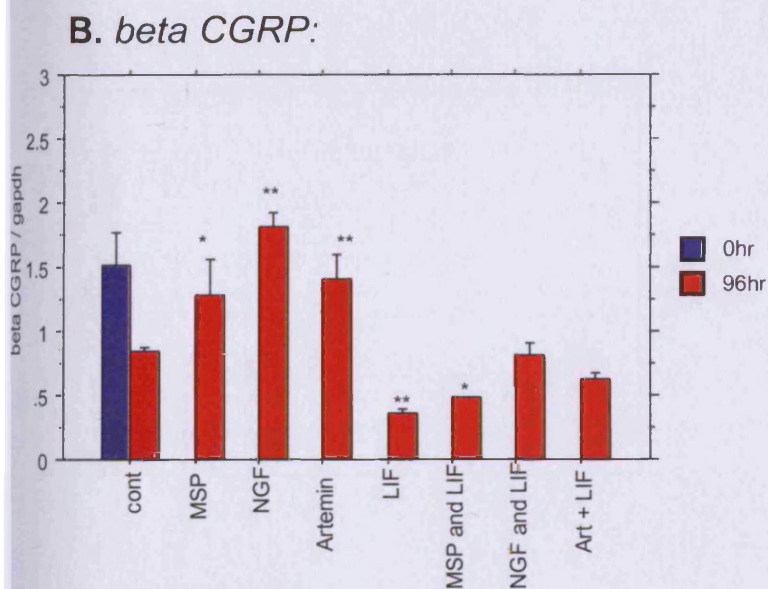
In contrast to its lack of effects on α -CGRP mRNA expression, the addition of 10ng/ml LIF to adult DRG cultures significantly enhanced the down-regulation of β -CGRP mRNA that is observed when adult DRG neurons were placed in culture in the absence of neurotrophic factor support (figure 4.22 B). Furthermore, 10ng/ml LIF significantly inhibited the positive regulatory effects that artemin, MSP and NGF have on β -CGRP mRNA expression. This not only suggests a role for endogenous LIF in regulating the injury induced changes in β -CGRP mRNA expression within DRG *in-vivo*, but also further demonstrates that the two CGRP mRNAs are expressed and regulated in different manners.

The effects of LIF on the expression of SP and VR1 mRNAs lie somewhere in between its contrasting effects on the regulation of α - and β -CGRP mRNAs. Supplementing adult DRG neuron cultures with 10ng/ml LIF did not alter the expression of SP or VR1 mRNAs compared to control cultures. However, LIF did appear to attenuate the expression enhancing effects of artemin, NGF and MSP on these mRNAs (figures 4.23A and B). In most cases the ability of LIF to attenuate neurotrophic factor enhanced expression of SP and VR1 mRNAs was significant, however LIF failed to significantly down-regulate the expression of VR1 mRNA in DRG cultures containing NGF and MSP.



C. alpha CGRP:

	P value	Significance
MSP, MSP + LIF	0.0862	
NGF, NGF + LIF	0.1369	
Art, Artemin + LIF	0.3734	
LIF, MSP + LIF	0.0494	*
LIF, NGF + LIF	<0.0001	**
LIF, Artemin + LIF	0.0182	*



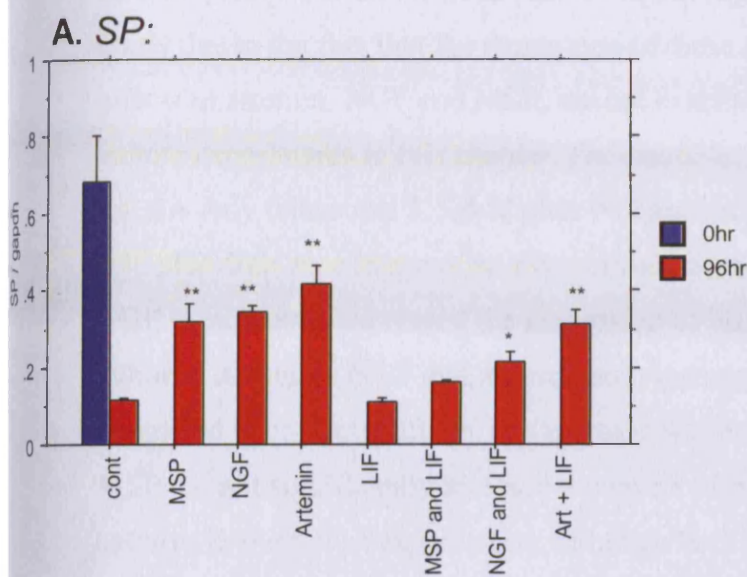
D. beta CGRP:

	P value	Significance
MSP, MSP + LIF	0.0002	**
NGF, NGF + LIF	<0.0001	**
Art, Artemin + LIF	<0.0001	**
LIF, MSP + LIF	0.4463	
LIF, NGF + LIF	0.0167	*
LIF, Artemin + LIF	0.1316	

Figure 4.22. Effect of LIF in combination with various neurotrophic factors on the expression of alpha and beta CGRP mRNAs in cultured adult mouse DRG neurons

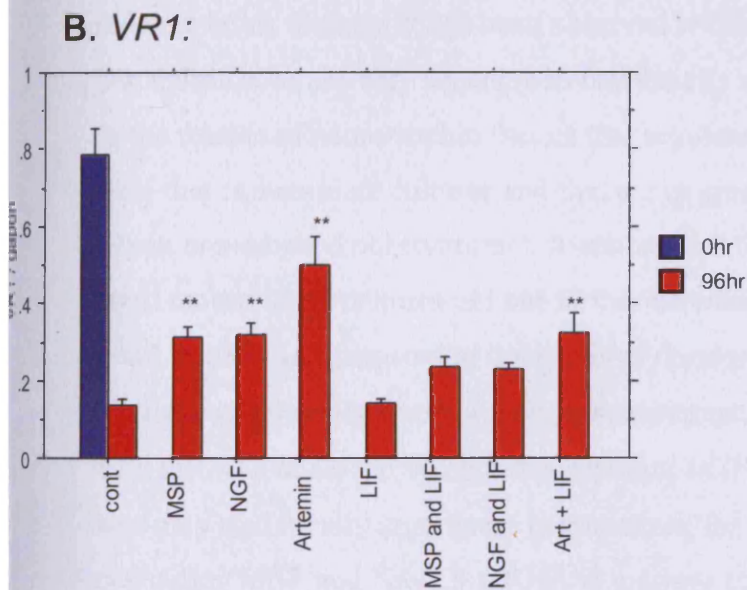
Adult mouse DRG neurons were cultured in the presence or absence of NGF (10ng/ml), MSP (50ng/ml), artemin (10ng/ml), LIF (50ng/ml) or LIF in combination with one of the fore mentioned neurotrophic factors. Expression of alpha CGRP (A) and beta CGRP (B) mRNAs were quantified using real-time QPCR. Significant differences in expression o that at 'control 96 hours' are indicated directly above bars, other relevant comparisons are outlined in C (alpha CGRP) and D (beta CGRP). * = $p < 0.05$ ** = $p < 0.01$.

Error bars = +/- standard error, n = 4



C. SP:

	P value	Significance
MSP, MSP + LIF	0.0017	**
NGF, NGF + LIF	0.0075	**
Art, Artemin + LIF	0.0168	*
LIF, MSP + LIF	0.2081	
LIF, NGF + LIF	0.0276	*
LIF, Artemin + LIF	<0.0001	**



D. VR1:

	P value	Significance
MSP, MSP + LIF	0.2032	
NGF, NGF + LIF	0.1345	
Art, Artemin + LIF	0.0043	**
LIF, MSP + LIF	0.0494	*
LIF, NGF + LIF	<0.0001	**
LIF, Artemin + LIF	0.0182	*

Figure 4.23. Effect of LIF in combination with various neurotrophic factors on the expression of SP and VR1 mRNAs in cultured adult mouse DRG neurons

Adult mouse DRG neurons were cultured in the presence or absence of NGF (10ng/ml), MSP (50ng/ml), artemin (10ng/ml), LIF (50ng/ml) or LIF in combination with one of the fore mentioned neurotrophic factors. Expression of SP (A) and VR1 (B) mRNAs were quantified using real-time QPCR. Significant differences in expression to that at 'control 96 hours' are indicated directly above bars, other relevant comparisons are outlined in C (SP) and D (VR1). * = $p < 0.05$ ** = $p < 0.01$.

Error bars = +/- standard error, n = 4

The effects of LIF on the expression of the two TTX-resistant sodium channel mRNAs, Nav1.8 and Nav1.9 are not clear cut (figures 4.24 A and B). This is probably partly due to the fact that the expression of these two mRNAs, and the regulatory effects of artemin, NGF and MSP, are not exactly the same as that seen in previous culture experiments in this chapter. For example, the expression levels of Nav1.8 mRNA only falls some 3.5-fold after 96 hours in control cultures compared to the 10-fold plus drop seen in previous experiments. Moreover, in this set of experiments only MSP significantly increased the expression of Nav1.8 mRNA compared to control cultures. Although NGF and artemin both increased the expression of this mRNA compared to control cultures, this increase was not statistically significant. Similarly, MSP did not significantly increase the levels of Nav1.9 mRNA compared to control cultures in this set of experiments, although both NGF and artemin did. The reasons behind these discrepancies are unclear, but it is possible that neuronal density in the cultures holds the key. It has been observed within our lab that the expression of certain mRNAs are very sensitive to cell density in adult DRG cultures, probably due to the release of neurotrophic factors that regulate mRNA expression from Schwann cells that contaminate cultures and that are in greater numbers in dense cell cultures (S Wyatt unpublished observations). It seems clear that the addition of 10ng/ml LIF to adult mouse DRG cultures did not further decrease the expression of either Nav1.8 or Nav1.9 mRNAs compared to the levels of these mRNAs found in control cultures. Whilst it is generally true that LIF acts to attenuate the increases in the expression of both mRNAs observed when either artemin, NGF or MSP are added to cultures, this was only statistically significant in two cases; for Nav1.8 mRNA in cultures containing MSP and Nav1.9 mRNA in cultures containing NGF.

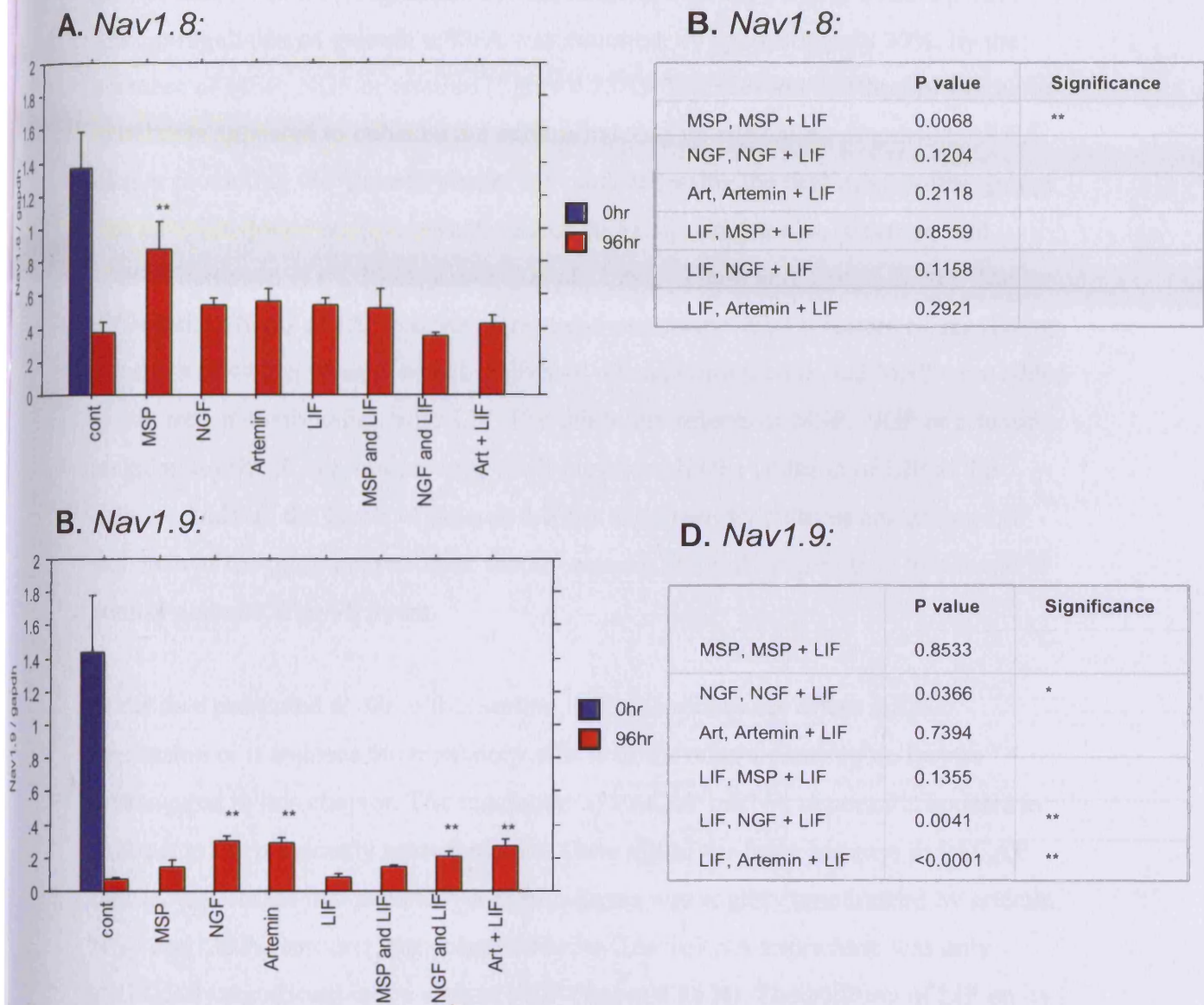


Figure 4.24. Effect of LIF in combination with various neurotrophic factors on the expression of Nav1.8 and Nav1.9 mRNAs in cultured adult mouse DRG neurons

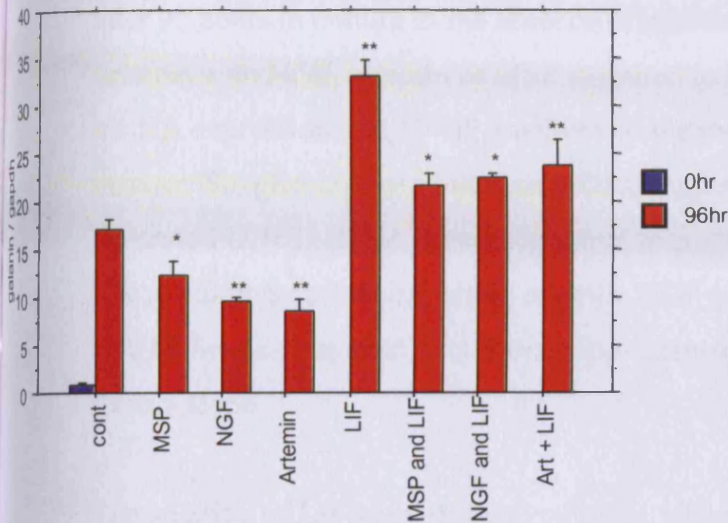
Adult mouse DRG neurons were cultured in the presence or absence of NGF (10ng/ml), MSP (50ng/ml), artemin (10ng/ml), LIF (50ng/ml) or LIF in combination with one of the fore mentioned neurotrophic factors. Expression of Scn10a (Nav1.8) (A) and Scn11a (Nav1.9) (B) mRNAs were quantified using real-time QPCR. Significant differences in expression to that at 'control 96 hours' are indicated directly above bars, other relevant comparisons are outlined in C (Nav1.8) and D (Nav1.9). * = $p < 0.05$ ** = $p < 0.01$.

Error bars = +/- standard error, n = 4

Figure 4.25 shows the expression of galanin and PACAP mRNAs and illustrates a greater than 10 fold up-regulation of both mRNAs over the 96 hour culture period. The up-regulation of galanin mRNA was inhibited, by approximately 30%, by the presence of MSP, NGF or artemin (figure 4.25A). The addition of 10ng/ml LIF alone to cultures appeared to enhance the culture-induced up-regulation of galanin mRNA, further promoting the 'injured phenotype', and reflecting the findings of other groups that have illustrated positive effects of LIF on galanin expression (Corness et al., 1996; Thompson et al., 1998; Kerekes et al., 1999; Ozturk and Tonge, 2001). The differential effects of LIF and the afore-mentioned neurotrophic factors on regulating galanin mRNA expression were highlighted when artemin, NGF and MSP were added to cultures in combination with LIF. The inhibitory effects of MSP, NGF or artemin on galanin mRNA expression were totally reversed by the addition of LIF to the cultures. Indeed, the levels of galanin mRNA expressed by cultures containing LIF and each of the other neurotrophic factors was significantly higher than that found in control cultures after 96 hours.

In the data presented so far in this section, LIF either does not effect mRNA expression or it apposes the regulatory effects of the other neurotrophic factors investigated in this chapter. The regulation of PACAP mRNA expression appears to contrast to the previously presented data. Once again, the large increase in PACAP mRNA expression that occurs in control cultures was slightly ameliorated by artemin, NGF and MSP. However, the reduction in PACAP mRNA expression was only statistically significant in the case of NGF (figure 4.25 B). The addition of LIF on its own to cultures also significantly attenuated the culture-induced increase in PACAP mRNA expression. Moreover, adding 10ng/ml LIF to cultures that also contain either NGF, artemin or MSP further enhanced the effects of these neurotrophic factors in reducing PACAP mRNA expression. Whilst the effects of LIF in reducing PACAP mRNA expression in cultures also containing either artemin, NGF or MSP did not quite reach statistical significance compared to cultures containing these factors in the absence of LIF, the data is significant compared to non-supplemented control cultures.

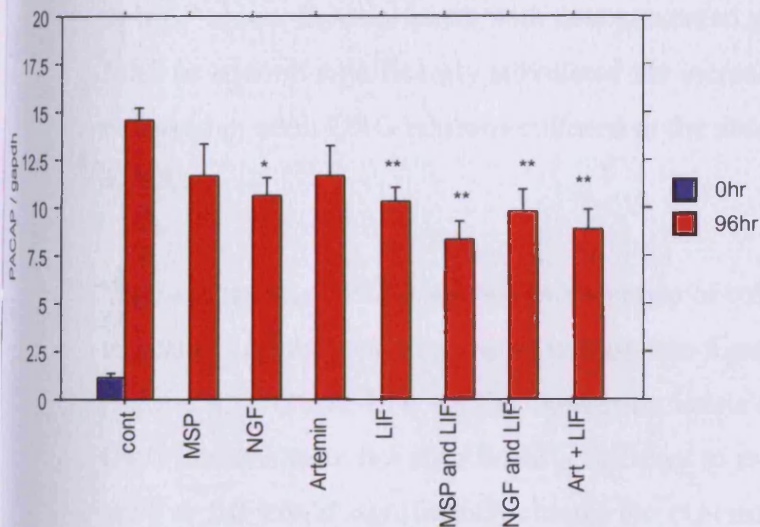
A. galanin:



C. galanin:

	P value	Significance
MSP, MSP + LIF	0.0005	**
NGF, NGF + LIF	<0.0001	**
Art, Artemin + LIF	<0.0001	**
LIF, MSP + LIF	<0.0001	**
LIF, NGF + LIF	0.0001	**
LIF, Artemin + LIF	0.0002	**

B. PACAP:



D. PACAP:

	P value	Significance
MSP, MSP + LIF	0.0512	
NGF, NGF + LIF	0.6098	
Art, Artemin + LIF	0.0688	
LIF, MSP + LIF	0.1953	
LIF, NGF + LIF	0.7429	
LIF, Artemin + LIF	0.3288	

Figure 4.25. Effect of LIF in combination with various neurotrophic factors on the expression of galanin and PACAP mRNAs in cultured adult mouse DRG neurons

Adult mouse DRG neurons were cultured in the presence or absence of NGF (10ng/ml), MSP (50ng/ml), artemin (10ng/ml), LIF (50ng/ml) or LIF in combination with one of the fore mentioned neurotrophic factors. Expression of galanin (A) and PACAP (B) mRNAs were quantified using real-time QPCR. Significant differences in expression to that at 'control 96 hours' are indicated directly above bars, other relevant comparisons are outlined in C (galanin) and D (PACAP).

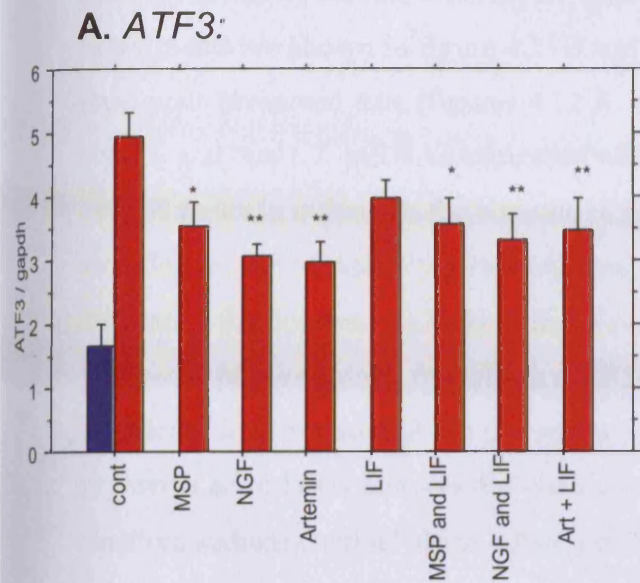
* = $p < 0.05$ ** = $p < 0.01$.

Error bars = +/- standard error, n = 4

The effects of LIF on DINE mRNA expression were very similar to its effects on PACAP mRNA expression (figure 4.26B) As in previous adult DRG culture experiments, DINE mRNA displayed an approximately 50-fold increase in levels after 96 hours in culture in the absence of neurotrophic factors. Whilst supplementing cultures with NGF, artemin or MSP appeared to attenuate the increase in DINE mRNA expression, only NGF ameliorated the increase in a statistically significant manner. Supplementing adult mouse DRG cultures with 10ng/ml LIF significantly decreased DINE mRNA levels compared to control cultures. Similarly, the addition of LIF to cultures containing either artemin, NGF or MSP significantly decreased DINE mRNA levels compared to cultures supplemented with any one of these neurotrophic factors alone.

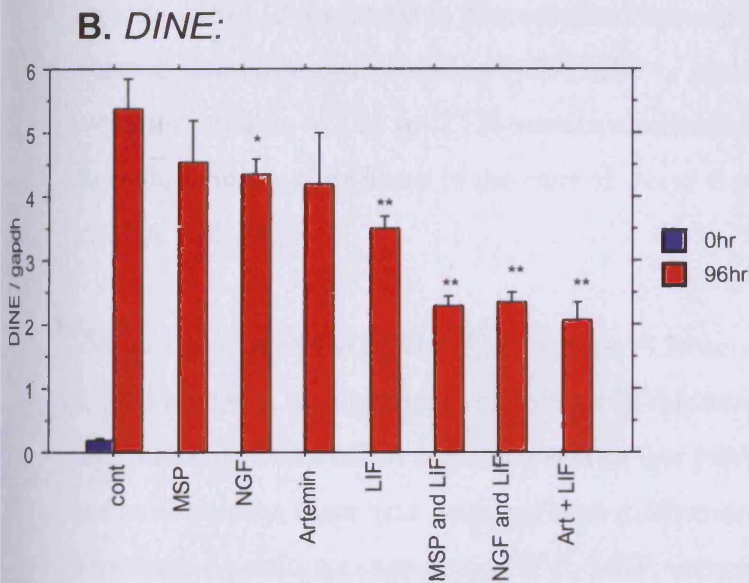
The addition of LIF to adult DRG cultures, either alone or in combination with the other three neurotrophic factors, did not alter the expression of ATF-3 mRNA compared to either control DRG cultures or cultures containing either artemin, NGF or MSP alone. In accordance with data presented previously in this chapter, NGF, MSP or artemin significantly attenuated the increase in ATF3 mRNA expression that occurred in adult DRG neurons cultured in the absence of neurotrophic factors (figure 4.26A).

The expression of P2X3 mRNA in the series of cultures set up to investigate the effects of LIF on gene expression is shown in figure 4.27A. As for previous cultures (figures 4.12 C and 4.19 A), the expression levels of P2X3 mRNA within cultured DRG neurons were not significantly different to those *in-vivo*. Neither artemin, NGF, MSP or LIF could significantly change the expression of P2X3 mRNA compared to control cultures.



C. ATF3:

	P value	Significance
MSP, MSP + LIF	0.9940	
NGF, NGF + LIF	0.6439	
Art, Artemin + LIF	0.3562	
LIF, MSP + LIF	0.4471	
LIF, NGF + LIF	0.2824	
LIF, Artemin + LIF	0.3627	



D. DINE:

	P value	Significance
MSP, MSP + LIF	0.0020	**
NGF, NGF + LIF	0.0049	**
Art, Artemin + LIF	0.0018	**
LIF, MSP + LIF	0.0524	
LIF, NGF + LIF	0.0885	
LIF, Artemin + LIF	0.0258	*

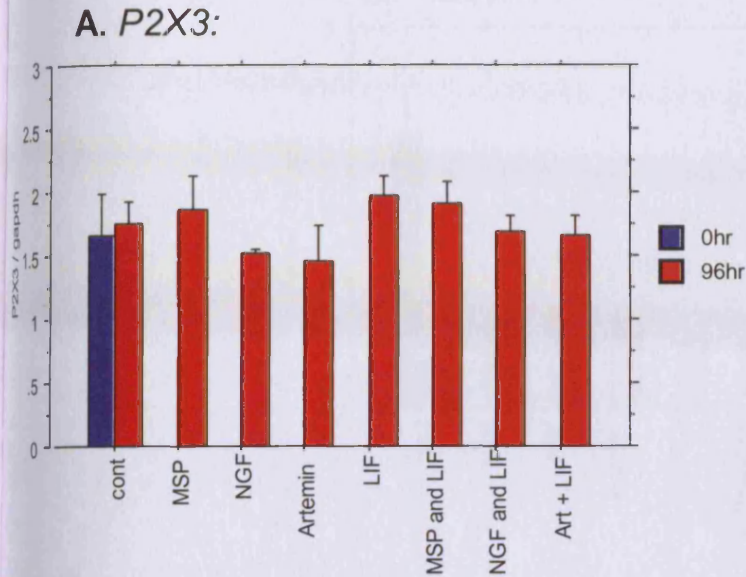
Figure 4.26. Effect of LIF in combination with various neurotrophic factors on the expression of ATF3 and DINE mRNAs in cultured adult mouse DRG neurons

Adult mouse DRG neurons were cultured in the presence or absence of NGF (10ng/ml), MSP (50ng/ml), artemin (10ng/ml), LIF (50ng/ml) or LIF in combination with one of the fore mentioned neurotrophic factors. Expression of ATF3 (A) and DINE (B) mRNAs were quantified using real-time QPCR. Significant differences in expression to that at 'control 96 hours' are indicated directly above bars, other relevant comparisons are outlined in C (ATF3) and D (DINE). * = $p < 0.05$ ** = $p < 0.01$.

Error bars = +/- standard error, n = 4

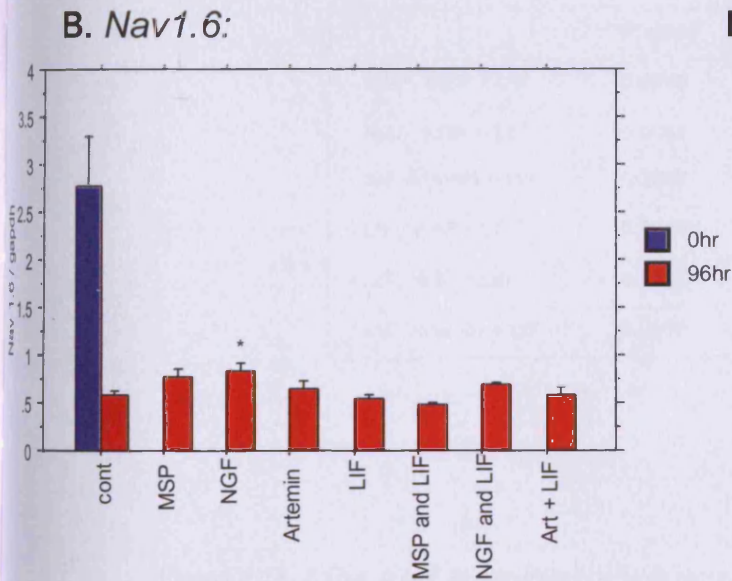
The expression of Nav1.6 and Nav1.7 mRNAs in the “LIF culture” series of experiments are shown in figure 4.27B and 4.28, respectively. In agreement with previously presented data (figures 4.12 A, 4.12 B, 4.19 B and 4.20), the amount of Nav1.6 and Nav1.7 mRNAs expressed within adult DRG neurons decreased greatly over 96 hours in culture in the absence of neurotrophic factor support. Also, in accordance with previously presented data, MSP and NGF had a tendency to slightly ameliorate the decrease in Nav1.6 and Nav1.7 mRNA expression. However, in contrast to previous data, the effects of NGF, but not MSP, were statistically significant for both mRNAs in this series of cultures. The addition of 10ng/ml LIF on its own to adult DRG cultures did not significantly alter the expression of either TTX-sensitive sodium channel alpha-subunit mRNAs compared to control cultures. In contrast, supplementing cultures containing either one of the other neurotrophic factors, with LIF appeared to decrease the expression of both sodium channel mRNAs compared to cultures containing NGF, MSP or artemin alone. However, the inhibitory effects of LIF on TTX-sensitive sodium channel mRNA expression are only statistically significant in the case of Nav1.6 mRNA with MSP and Nav1.7 mRNA with NGF.

The expression of GAPDH mRNA for the “LIF series” of cultures is shown in figure 4.29. There was no significant difference in this series of cultures between the time 0 levels of GAPDH mRNA and the levels of this mRNA in control cultures after 96 hours. Similarly, there was no significant difference in the amount of GAPDH mRNA expressed by cultures containing NGF, MSP, artemin or LIF compared to control cultures. The addition of LIF to adult DRG cultures that also contained NGF, MSP or artemin appeared to slightly increase the expression of GAPDH mRNA compared to cultures containing only NGF, MSP or artemin.



C. P2X3:

	P value	Significance
MSP, MSP + LIF	0.8357	
NGF, NGF + LIF	0.5294	
Art, Artemin + LIF	0.4153	
LIF, MSP + LIF	0.7972	
LIF, NGF + LIF	0.3025	
LIF, Artemin + LIF	0.2243	



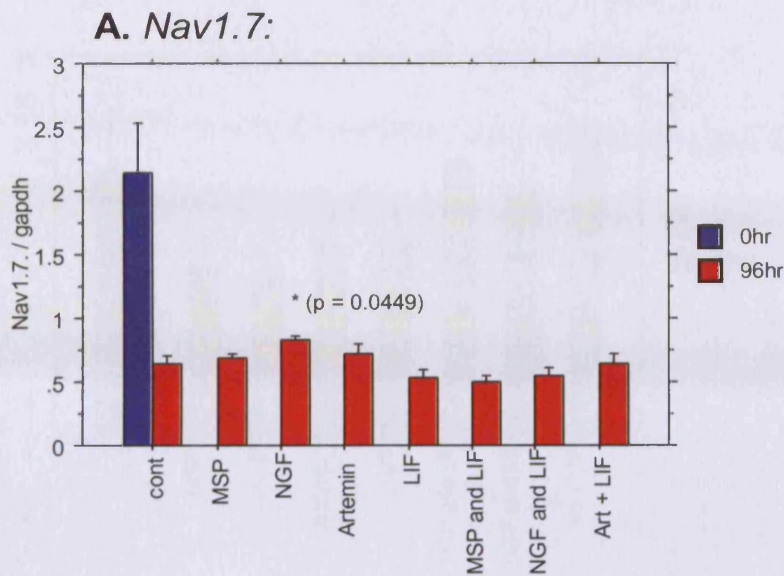
D. Nav1.6:

	P value	Significance
MSP, MSP + LIF	0.0040	**
NGF, NGF + LIF	0.1610	
Art, Artemin + LIF	0.5191	
LIF, MSP + LIF	0.5013	
LIF, NGF + LIF	0.1108	
LIF, Artemin + LIF	0.5191	

Figure 4.27. Effect of LIF in combination with various neurotrophic factors on the expression of P2X3 and Nav1.6 mRNAs in cultured adult mouse DRG neurons

Adult mouse DRG neurons were cultured in the presence or absence of NGF (10ng/ml), MSP (50ng/ml), artemin (10ng/ml), LIF (50ng/ml) or LIF in combination with one of the fore mentioned neurotrophic factors. Expression of P2X3 (A) and Nav1.6 (B) mRNAs were quantified using real-time QPCR. Significant differences in expression to that at 'control 96 hours' are indicated directly above bars, other relevant comparisons are outlined in C (P2X3) and D (Nav1.6). * = $p < 0.05$ ** = $p < 0.01$.

Error bars = +/- standard error, n = 4



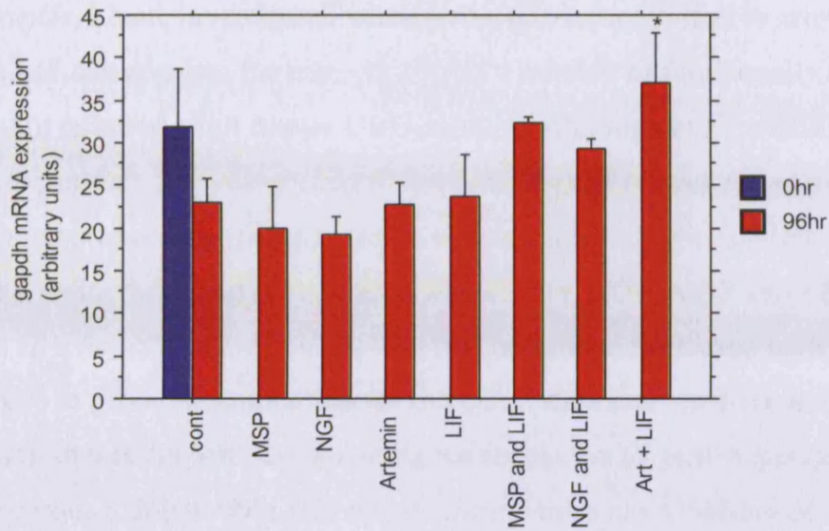
B. Nav1.7:

	P value	Significance
MSP, MSP + LIF	0.0543	
NGF, NGF + LIF	0.0061	**
Art, Artemin + LIF	0.2992	
LIF, MSP + LIF	0.6620	
LIF, NGF + LIF	0.8712	
LIF, Artemin + LIF	0.2500	

Figure 4.28. Effect of LIF in combination with various neurotrophic factors on the expression of Nav1.7 mRNA in cultured adult mouse DRG neurons

Adult mouse DRG neurons were cultured in the presence or absence of NGF (10ng/ml), MSP (50ng/ml), artemin (10ng/ml), LIF (50ng/ml) or LIF in combination with one of the fore mentioned neurotrophic factors. Expression of Nav1.7 (A) mRNAs was quantified using real-time QPCR. Significant differences in expression to that at 'control 96 hours' are indicated directly above bars, other relevant comparisons are outlined in B (Nav1.7). * = $p < 0.05$ ** = $p < 0.01$.

Error bars = +/- standard error, n = 4



	P value	Significance
MSP, MSP + LIF	0.0310	*
NGF, NGF + LIF	0.0747	
Art, Artemin + LIF	0.0082	**
LIF, MSP + LIF	0.0977	
LIF, NGF + LIF	0.3119	
LIF, Artemin + LIF	0.0129	**

Figure 4.29. Effect of LIF in combination with various neurotrophic factors on the expression of GAPDH mRNA in cultured adult mouse DRG neurons

Adult mouse DRG neurons were cultured in the presence or absence of NGF (10ng/ml), MSP (50ng/ml), artemin (10ng/ml), LIF (50ng/ml) or LIF in combination with one of the fore mentioned neurotrophic factors. Expression of GAPDH mRNA was quantified using real-time QPCR and all other results normalised against these values.

Error bars = +/- standard error, n = 4

4.4. Discussion

In this chapter, I have investigated whether the neurotrophic factors artemin, MSP, NGF and LIF can regulate the transcription of a number of functionally important genes within cultured adult mouse DRG neurons. Although extrapolating data from culture experiments and interpreting their physiological relevance has a number of caveats, *in-vivo* experimental approaches to investigating gene regulation also have drawbacks. Mice with null mutations of the artemin, NGF, MSP and LIF receptor genes are all available and would survive into adulthood if crossed with Bax null-mutant mice to produce double-null mutant lines. However, as discussed in the introduction to this chapter, investigating the regulation of gene expression in adult sensory neurons using double-null mutant mouse lines has a number of limitations. These factors should be borne in mind as may cause misleading interpretation of data. In addition, generating sufficient numbers of double-null mutant adult animals to produce statistically significant results would be an extremely time consuming and costly exercise that would lead to unacceptably high levels of animal wastage. The *in-vivo* administration of either neurotrophic factors or neurotrophic factor receptor blocking reagents to the peripheral or central target field of DRG neurons could also have been used to investigate the efficacy of neurotrophic factors in regulating neuronal gene expression. However, this approach also has a number of pitfalls that were discussed in the introduction to this chapter.

The DRG cultures used to investigate transcriptional gene regulation by NGF, artemin, MSP and LIF were mixed cultures containing both neurons and glial cells. In fact, non-neuronal cells typically significantly outnumbered neurons at the start of the culture period. After 96 hours, there were many fewer non-neuronal cells in the adult DRG cultures than at the start of the culture period, presumably because the non-neuronal cells did not survive very well in the minimal medium used for the cultures. However, despite the time-dependent decrease in the number of non-neuronal cells surviving in DRG cultures, significant numbers were still present after 96 hours of culture. The level of glial cell contamination in DRG cultures clearly varied from one set of cultures to another, but was uniform across all dishes in any one experiment. *In-*

vivo, the majority of mRNAs that were assayed in this chapter are predominantly or exclusively expressed by neurons within DRG. Since the same is likely to be true in culture, the expression of the assayed mRNAs by contaminating glia is unlikely to significantly alter the interpretation of data. Neurons and glial cells both express GAPDH mRNA. However, the level of GAPDH mRNA expressed by neurons is several orders of magnitude greater than that expressed by glia, reflecting the size difference between the two cell types (discussed in previous chapters). Therefore, GAPDH mRNA expression by non-neuronal cells is unlikely to significantly affect the accuracy of neuronal mRNA quantification that is normalised to GAPDH mRNA levels. Especially since the level of non-neuronal cell contamination in each dish of any one set of cultures is likely to be similar. It is well established that glial cells, and Schwann cells in particular, can synthesise and secrete neurotrophic factors, both *in-vivo* and *in-vitro*. Therefore, it is possible that neurotrophic factors secreted by glia may either conceal or exacerbate the effects of exogenous neurotrophic factors in regulating transcriptional expression in some cultures. This is a possibility that must be borne in mind when interpreting the expression data presented in this chapter.

Below, I will discuss the transcriptional regulation of the mRNAs investigated in this chapter in turn, followed by summing up the significance of the data in terms of normal nociceptive thresholds and the response of sensory neurons to nerve lesion.

4.4.1. α - and β -CGRP

In chapter two of this thesis, an analysis of trigeminal and dorsal root ganglia from transgenic embryos demonstrated the differential regulation of α - and β - CGRP mRNAs by NGF/TrkA signalling in embryonic neural crest-derived sensory neurons. In chapter three, this research was extended to investigate the *in-vitro* and *in-vivo* regulation of α - and β - CGRP mRNAs by both NGF/TrkA and NT-3 dependent signalling in neonatal neural-crest and placode-derived sensory neurons. Results demonstrated that NGF/TrkA signalling was essential to achieve and maintain the correct expression levels of both α - and β - CGRP mRNAs in neonatal trigeminal, nodose and dorsal root ganglion neurons *in-vivo*. It was also revealed that NGF could maintain the expression of α -CGRP mRNA in cultured neonatal trigeminal neurons

for 48 hours, preventing the marked drop in the expression of this neuropeptide mRNA that occurred in control cultures. In contrast to α -CGRP mRNA, my data from chapter three showed that β -CGRP mRNA levels did not change significantly in neonatal trigeminal neurons cultured for 48 hours without neurotrophic factor support.

Figures 4.8, 4.14, and 4.22 reveal that the levels of α - and β -CGRP mRNAs, expressed by cultured adult mouse DRG neurons, fell 2- to 3-fold over 96 hours in the absence of neurotrophic factor support. The reduction in α -CGRP mRNA levels was consistently more pronounced than the fall in β -CGRP mRNA levels, an observation that is in agreement with the data obtained from neonatal trigeminal neuron cultures in the previous chapter. If one regards *in-vitro* culture as a model of nerve injury, then the culture data is in accordance with a number of previous publications demonstrating that CGRP mRNA and peptide expression falls sharply within damaged rat and mouse lumbar DRG neurons *in-vivo* following sciatic nerve transection (Noguchi et al., 1990; Verge et al, 1995; Mulder et al., 1997; Sterne et al., 1998; Shi et al., 2001; Shadiack et al., 2001). The studies by Noguchi et al and Verge et al are particularly relevant, since, like the data presented in this chapter, they discriminate between α - and β -CGRP mRNAs.

NGF, artemin and MSP were all able to significantly attenuate the culture-induced decrease in α - and β -CGRP mRNA expression in adult mouse DRG neurons (figures 4.8, 4.14 and 4.22). NGF and artemin appeared to have similar efficacies in their ability to regulate the expression of α - and β -CGRP mRNAs, whilst MSP was slightly less effective in promoting CGRP mRNA expression than the other two neurotrophic factors. The ability of NGF to positively regulate the expression of CGRP mRNA and peptide within rodent sensory neurons, both *in-vitro* and *in-vivo*, has been previously well documented (e.g. Lindsay et al., 1989; Verge et al, 1995; Jiang and Smith, 1995; Price et al, 2005). Likewise, artemin has also been shown to partially reverse the down-regulation of CGRP peptide within adult rat DRG following sciatic nerve section or ligation.(Gardell et al, 2003). The demonstration that MSP was able to promote the expression of α - and β -CGRP mRNAs in cultured adult mouse DRG neurons, thereby partially reversing the axotomized phenotype, is a novel and potentially important observation that will be discussed more fully below. The data

suggests that MSP may play a role in establishing the level of α - and β -CGRP mRNAs expressed by nociceptive sensory neurons *in-vivo*, although additional studies, preferably using conditional null-mutant transgenic mice, would be required to clarify this hypothesis.

The observations that MSP and artemin could regulate the expression of CGRP mRNAs in adult DRG cultures raises the possibility that these neurotrophic factors may play a role in the initial induction and developmental regulation of CGRP expression in developing sensory neurons, in a similar manner to NGF. An analysis of gene expression within sensory ganglia of embryonic and neonatal conditional null-mutants for the MSP receptor, RON, or the artemin receptor, GFR- α 3, would help to determine whether this is the case. A similar approach would also to determine whether artemin and/or MSP are also involved in regulating the *in-vivo* developmental expression of the many other genes that were found in this chapter to be regulated by these neurotrophic factors in cultured adult DRG neurons (see below).

The combined addition of saturating concentrations of NGF and artemin to adult DRG cultures significantly increased the expression of both α - and β -CGRP mRNAs to higher levels than those found with either factor alone (figures 4.14 A and B). The levels of both CGRP mRNAs in cultures containing a combination of both neurotrophic factors was approximately 20% greater than cultures containing a single neurotrophic factor, and restored CGRP mRNA levels to time 0 (*in-vivo*) levels. The increase in CGRP mRNA expression may reflect the possibility that both neurotrophic factors use different intracellular signal transduction pathways to increase the expression of CGRP mRNAs within individual neurons that express CGRP and both neurotrophic factor receptors. However, the simplest interpretation of the data is that not all CGRP mRNA-positive DRG neurons express receptors for both NGF and artemin in culture. In adult rodents, approximately 35% of neurons in L4/L5 DRG express TrkA. Most TrkA positive neurons are C- or A- δ fibre nociceptors that also express CGRP. Around 35% of this population also express the GDNF family signal transducing receptor, Ret (Averill et al, 1995; Molliver et al, 1997; Michael and Priestley, 1999; Orozco et al, 2001). GFR- α 3 expression is restricted to approximately 20% of L4/L5 DRG neurons. GFR- α 3-positive DRG neurons

predominantly represent the sub-population of TrkA-positive, CGRP-positive, non-myelinated C-fibre neurons that express Ret. However, 8% of GFR-alpha 3 expressing neurons (1.6% of total neurons in L4/L5 DRG and approximately 5% of CGRP-positive neurons) are Ret- and CGRP-positive but TrkA-negative (Orozco et al, 2001). Thus, in the absence of nerve injury, approximately 5% of CGRP-positive DRG neurons are responsive to artemin and not NGF, 60% are responsive to NGF but not artemin and 35% of CGRP-positive DRG neurons are responsive to both factors. This is illustrated in figure 4.30.

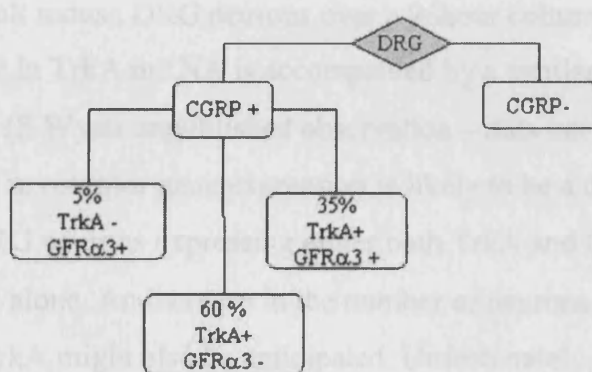


Figure 4.30. proportions of CGRP positive DRG neurons that express NGF and artemin receptors

Based on the expression patterns of NGF and artemin receptors in uninjured DRG, it may be predicted that NGF would be more effective than artemin at regulating CGRP mRNA expression *in-vitro*. It might also be anticipated that the addition of artemin to cultures containing NGF would not significantly increase the expression of CGRP mRNAs compared to cultures containing NGF alone. However, peripheral nerve lesion and *in-vitro* culture induce many similar changes in neuronal gene expression, including changes in neurotrophic factor receptor expression. The number of rat lumbar DRG neurons expressing detectable TrkA mRNA decreases by 50% at 7 days

following sciatic nerve crush, ligation or transection (Krekoski et al, 1996; Kashiba et al, 1998; Shen et al, 1999). Conversely, the number of rat lumbar DRG neurons expressing GFR- α 3 protein almost doubles by 14 days following spinal nerve ligation, so that virtually all C-fibre, peripherin-positive lumbar DRG neurons are GFR- α 3-positive (Gardell et al, 2003). It is not clear whether similar changes in the expression of artemin and NGF receptors occur in DRG neurons at all levels of the rat spinal cord following sciatic nerve lesion. Similarly, it is not clear from the published literature whether changes in TrkA or GFR- α 3 expression occur within mouse DRG, either *in-vivo*, following sciatic nerve lesion, or *in-vitro*. Unpublished work from within my laboratory has revealed a 20% decrease in the levels of TrkA mRNA expressed by adult mouse DRG neurons over a 96hour culture period. The culture-induced decrease in TrkA mRNA is accompanied by a similar increase in the levels of GFR- α 3 mRNA (S Wyatt unpublished observation – data not shown). The net result of these changes in receptor gene expression is likely to be a decrease in the proportion of DRG neurons expressing either both TrkA and GFR- α 3 together, or expressing TrkA alone. An increase in the number of neurons expressing GFR- α 3 in the absence of TrkA might also be anticipated. Unfortunately, immunocytochemistry data is not available to substantiate this hypothesis. Nonetheless, this possibility could account for the, better than expected, efficacy of artemin in regulating the expression of CGRP mRNAs in comparison to NGF. It may also explain the partially “additive” ability of NGF and artemin to increase CGRP mRNA expression.

In contrast to cultures containing both NGF and artemin, the addition of saturating levels of MSP to cultures containing saturating levels of either NGF or artemin did not increase the expression levels of either α - or β -CGRP mRNAs above those found in cultures containing just NGF or artemin alone. To date, the distribution of RON amongst adult DRG neurons *in-vivo* has not been determined. Likewise, the identity of the cultured adult mouse DRG neurons that express RON has not been established. The data presented here from the “additive” culture experiments suggests that the majority of cultured DRG neurons that express both RON and CGRP also express TrkA and GFR- α 3.

The addition of 50ng/ml LIF to adult mouse DRG cultures affected the expression of α - and β -CGRP mRNAs in markedly different ways. Whilst LIF did not significantly alter the expression of α -CGRP mRNA, either alone or in combination with the other three neurotrophic factors (figure 4.22 A), it did have a marked effect on the expression of β -CGRP mRNA (figure 4.22B). The addition of LIF to cultures significantly reduced the expression of β -CGRP mRNA compared to control cultures. Moreover, LIF blocked the ability of MSP, artemin and NGF to ameliorate the culture induced down-regulation of β -CGRP mRNA expression. LIF mRNA and protein has been shown to be up-regulated, and functional LIF released, by de-differentiating Schwann cells at the site of nerve damage following nerve ligation, transection or crush (Banner and Paterson, 1994; Bolin et al., 1995; Sun and Zigmond, 1996; Hirota et al, 1996; Kurek et al., 1996; Ito et al., 1998; Thomson and Majithia, 1998). Functional LIF receptors are predominantly expressed by small nociceptive sensory neurons, the majority of which are CGRP-positive (Thompson et al, 1997). It has previously been postulated that the injury-induced release of LIF by Schwann cells may play a role in orchestrating some of the widespread changes in gene expression that occur in sensory neurons following peripheral nerve trauma. For example, LIF appears to play a role in the up-regulation of galanin and DINE expression following nerve injury (Corness et al., 1996; 1998; Ozturk and Tonge, 2001; Kerekes et al., 1999; Sun and Zigmond, 1996; Kato et al, 2002). The data presented for β -CGRP mRNA in figure 4.22 B supports this hypothesis and is the first observation that LIF can regulate β -CGRP mRNA expression in dissociated DRG cultures.

Curiously, it has previously been reported that the addition of exogenous LIF to mouse lumbar DRG explants increases the expression of CGRP peptide compared to explants cultured in the absence of growth factors (Ozturk et al., 2002). However, neurons within explanted lumbar DRGs, behave very differently to those in dissociated cultures and do not appear to mimic changes in gene expression that occur following sciatic nerve lesion. For example, the number of CGRP immuno-positive neurons that are present within lumbar DRG explants do not decrease over time. This observation is in marked contrast to the rapid drop in CGRP mRNA and peptide expression in both dissociated DRG cultures and lumbar DRG subjected to *in-vivo* sciatic nerve lesion (e.g. Lindsay et al., 1989; Verge et al., 1995; Jiang and Smith,

1995). Moreover, NGF does not appear to enhance CGRP expression in explanted DRG (Ozturk et al., 2002), despite the wealth of published data demonstrating that NGF can increase the expression of CGRP mRNA and peptide within DRG neurons both in culture and following *in-vivo* peripheral nerve lesion (for references see above).

The observation that exogenous LIF could decrease the expression of β -CGRP mRNA in adult mouse DRG cultures, whereas it did not affect the expression of α -CGRP mRNA, may reflect a genuine difference in the transcriptional regulation of the two different CGRP genes. Alternatively, it may reflect a difference in the sensitivity of each gene to regulation by LIF. Adult DRG cultures contain Schwann cells that are likely to begin synthesizing LIF after they have been removed from their normal *in-vivo* environment. This raises the possibility that LIF, released by Schwann cells within DRG cultures, plays a role in promoting some of the culture-induced changes in gene expression observed in this chapter. If the α -CGRP gene is very sensitive to regulation by LIF, the amounts of LIF present in control cultures may be saturating with regard to the regulation of α -CGRP mRNA expression. If the β -CGRP gene is less sensitive to regulation by LIF, the amount of LIF in control cultures might not have been high enough to significantly reduce β -CGRP mRNA expression. Indeed, this may explain why the drop in α -CGRP mRNA levels when DRG neurons were placed in culture was consistently greater than the drop in β -CGRP mRNA levels. Culturing DRG neurons with and without an antibody that either blocks the function of the signal- transducing component of the LIF receptor, gp130, or sequesters and inactivates LIF would address this hypothesis. This approach may also shed further light on the ability of LIF to regulate the expression of other mRNAs within cultured DRG neurons. Unfortunately, time constraints prevented me from carrying out these experiments.

4.4.2. Substance P

Substance P (SP) mRNA showed a very similar pattern of regulation to that of α - and β -CGRP mRNAs in cultured adult mouse DRG neurons, reflecting the co-expression

of SP in a sub-population (around 50%) of CGRP-positive, TrkA-positive, adult rodent DRG neurons (Lundberg et al., 1985; Lee et al., 1985; Skofitsch and Jacobowitz, 1985; Kashiba et al., 1996; Gardell et al., 2003).

SP mRNA expression levels dropped 8-to 10-fold over a 96 hour culture period in all cultures of adult mouse DRG neurons analysed (figures 4.7A, 4.8C, 4.15A and 4.23A). This data is in accordance with previous studies on cultured adult rat DRG neurons (Mulderry, 1994; Lindsay et al., 1989). A marked drop in the expression of SP mRNA over time in culture was also observed in Chapter three for neonatal trigeminal neurons. Similarly, a marked reduction in the expression of SP mRNA and peptide within rat lumbar DRG has also been reported following sciatic or spinal nerve transection, ligation or crush (e.g. Nielsch et al., 1987; Zhang et al., 1995; Ji et al., 1996; Sterne et al., 1998; Gardell et al., 2003). The reduction in SP expression following peripheral nerve lesion has been directly attributed to a reduction in the supply of target field-derived NGF to neuronal cell bodies within DRG (Shadiack et al., 2001). The data presented in this chapter suggests that NGF may not be the only target field derived neurotrophin that regulates the steady state expression levels of SP within adult DRG neurons.

In chapters two and three of this thesis, I demonstrated that NGF is required for the induction and correct developmental regulation of SP mRNA in mouse sensory neurons. I also demonstrated that NGF can ameliorate the culture-induced down-regulation of SP mRNA in neonatal mouse trigeminal neurons. In accordance with this data, NGF could reduce the culture induced drop in SP mRNA expression within adult mouse DRG neurons by almost 50% (figure 4.8C). This finding is not novel, since NGF has previously been shown to promote the expression of SP in cultured adult mouse and rat DRG neurons (Lindsay et al., 1989; Mulderry, 1994; Zhang et al., 1995; Skoff et al., 2006), and ameliorate the drop in SP expression within rat lumbar DRG neurons that occurs following sciatic nerve lesion (Zhang et al., 1995; Ji et al., 1996). Moreover, transgenic mice ectopically expressing NGF in the spinal cord contain significantly more SP-positive neurons within DRG than wild type mice (Ma et al., 1995).

Artemin could also significantly attenuate the culture-induced drop in SP mRNA levels in adult mouse DRG neurons (figures 4.7A, 4.8C, 4.15A and 4.23A). Perhaps surprisingly, given the relative distribution of artemin and NGF receptors within rodent DRG *in-vivo* (Averill et al., 1995; Molliver et al., 1997; Michael and Priestley, 1999; Orozco et al., 2001, and illustrated in figure 4.30 above), artemin is significantly more effective than NGF in supporting the expression of SP mRNA in cultured adult mouse DRG neurons. However, as discussed above, it seems likely that the number of TrkA-positive mouse DRG neurons decreases over time in culture, whilst the number of neurons expressing the artemin receptor, GFR- α 3, increases over time in culture. The data for SP, presented in this chapter, would tend to suggest that a proportion of SP-positive DRG neurons lose TrkA expression and gain GFR- α 3 expression during the culture period. The observation that artemin could positively regulate the expression of SP is also not novel, since it has previously been demonstrated that systemic artemin can ameliorate the fall in SP peptide within adult rat DRG neurons following spinal nerve ligation (Gardell et al., 2003). However, the observation that MSP was as effective as NGF in reducing the culture-induced down-regulation of SP mRNA expression in adult mouse DRG sensory neurons (figures 4.7A, 4.8C, 4.15A and 4.23A) is a novel finding. This result suggests that a significant number of SP-positive mouse DRG neurons express functional MSP receptors in culture. Moreover, this data, together with the data from CGRP mRNA expression, suggests that MSP may play a role in regulating neuropeptide expression in adult DRG neurons *in-vivo*. Hence MSP may be involved in regulating sensory thresholds in nociceptive neurons.

Once again, as in the case of α - and β -CGRP mRNAs, the addition of saturating concentrations of NGF together with saturating concentrations of artemin to cultures increased the expression of SP mRNA to significantly higher levels than in cultures containing either NGF or artemin alone (figure 4.15A). Moreover, culturing adult mouse DRG neurons with both these neurotrophic factors totally prevented the down-regulation of SP mRNA over time in culture. This raises the possibility that not all DRG neurons that express SP in culture express both TrkA and GFR- α 3. Rather, since the effects of both factors on SP mRNA expression were not numerically the sum of each individual factor alone, it strongly suggests that adult mouse DRG

neurons that express SP in culture can be divided into three sub-populations with regards to the expression of TrkA and GFR- α 3. These populations being: A sub-population that expresses SP together with TrkA and GFR- α 3; a sub-population that expresses SP and just TrkA; a sub-population that expresses SP and just GFR- α 3.

The addition of both artemin and MSP to DRG cultures also increased the expression of SP mRNA to higher levels than with either neurotrophic factor alone. In fact, this combination of neurotrophic factors was as effective as the combination of artemin and NGF together, totally preventing the culture-induced down-regulation of SP mRNA (figure 4.15A). In contrast, the addition of MSP and NGF together, to adult mouse DRG cultures, only slightly increased the neuronal expression of SP mRNA in comparison to cultures containing either NGF or MSP alone. Moreover, the increase in SP mRNA expression with both NGF and MSP only reached statistical significance when compared to NGF alone. Significantly, the combination of NGF and MSP together is markedly less effective in promoting SP mRNA expression than the combinations of NGF or MSP with artemin. The most parsimonious explanation for this, especially since the efficacies of MSP and NGF individually in promoting SP mRNA expression are very similar, is that the majority of SP-positive neurons that express RON in culture also express TrkA. The hypothesis that RON expression is almost entirely restricted to a subset of NGF-responsive cultured adult mouse DRG neurons is supported by much of the other expression data presented in chapter 4 (see below).

The addition of 50ng/ml LIF to adult mouse DRG cultures did not alter the levels of SP mRNA compared to control cultures. However, LIF significantly attenuated the ability of NGF, artemin and MSP to promote the expression of SP (figure 4.23A). This data is in agreement with the previous observation that LIF can inhibit the NGF-induced up-regulation of SP mRNA in cultured adult rat DRG neurons (Mulderry, 1994). Whilst my data, and the data from Mulderry, suggests that the release of LIF by Schwann cells partly underlies the nerve injury-induced reduction in SP expression, two previously published articles present data that opposes this hypothesis. First, it has been reported that the, sciatic nerve ligation-induced, down-regulation of SP mRNA expression occurs to the same extent in LIF^{-/-} mice as wild-

type mice, suggesting that nerve lesion-induced reductions in SP mRNA expression within DRG neurons are independent of LIF (Sun and Zigmond, 1996). However, LIF belongs to a family of cytokines that all signal through a common receptor component, gp130 (reviewed in Taga, 1996; Murphy et al., 1997). This raises the possibility that other cytokines replace the functions of LIF in LIF^{-/-} mice. CNTF and IL-6 are two potential candidates for factors that may compensate for the lack of LIF in LIF^{-/-} mice. CNTF is expressed within Schwann cells surrounding peripheral nerves. Since CNTF lacks a signal peptide sequence it is only thought to be released from cells following nerve damage. Indeed, sciatic nerve lesion leads to the release of CNTF at the site of lesion resulting in increased retrograde transport in damaged sensory neurons (Curtis et al., 1993). IL-6, which like CNTF signals through a receptor complex containing gp130, is rapidly up-regulated in peripheral nerves following nerve injury (Ito et al, 1998). Second, application of LIF to the proximal transected sciatic nerve stump reduces the axotomy-induced down-regulation of SP mRNA in rat lumbar DRG neurons (Zhang et al., 1995). However, LIF is only effective in increasing the expression of SP within rat DRG neurons when very large amounts are applied to the transected nerve stump. Numerous previous unpublished observations within my laboratory demonstrate that many neurotrophic factors display a bell-shaped dose response curve in relation to promoting neuronal survival, neurite outgrowth and modulating gene expression. This raises the possibility that the addition of super-saturating concentrations of LIF to the proximal stump of transected sciatic nerves partially reverses the down-regulation of SP mRNA that is induced by the release of lower, more physiologically relevant, amounts of LIF from differentiating Schwann cells at the site of lesion. More research, perhaps using mice lacking functional gp130 expression in sensory neurons, is required to clarify the role of injury released LIF in regulating SP expression in DRG neurons following nerve injury

4.4.3. VR1.

In chapter three of this thesis, I demonstrated that NGF/TrkA signalling did not appear to be required for the developmental regulation of VR1 mRNA expression in neural crest-derived sensory ganglia *in-vivo*. However, NGF was capable of totally

preventing the culture-induced down-regulation of VR1 mRNA expression that occurs in neonatal trigeminal neurons. I also presented data suggesting that NT-3 suppresses the expression of VR1 mRNA in developing neural crest-derived sensory neurons.

Adult mouse DRG neurons also exhibited a marked drop in VR1 mRNA expression over time in culture (figures 4.9 C, 4.15 B and 4.23 B). The culture-induced reduction in VR1 mRNA expression mirrors the changes that occur in rodent DRG neurons *in-vivo* following sciatic nerve injury (Michael and Priestly, 1999; Michael and Priestly 2002; Fukuoka T et al., 2002; Wendland et al., 2003). Saturating concentrations of NGF reduced the culture-induced loss of VR1 mRNA expression by around 30% (figures 4.9 C, 4.15 B and 4.23 B). This observation is in accordance with previously published data demonstrating that exogenous NGF can promote VR1 mRNA expression in adult rodent DRG neurons *in-vitro* and *in-vivo* (Michael and Priestley, 1999; Shu and Mendell, 1999; Winston et al., 2001). In addition, CFA-induced inflammation has been shown to increase the levels of VR1 protein expressed by TrkA-positive DRG neurons via an increase in the expression of NGF at the site of inflammation (Amaya et al., 2004). It is curious that NGF could completely prevent the culture-induced down-regulation of VR1 mRNA in neonatal trigeminal neurons (chapter three), but could only prevent 30% of the decrease in cultured adult mouse DRG neurons, especially since VR1 expression is almost entirely restricted to a sub-population of TrkA- and CGRP- positive neurons in adult mouse L4/L5 DRG (Zwick et al., 2002). It is possible that the pattern of VR1 expression differs in mouse DRG at other axial levels (as represented in my DRG cultures) and/or in culture, being expressed in both TrkA-positive and TrkA-negative nociceptive neurons. Another explanation to account for the relatively poor ability of NGF to rescue the expression of VR1 mRNA in cultured adult mouse DRG neurons is that the proportion of TrkA-positive, and hence NGF-responsive, neurons decreases in adult DRG cultures over time (see above).

Artemin was also capable of preventing the culture-induced decrease in VR1 mRNA expression in adult mouse DRG cultures, with an efficacy slightly greater than NGF (figures 4.9 C, 4.15 B and 4.23 B). This would suggest, in culture at least, that VR1 mRNA is expressed in a sub-population of neurons that predominantly express both

TrkA and GFR- α 3. Further evidence for this hypothesis is found in figure 4.15B. This figure demonstrates that the addition of saturating levels of both artemin and NGF to cultures did not significantly increase the expression of VR1 mRNA compared to cultures containing either NGF alone or artemin alone. The data presented in this chapter is the first demonstration that artemin can regulate the expression of VR1 in cultured sensory neurons, and raises the possibility that it modulates the neuronal expression of this important determinant of thermal and chemical nociceptive thresholds *in-vivo*.

MSP proved to be as effective as NGF in ameliorating the drop in VR1 mRNA levels in cultured DRG neurons (figures 4.9 C, 4.15 B and 4.23 B). This is the first demonstration that MSP can regulate the expression of VR1 in sensory neurons and adds to the number of functionally important sensory neuron mRNAs that can be transcriptionally regulated by this novel neurotrophic factor. Interestingly, the addition of saturating concentrations of NGF and MSP together to cultures did not enhance VR1 mRNA expression compared to either factor alone, suggesting that all DRG neurons that express TrkA and VR1 mRNA in culture also express RON. The combined effects of MSP and artemin on VR1 mRNA expression were also no greater than either of these two factors alone. In fact, the ability of all three neurotrophic factors combined to regulate the *in-vitro* expression of VR1 mRNA was no greater than any single neurotrophic factor on its own (figure 4.15 B). Since all three neurotrophic factors have a similar efficacy in terms of regulating the expression of VR1 mRNA, this raises the possibility that virtually all adult mouse DRG neurons, that express VR1 mRNA in culture, also express the receptors for all three neurotrophic factors.

The fact that the addition of all three neurotrophic factors together only prevented about 30% of the culture-induced reduction in VR1 mRNA expression raises the possibility that other neurotrophic factors may additionally regulate the expression of VR1 mRNA, both *in-vitro* and *in-vivo*. In the rat, VR1 is expressed in both the NGF-responsive, peptidergic sub-population of nociceptive neurons and the IB4-binding, non-peptidergic, sub-population of nociceptive neurons (Michael and Priestley, 1999; Guo et al., 1999). GDNF has been shown to play a role in the inflammation-induced

up-regulation of VR1 expression in rat DRG (Amaya et al., 2004), and can also ameliorate the culture-induced down-regulation of VR1 mRNA in adult rat DRG neurons (Ogun-Muyiwa et al., 1999; Wendland et al., 2003). In the rat, the GDNF receptor, GFR- α 1 is expressed in 40% of lumbar DRG neurons, and this number increases to 60% following sciatic nerve transection (Kashiba et al., 1998; Bennett et al., 2000). It is not clear whether an up-regulation in GFR- α 1 expression occurs in cultures of rat DRG neurons. It is also not known whether it occurs in the mouse, either following peripheral nerve lesion, or in culture. However, it is possible that some VR1-positive cultured adult mouse DRG neurons expressing TrkA, lose NGF responsiveness over time in culture and gain expression of GFR- α 1, and hence GDNF responsiveness. It is interesting to speculate that the addition of GDNF to adult mouse DRG cultures containing NGF and/or artemin and/or MSP would restore the expression of VR1 mRNA to *in-vivo* levels. Another candidate for a neurotrophic factor that may positively regulate the expression of VR1 mRNA is NT4/5. NT4/5 has been shown to increase the capsaicin sensitivity of cultured adult rat DRG neurons (Shu and Mendell, 1999), presumably by increasing the expression of VR1 (although changes in the phosphorylation status of VR1 can alter its sensitivity to ligands, as discussed in the introduction to chapter 3). NT4/5 signals through the tyrosine kinase receptor TrkB (Klein et al., 1992; Ip et al., 1992), a receptor that is not normally expressed on most VR1-positive sensory neurons. However, NT4/5 can also activate TrkA, albeit with low efficiency (Berkemeier et al., 1991; Hallbook et al., 1991; Ip et al., 1992), and can also signal through the common neurotrophin receptor, p75 (Rodriguez-Tébar et al., 1992), providing a theoretical mechanism whereby it could increase the expression of VR1 in cultured adult mouse DRG neurons.

The effects of LIF on the expression of VR1 mRNA were very similar to the effects of LIF on SP mRNA expression. The addition of LIF alone to cultures did not alter VR1 mRNA expression levels compared to controls. However, LIF could attenuate the ability of NGF, artemin and MSP to promote VR1 mRNA expression, although this is only statistically significant in the case of artemin (figure 4.23 B). This data supports the idea that injury-induced LIF production by Schwann cells may drive some of the changes in gene expression within DRG neurons that accompany peripheral nerve injury.

LIF may not be the only injury-induced factor that drives changes in the expression of VR1 mRNA expression, both *in-vivo* and *in-vitro*. The *in-vivo* and *in-vitro* data presented in chapter three suggested that NT-3 may suppress the expression of VR1 mRNA in neonatal neural crest-derived sensory neurons. This hypothesis is supported by the observation that NT-3 can reverse the thermal hyperalgesia-inducing increase in VR1 mRNA and protein expression that occurs following chronic constriction injury to the adult rat sciatic nerve (Wilson-Gerwing et al., 2005). Indeed, exogenous NT-3 can regulate the expression of a number of genes following axotomy, often in an antagonistic manner to NGF, and can prevent the loss of neurons and satellite cells within DRG following nerve lesion (Karchewski et al., 2002; Jongsma Wallin et al., 2001; Park et al., 2003; Sterne et al., 1998; Wilson-Gerwing et al., 2006; Kuo et al., 2005; Groves et al., 1999). Whilst sciatic nerve lesion reduces the expression of NT-3 within peripheral nerves (Funakoshi et al., 1993; Cai et al., 1998), macrophages and activated T-cells invading the lesion site express NT-3 (Moalem et al, 2000; Sobue et al, 1998), raising the possibility that endogenous NT-3 may play a role in orchestrating the response of DRG neurons to injury. NT-3 may be particularly efficacious as an injury response agent, since it can activate all neurotrophic factor receptors to some degree and can thus exert effects on virtually all DRG neurons (see previous introductory sections for details). Schwann cells in culture express NT-3 (Meier et al, 1999; Cai et al, 1999), as well as LIF, raising the possibility that NT-3 may play a role in regulating the numerous culture-induced changes in gene expression observed in adult mouse DRG cultures.

4.4.4. TTX-resistant sodium channels

In chapter two of this thesis, I demonstrated that NGF/TrkA signalling was required for the initial induction of Nav1.8 and Nav1.9 mRNA expression in neural crest-derived sensory ganglia *in-vivo*. In chapter three, I showed that NGF is required for the correct developmental expression of both TTX-resistant sodium channel mRNAs in neural crest-derived sensory ganglia, but not placode-derived nodose ganglia. Moreover, I demonstrated that NGF could partially prevent the culture-induced drop in Nav1.8 and Nav1.9 mRNA expression in neonatal trigeminal and nodose neurons.

In agreement with my previous data, NGF could ameliorate the reduction in TTX-resistant sodium channel mRNAs that also occurred in cultured adult mouse DRG neurons, thereby partially restoring the “un-injured” phenotype to cultured nociceptive neurons (figures 4.9A and B, 4.16A and B and 4.24A and B). The culture induced drop in Nav1.8 and Nav1.9 expression mirrors the effects of peripheral nerve lesion *in-vivo* (Okuse et al., 1997; Cummins et al., 2000; Tate et al., 1998; Dib-hajj et al., 1998a, 1999; Sleeper et al., 2000; Decosterd et al., 2002). My data is in agreement with previously published data demonstrating that NGF can positively regulate the expression of Nav1.8 mRNA and peptide both in adult rat DRG cultures (Black et al., 1997; Fjell et al., 1999a) and *in-vivo* (Dib-Hajj et al., 1998b Fjell et al., 1999b and c). A previous publication failed to demonstrate that NGF could regulate the expression of Nav1.9 mRNA (Fjell et al., 1999a). However, the authors used a semi-quantitative method for measuring Nav1.9 mRNA expression that does not have sufficient resolution to determine small changes in gene expression. In contrast, the same authors have demonstrated an increased expression of Nav1.9 mRNA within DRG in mice over-expressing NGF in the skin (Fjell et al., 1999b).

NGF could almost completely prevent the culture-induced drop in Nav1.8 mRNA expression in neonatal trigeminal neurons (chapter three of this thesis) In contrast, saturating levels of NGF were only capable of preventing 15% of the drop in Nav1.8 mRNA expression levels that occurs within cultured adult DRG neurons. The time course data that I presented in chapter three suggests that Nav1.8 mRNA is significantly more widely expressed in neonatal neural-crest derived sensory ganglia compared to adult ganglia. P0 sensory ganglia also contain more NGF-responsive neurons than adult ganglia (Molliver and Snider, 1997; Molliver et al., 1997) suggesting a considerable overlap between TrkA- and Nav1.8-positive neurons in neonatal sensory ganglia. In adult DRG, Nav1.8 mRNA is expressed in virtually all A- δ and C-fibre (and some A- β fibre) nociceptive neurons *in-vivo* (around 65% of DRG neurons), whereas TrkA is only expressed in 35% of neurons. The majority of Nav1.8-positive adult DRG neurons that do not express TrkA are IB4-positive, express Ret and are responsive to members of the GDNF family of neurotrophic factors (Djoughri et al., 2003; Fang et al., 2005; Averill et al., 1995; Molliver et al.,

1997; Michael and Priestley, 1999; Fjell et al., 1999 (a); Amaya et al., 2000; Orozco et al., 2001). Indeed, GDNF has been shown to up-regulate the expression of Nav1.8 mRNA, both *in-vitro* and also *in-vivo* following sciatic nerve lesion (Fjell et al., 1999a; Cummins et al., 2000). Therefore, it is not surprising that NGF is unable to restore Nav1.8 mRNA levels to *in-vivo* levels in adult mouse DRG cultures, especially in light of the changes in neurotrophic factor receptor expression that are likely to occur in culture. As outlined above such changes include a decrease in NGF responsive cells and an increase in GDNF responsive cells over the culture period. It is interesting to speculate that a combination of NGF and GDNF would maintain Nav1.8 mRNA expression at *in-vivo* levels in cultured adult mouse DRG neurons.

NGF appeared to be even less effective in maintaining Nav1.9 mRNA levels at time 0 levels in culture than it was in maintaining Nav1.8 mRNA expression. A situation that mirrors the data from neonatal trigeminal neurons, presented in chapter three. In contrast to Nav1.8, Nav1.9 is exclusively expressed in nociceptive C-fibre neurons within sensory ganglia, with no expression in either TrkA-positive, A- δ nociceptors or the rare A- β nociceptors (Fang et al., 2006). Within C-fibre nociceptive neurons, Nav1.9 is expressed in both TrkA-positive and IB4-positive sub-populations (Amaya et al., 2000). However, Nav1.9 expression is positively correlated with IB4 expression, unlike Nav1.8 whose expression is positively correlated with TrkA expression (Fang et al., 2005 and 2006). Thus, there are significantly less TrkA-positive neurons expressing high levels of Nav1.9 mRNA than Nav1.8 mRNA *in-vivo*. Conversely, many GDNF responsive, IB4-positive neurons express high levels of Nav1.9 and are not responsive to NGF. The overlap between TrkA and Nav1.9 mRNA expression is likely to be further reduced *in-vitro*, accounting for the poor efficacy of NGF in promoting the expression of Nav1.9 mRNA in adult mouse DRG cultures. Once again, as in the case of Nav1.8, GDNF has been previously shown to positively regulate the expression of Nav1.9 (Fjell et al., 1999a; Cummins et al., 2000), raising the possibility that a combination of saturating concentrations of NGF and GDNF added to cultures could fully maintain Nav1.9 mRNA expression at *in-vivo* levels.

Artemin was also able to partially attenuate the culture-induced drop in TTX-resistant sodium channel mRNAs (figures 4.9A and B, 4.16A and B and 4.24A and B). The efficacy of artemin and NGF in promoting Nav1.8 and Nav1.9 mRNA expression is similar, reflecting the broad overlap in expression between TrkA and GFR- α 3 in adult mouse DRG neurons. Artemin has previously been shown to promote the expression of Nav1.8 protein following spinal nerve ligation (Gardell et al., 2003), however, my data is the first demonstration that artemin can modulate the expression of Nav1.9.

The demonstration that MSP could positively modulate the expression of both Nav1.8 and Nav1.9 mRNAs, is also a novel finding. On the whole MSP has a similar efficacy to NGF and artemin in regulating the expression of TTX-resistant sodium channel mRNAs, suggesting, as for previous genes above, that there is a fairly broad overlap in the expression of TrkA, GFR- α 3 and RON by cultured adult mouse DRG neurons. This hypothesis is substantiated by the results from experiments where different combinations of saturating concentrations of neurotrophic factors were added to adult mouse DRG cultures (figures 4.16A and B). Whilst the combination of artemin and NGF could significantly increase the expression of both Nav1.8 and Nav1.9 mRNAs compared to either factor alone, the increase in expression of both mRNAs compared to cultures with a single factor was only around 20%, suggesting that only a small proportion of Nav1.8- or Nav1.9-positive neurons express either TrkA in the absence of GFR- α 3 or GFR- α 3 in the absence of TrkA. The data for cultures containing a combination of artemin and MSP is very similar to the data for cultures containing NGF and artemin. Once again, adding both MSP and NGF to cultures did not significantly increase the expression of Nav1.8 or Nav1.9 mRNAs above cultures containing either MSP or NGF alone. This data adds weight to the hypothesis that TrkA and RON are co-expressed in almost entirely overlapping sub-populations on cultured adult mouse DRG neurons.

LIF did not reduce the expression of Nav1.8 or Nav1.9 mRNAs in adult mouse DRG neurons compared to control cultures (figure 4.24 A). Whilst LIF did have a tendency to reduce the ability of NGF, artemin and MSP to promote the expression of both TTX-resistant sodium channel mRNAs, this was only statistically significant in the

cases of Nav1.8 mRNA in cultures containing MSP and Nav1.9 mRNA in cultures containing NGF. Nonetheless, the data raises the possibility that LIF may play a role in regulating the nerve lesion -induced drop in TTX-resistant sodium channel mRNA *in-vivo*. As discussed above, the effects of LIF in culture may be masked by LIF secreted from Schwann cells within the cultures, especially if Nav1.8 and Nav1.9 mRNAs are very sensitive to regulation by LIF. Once again, culturing adult DRG neurons with a LIF or gp130 function blocking antibody may shed more light on the role of Schwann cell-derived LIF in regulating the expression of TTX-resistant sodium channel mRNAs both *in-vitro*, and *in-vivo*, following peripheral nerve lesion.

4.4.5. Galanin

In chapter three, the analysis of transgenic mice produced data suggesting that NGF may promote the expression of galanin mRNA in neonatal neural-crest derived sensory neurons within trigeminal and dorsal root ganglia. This data was surprising, since it contradicted a number of publications demonstrating that a reduction in the availability of target field-derived NGF was at least partially responsible for the dramatic increase in galanin expression in adult DRG neurons following peripheral nerve lesion. Moreover, exogenous NGF has been shown to reduce galanin expression in adult DRG neurons, both in culture and *in-vivo* following peripheral nerve lesion (Verge et al., 1995; Kerekes et al., 1997; Ozturk and Tonge, 2001; Shadiack et al., 2001).

In contrast to chapter three, the data presented in this chapter is in agreement with previously published data documenting the regulatory effects of NGF on galanin mRNA expression in adult DRG neurons. Galanin mRNA was expressed at very low levels in freshly dissociated adult mouse DRG (figures 4.10A, 4.17A and 4.25A), corresponding to the restricted expression of galanin in 2-5% of, mainly small-diameter, adult rodent DRG neurons *in-vivo* (Chang et al., 1985; Skotfitsch and Jacobowitz, 1985; Xu et al., 1996; Ma and Bisby, 1999). Galanin mRNA expression increased more than 20-fold in adult mouse DRG neurons over a 96 hour culture period. This is in accordance with previous *in-vitro* studies on adult mouse and rat DRG neurons (Kerekes et al., 1997; Ozturk and Tonge, 2001). A dramatic increase in

galanin mRNA and peptide expression is also observed in adult rodent DRG following peripheral nerve injury (e.g. Hokfelt et al., 1987; Villar et al., 1989; Ma and Bisby, 1997, 1999; Zhang et al., 1998). The up-regulation of galanin expression in rat DRG following sciatic nerve injury occurs in both injured and “spared” neurons of all sizes and modalities (Ma and Bisby, 1997; 1999). A number of elegant transgenic studies suggest that lesion-induced galanin acts as a neuroprotective, anti-nociceptive agent (reviewed in Holmes et al, 2005).

The addition of 10ng/ml NGF to cultures prevented about 40% of the culture-induced up-regulation of galanin mRNA (figures 4.10 A, 4.17 A and 4.25 A), an observation that is in agreement with previous literature documenting the regulation of galanin mRNA and peptide expression by NGF. The ability of NGF to prevent 40% of the culture-induced increase in galanin mRNA expression suggests that many of the small-diameter cells showing *de-novo* galanin expression in culture are TrkA-positive.

Artemin was also able to attenuate the increase in galanin mRNA expression in cultured DRG neurons, with an efficacy similar to NGF (figures 4.10A, 4.17A and 4.25A). The data suggests that most of the galanin-positive neurons that express TrkA and respond to NGF in culture also express GFR- α 3 and respond to artemin. This hypothesis is supported by the observation that the addition of saturating concentrations of both artemin and NGF together to adult mouse DRG cultures only marginally decreases the expression of galanin mRNA compared to either factor alone. Moreover, the further decrease in galanin expression in cultures containing both NGF and artemin is only significant compared to cultures containing NGF alone. The observation that artemin can ameliorate the culture-induced increase in galanin mRNA expression is in accordance with recent data demonstrating that artemin can attenuate the increase in galanin peptide expression that occurs in rat DRG neurons following spinal nerve ligation (Gardell et al., 2003)

MSP was also able to partially prevent the culture-induced up-regulation in galanin mRNA in adult mouse DRG neurons (figures 4.10 A, 4.17 A and 4.25 A), although with a slightly lower efficacy than NGF or artemin. The combination of artemin and MSP was significantly more effective in reducing galanin mRNA expression than

either factor alone, reducing the increase in galanin mRNA by around 75%. This suggests that a number of galanin mRNA-positive neurons exist in adult mouse DRG cultures that express either GFR- α 3 or RON (figure 4.17 A). In contrast, MSP-responsive neurons expressing galanin would appear to be entirely a subset of NGF-responsive, galanin-positive neurons, since the addition of both factors together to cultures is no more effective than NGF alone in reducing galanin mRNA levels.

The addition of all three neurotrophic factors to cultures also inhibits the culture-induced increase in galanin mRNA by around 75%. The residual increase in galanin mRNA levels in the presence of all three neurotrophic factors is probably due to a combination of increased galanin mRNA expression in IB4-positive, TrkA-negative C-fibre neurons together with increased galanin mRNA expression in medium to large-diameter, myelinated neurons (Ma and Bisby, 1997; 1999). The former neuronal population expresses functional receptors for GDNF *in-vivo* (Molliver and Snider, 1997; Molliver et al., 1997; Kashiba et al., 1998; Bennett et al., 2000) and may well respond to GDNF in culture by decreasing their expression of galanin mRNA. GFR- α 1 expression is up-regulated in a sub-population of medium to large-diameter DRG neurons following nerve injury (Kashiba et al., 1998; Bennett et al., 2000). If GFR- α 1 is also up-regulated in this neuronal sub-population in culture, GDNF may also be able to ameliorate the culture-induced increase in galanin mRNA in larger myelinated DRG neurons. Indeed, GDNF can attenuate the increase in galanin peptide that occurs in both large- and small-diameter rat DRG neurons following spinal nerve ligation (Wang et al., 2003).

Interestingly, BDNF has also been shown to ameliorate culture-induced increases in galanin mRNA expression in rat DRG neurons (Kerekes et al., 1997). The BDNF receptor, TrkB, is predominantly expressed by medium to large-diameter myelinated DRG neurons in the adult rodent (Karchewski et al., 1999; Kashiba et al., 2003), raising the possibility that BDNF attenuates increases in galanin mRNA expression in this population of neurons *in-vitro*.

NT-3 is also a candidate neurotrophic factor for attenuating the culture-induced increase in the expression of galanin mRNA in large, myelinated, TrkC-positive

proprioceptive neurons, since it has been shown to reduce the expression of galanin mRNA in adult rat DRG neurons following chronic constriction injury (Wilson-Gerwing and Verge, 2006).

The presence of LIF significantly enhanced the culture-induced expression of galanin mRNA in adult mouse DRG cultures compared to control cultures (figure 4.25A). LIF also significantly reduced the ability of NGF, artemin and MSP to attenuate the expression of galanin mRNA in culture. This data is in accordance with several previous publications that demonstrate that LIF increases the expression of galanin mRNA within adult DRG neurons, both *in-vitro* and *in-vivo* (Sun and Zigmond, 1996; Corness et al., 1996, 1998; Thompson et al., 1998; Kerekes et al., 1999; Ozturk and Tonge, 2001). Whilst these publications have demonstrated that LIF antagonises the effects of NGF in suppressing the expression of galanin, the data presented here is the first demonstration that LIF directly antagonises the ability of MSP and artemin to down-regulate the expression of galanin.

4.4.6. PACAP

The levels of PACAP mRNA expressed by adult mouse DRG neurons consistently increased more than ten-fold over 96 hours in culture (figures 4.10B, 4.17B and 4.25B). The low level of PACAP mRNA in DRG neurons that was observed at time 0 reflects the fact that PACAP is only expressed in 10% of adult rodent DRG neurons, predominantly in a sub-population of CGRP- and SP-positive small-diameter neurons (Moller et al., 1993; Mulder et al., 1994). The culture-induced increase in PACAP mRNA expression mirrors the effects of sciatic nerve transection and nerve compression injury (Zhang et al., 1995; Zhang et al., 1996; Jongsma-Wallin et al., 2001; Pettersson et al., 2004). The former injury leads to a marked increase in the number of medium- to large-diameter L4/L5 DRG neurons expressing PACAP mRNA and protein, whilst sciatic nerve compression increases the expression of PACAP mRNA and protein in both large and small lumbar DRG neurons.

The addition of 10ng/ml NGF to cultures attenuated the culture-induced increase in PACAP mRNA expression. However, the efficacy of NGF in reducing PACAP expression was low, and was not statistically significant in all series of culture experiments (figure 4.17 B). The ability of NGF to partially prevent the culture-induced increase in PACAP mRNA expression within adult DRG neurons was an unexpected result, as NGF has previously been shown to play a role in the inflammation-induced increase in PACAP expression in TrkA-positive nociceptive neurons (Zhang et al., 1998; Jongsma-Wallin et al., 2003). Moreover, exogenous NGF increases the expression of PACAP mRNA and protein within rat TrkA-positive lumbar DRG neurons, both in the intact animal and following sciatic nerve transection (Jongsma-Wallin et al., 2001). However, NGF attenuates the increase in PACAP mRNA and peptide that occurs in TrkC-positive proprioceptive neurons of L4/L5 DRG following sciatic nerve transection (Jongsma-Wallin et al., 2001). Presumably, NGF regulates PACAP expression in TrkC-positive neurons via the common neurotrophin receptor, p75, since there are no reports of *de-novo* TrkA expression within proprioceptive neurons following nerve injury. Alternatively, NGF may regulate PACAP mRNA expression within proprioceptive neurons by a paracrine mechanism. Whatever the mechanism, the fact that NGF down-regulates PACAP expression in proprioceptive neurons following nerve injury, explains the data presented in figures 4.10B, 4.17B and 4.25 B. It is likely that only 10% of DRG neurons express TrkA and PACAP mRNA in culture (Moller et al., 1993; Mulder et al., 1994), limiting the ability of NGF to increase the expression of PACAP mRNA within RNA samples extracted and purified from all cultured neurons. The increase in PACAP mRNA within TrkA-positive nociceptive neurons observed may be more than offset by the ability of NGF to attenuate the increase in PACAP mRNA within cultured proprioceptive neurons. A simple way to test this hypothesis would be to culture L4/L5 DRG neurons alongside DRG neurons from lower-thoracic axial levels. If the hypothesis is correct, PACAP mRNA levels should be increased by NGF in the cultures from thoracic DRG, containing few proprioceptive neurons, and decreased by NGF in cultures from L4/L5 DRG that contain relatively high numbers of proprioceptive neurons.

Artemin and MSP reduced PACAP mRNA expression in adult mouse DRG cultures by 15% compared to control cultures. This reduction, however, does not reach

statistical significance in any set of cultures. The limited efficacy of MSP and artemin in reducing PACAP mRNA expression is in accordance with the observation that PACAP mRNA expression is restricted to a small subset of nociceptive neurons in adult rodent DRG and its expression is predominantly up-regulated within medium- to large-diameter myelinated neurons following peripheral nerve transection (Moller et al., 1993; Mulder et al., 1994; Zhang et al., 1995; Zhang et al., 1996; Jongsma-Wallin et al., 2001). The majority of larger myelinated neurons do not express GFR- α 3 and the data presented in this chapter supports the idea that RON expression is also restricted to nociceptive neurons. The data suggests that a sub-population of small-diameter nociceptive neurons express PACAP mRNA and receptors for artemin and /or MSP in control DRG cultures, and that artemin and MSP can significantly attenuate PACAP mRNA expression within this sub-population of neurons. If this were the case, this would be the first example of artemin and MSP having a different effect to NGF on regulating gene expression within nociceptive sensory neurons. Whilst this hypothesis would require a detailed immuno-cytochemistry study for verification, the data from the “additive” experiments gives it support. A combination of saturating concentrations of artemin and MSP reduced the culture-induced increase in PACAP mRNA expression by a statistically significant 30% (figure 4.17B). The additive effect of MSP and artemin suggests that most nociceptive DRG neurons expressing PACAP mRNA in culture express either GFR- α 3 or RON, but not both (or, of course, neither receptor).

The addition of 50 ng/ml LIF to adult DRG cultures reduced the expression of PACAP mRNA by 25% compared to control cultures, a reduction that is statistically significant (figure 4.25B). Moreover, LIF enhances the limited ability of artemin and MSP, but not NGF, to attenuate the culture-induced up-regulation of PACAP mRNA, although this effect does not quite reach statistical significance. The ability of LIF to reverse the “axotomised phenotype” in the case of PACAP mRNA is markedly different to the effects that it exerts on the majority of other genes investigated in this chapter. Functional receptors for LIF are not expressed on the myelinated, larger-diameter DRG neurons that predominantly up-regulate PACAP mRNA expression following nerve transection (Zhang et al., 1995; Zhang et al., 1996; Jongsma-Wallin et al., 2001; Thompson et al., 1997). However, the data from cultures containing

artemin and MSP suggests that a sub-population of small-diameter nociceptive neurons express PACAP mRNA in control DRG cultures. This raises the possibility that LIF decreases the expression of PACAP mRNA in this neuronal sub-population. The possibility also exists that LIF attenuates the culture-induced *de-novo* expression of PACAP mRNA within large-diameter myelinated DRG neurons via a paracrine mechanism involving the release of a second neurotrophic factor from small-diameter DRG neurons.

4.4.7. ATF3 and DINE

ATF3 and DINE are both ostensibly injury induced factors in the adult peripheral sensory nervous system. Both are expressed at extremely low levels in undamaged adult DRG neurons, but are rapidly induced following various peripheral nerve lesions. DINE mRNA is predominantly up-regulated in TrkA-positive, IB4-negative nociceptive neurons, and DINE expression is often coincident with *de-novo* galanin expression. No detectable DINE expression is found in glial cells following nerve injury (Kiryu-Seo et al., 2000; Kato et al., 2002; Ohba et al., 2004; Nagata et al., 2006). In contrast, ATF3 mRNA and protein expression is induced within the majority of damaged neurons, of all sizes and modalities, following peripheral nerve damage (Tsujino et al., 2000; Tsuzuki et al., 2001; Obata et al., 2003; Wang et al., 2003; Averill et al., 2004) ATF3 is also induced within Schwann cells following nerve lesion (Hunt et al., 2004).

The developmental time course data for ATF3 and DINE mRNAs reinforces the hypothesis that both factors play a negligible role in maintaining the functions and phenotype of healthy sensory neurons (figures 4.1B and C) Both mRNAs were barely detectable in either neural crest- or placode-derived sensory neurons at ages between E16 and the adult.

In accordance with the *in-vivo* nerve injury data, ATF3 and DINE mRNAs were both markedly up-regulated in cultured adult mouse DRG neurons over a 96 hour culture period (figures 4.11A and B, 4.18A and B and 4.26A and B). This is the first data showing that these two genes are induced in adult DRG neurons by cell culture. The

culture-induced increase in ATF3 mRNA over the 96 hour culture period (2- to 3-fold) was significantly less than the culture induced increase in DINE mRNA (30-fold). This observation is a little surprising, since, based on *in-vivo* nerve injury studies, DINE mRNA is only likely to be up-regulated in a relatively small sub-population of cultured DRG neurons, whereas ATF3 is likely to be up-regulated in the majority of neurons and contaminating Schwann cells. Figure 4.7C provides a partial explanation for this observation. The rise in ATF3 mRNA levels appears to be short lived, peaking at 48 hours of culture and falling by 96 hours in culture. This is not the case for DINE mRNA (data not shown). Indeed, ATF3 mRNA was the only example amongst all of the mRNAs assayed showing a greater culture-induced change in gene expression at 48 hours than 96 hours (figures 4.7A and B and data not shown). ATF3 mRNA was also the only mRNA assayed that showed significantly higher expression (> 5-fold) in freshly dissociated neurons compared to the intact DRG (data not shown), suggesting that the dissection and dissociation procedure initiates rapid ATF3 mRNA induction. Thus, if the induction of ATF3 mRNA was assessed between intact DRG and neurons after 48 hours in culture the magnitude of the culture-induced induction would be greater than that of DINE mRNA.

NGF, artemin and MSP were all able to partially attenuate the culture-induced up-regulation of ATF3 and DINE mRNAs, although in the case of DINE mRNA the effects of the three neurotrophic factors are not always statistically significant (figures 4.11A and B, 4.18A and B and 4.26A and B). Exogenous NGF has previously been shown to reduce ATF3 protein expression in small-diameter lumbar DRG neurons following sciatic nerve transection (Averill et al., 2004). My data is the first demonstration that NGF can regulate the expression of ATF3 mRNA in cultured adult rodent sensory neurons. Although it has not been established whether NGF can ameliorate DINE mRNA expression in nociceptive DRG neurons following peripheral nerve lesion, sequestering target field-derived NGF with an NGF blocking antibody is sufficient to induce DINE mRNA expression within small-diameter DRG neurons in the adult rat (Kato et al., 2002). Withdrawal of NGF from neonatal rat DRG explant cultures also induces DINE mRNA expression (Kato et al., 2002). Neither artemin nor MSP have been previously shown to regulate the expression of either ATF3 or DINE mRNAs in adult rodent DRG neurons *in-vitro* or *in-vivo*.

Artemin was consistently the most effective of the three neurotrophic factors in reducing both DINE and ATF3 mRNA levels in all experiments here. Whilst both TrkA and GFR- α 3 are predominantly expressed by small-diameter nociceptive neurons *in-vivo*, the number of DRG neurons expressing TrkA is double the number of neurons expressing GFR- α 3. It might therefore be anticipated that NGF would be more effective than artemin in regulating the expression of neuronal genes (like DINE) whose expression is mainly restricted to nociceptive neurons (Averill et al., 1995; Molliver et al., 1997; Michael and Priestley, 1999; Orozco et al., 2001). However, as discussed above, unpublished data from my laboratory suggests that the number of NGF responsive neurons decreases in adult mouse DRG cultures, whilst the number of artemin responsive neurons increases. This phenomenon may partially account for the better than expected efficacy of artemin in regulating DINE and ATF3 mRNA compared to NGF. NGF and MSP appeared to show a similar efficacy in reversing culture-induced changes in ATF3 and DINE mRNA expression.

The addition of saturating combinations of either artemin and NGF or artemin and MSP to adult mouse DRG cultures did not significantly decrease the levels of ATF3 mRNA or DINE mRNA beyond the levels found with artemin alone (figure 4.18A). Similarly, the addition of all three neurotrophic factors to cultures is no more effective in attenuating the culture induced increase in DINE and ATF3 mRNAs than artemin alone. This data suggests that more ATF3 and DINE expressing cells express GFR- α 3 than express TrkA or RON. It could be postulated therefore that NGF- and MSP-responsive cells expressing these two injury inducible factor mRNAs are a subset of artemin responsive cells. The addition of saturating concentrations of NGF and MSP to cultures was also no more effective at reducing ATF3 and DINE mRNA levels than either factor alone. The similar efficacy of NGF and MSP in regulating gene expression, and the lack of an additive effect of the two neurotrophic factors when added to cultures together, is a recurrent theme of the data presented in this chapter and suggests that NGF and MSP responsive adult mouse DRG neurons are a broadly over-lapping population, at least in culture.

The addition of either artemin alone or artemin together with any combination of the other two neurotrophic factors reduced the levels of ATF3 mRNA to time 0 levels. However, based on *in-vivo* data, ATF3 is likely to be up-regulated in the majority of cultured adult mouse DRG neurons (Averill et al., 2004), whereas GFR- α 3 expression, and hence artemin responsiveness, is likely to be restricted to a sub-population of small-diameter nociceptive neurons (Gardell et al, 2003). This raises the possibility that the apparent 100% efficacy of artemin in preventing the culture-induced up-regulation of ATF3 mRNA is an artifact of the fact that dissection and dissociation of DRG induces significant ATF3 mRNA expression, thereby giving an artificially high time 0 level of ATF3 mRNA expression. GDNF has been shown to reduce the levels of ATF3 in both small- and large-diameter DRG neurons following peripheral nerve lesion (Wang et al., 2003; Kato et al., 2004), and is therefore a good candidate neurotrophic factor for reducing ATF3 mRNA expression in cultured DRG neurons that are not responsive to NGF, artemin or MSP.

DINE mRNA has been reported to be induced in mainly TrkA-positive, nociceptive DRG neurons following peripheral nerve injury (Kato et al., 2002). Since this neuronal sub-population is responsive to NGF and artemin in culture (for example, see CGRP mRNA expression data in this chapter), it is curious that a combination of these neurotrophic factors were not more effective in reducing the culture-induced increase in DINE mRNA expression. It seems likely that the up-regulation in DINE mRNA is not entirely restricted to TrkA-positive nociceptive neurons in culture.

The addition of 50ng/ml LIF to adult mouse DRG cultures did not alter the levels of ATF3 mRNA compared to control cultures (figure 4.26 A). Similarly, the addition of LIF to cultures containing NGF, artemin or MSP did not alter ATF3 mRNA compared to cultures containing either of the three neurotrophic factors alone. The data suggests that LIF does not play a role in inducing ATF3 mRNA expression following nerve injury. This observation is not that surprising, since functional LIF receptors are restricted to small-diameter, nociceptive neurons in the adult rodent (Thompson et al., 1997), whereas ATF3 is induced in neurons of all sizes and modalities following peripheral nerve lesion (Averill et al., 2004). In the absence of published data demonstrating that ATF3 expression is positively-regulated by another injury-induced

neurotrophic factor following nerve lesion, it would seem that neurotrophic factor withdrawal drives ATF3 expression. However, this hypothesis does not fit easily with the extremely rapid onset of ATF3 mRNA induction compared to the other genes analysed in this chapter. Further work needs to be done to establish the factors that drive the expression of ATF3 in damaged neurons.

Surprisingly, the addition of LIF to adult mouse DRG cultures partially prevented the culture-induced up-regulation of DINE mRNA observed in control cultures. Moreover, LIF significantly increased the effectiveness of artemin, NGF and MSP in ameliorating the increase in DINE mRNA expression (figure 4.26 B). This is the first data suggesting that LIF attenuates axotomy/culture induced changes in gene expression in nociceptive adult DRG neurons that express functional LIF receptors. This observation is a marked contrast to the apparent ability of LIF to enhance or drive the “axotomised phenotype” in nociceptive neurons that has been observed for many of the genes analysed in this chapter and has also been reported in several previous publications (Mulderry, 1994; Corness et al., 1996, 1998; Ozturk and Tonge, 2001; Kerekes et al., 1999; Sun and Zigmond, 1996).

The data presented in figure 4.26 B conflicts with previous data demonstrating that the application of LIF to rat sciatic nerves induced DINE mRNA expression in lumbar DRG, as measured by semi-quantitative RT-PCR and in-situ hybridization (Kato et al., 2002). The application of a gp130 blocking antibody was also shown to reduce DINE mRNA expression in DRG following sciatic nerve transection. Additionally, *in-vitro* experiments, using explanted 1day old rat DRG, demonstrated that LIF could increase DINE mRNA expression and that the increased DINE mRNA expression could be antagonised by a gp130 blocking antibody. The data in this publication was not convincing, since merely exposing the sciatic nerve to allow the application of LIF induced significant DINE mRNA expression and LIF only increased this expression 1.3-fold. Similarly, the application of a blocking gp130 antibody to transected sciatic nerve only reduced DINE mRNA expression by 20% compared to controls. As discussed above, explant cultures are not a good model of axotomy and nerve injury in the adult compared to dissociated cultures, especially with neonatal ganglia. Moreover, the changes in DINE mRNA expression in explant cultures containing LIF were also only 1.2- to 1.3-fold, in accordance with the *in-vivo*

experiments. Neither semi-quantitative RT-PCR nor in-situ hybridization are accurate enough techniques to measure the small changes in DINE mRNA expression that occurred in both the *in-vivo* and *in-vitro* studies presented in this publication.

The interpretation of the data presented in figure 4.26 B is complicated by the likelihood that control cultures will contain LIF as a result of contaminating Schwann cells. Clearly, further experiments are needed, using LIF together with anti-LIF and anti-gp130 blocking antibodies, to determine whether LIF really does antagonise the culture-induced up-regulation of DINE mRNA in adult mouse DRG neurons.

4.4.8. P2X3

The time course of P2X3 mRNA expression revealed that P2X3 mRNA is expressed in a similar developmental pattern in nodose, trigeminal and dorsal root ganglia (figure 4.1 A). In all three ganglia, P2X3 mRNA levels were highest in the embryonic and neonatal period and a significant drop in expression occurred between P5 and the adult. The drop in the expression of P2X3 mRNA was most marked in the two neural-crest derived sensory ganglia. This data is in agreement with previously published observations of P2X3 mRNA and protein expression in the developing and adult peripheral sensory nervous system. P2X3 protein is found in the majority of sensory neurons within embryonic trigeminal, dorsal root and nodose ganglia (Boldogkoi et al., 2002; Ruan et al., 2004). In contrast, within lumbar DRG of the *adult* rodent, P2X3 protein and mRNA are localised to a sub-population of IB4-positive, small-diameter neurons. Additional P2X3 expression is also observed within a sub-population TrkA- and CGRP-positive C- and A- δ fibre nociceptors in more rostral DRG. P2X3 expression is also restricted to predominantly small-diameter, IB4-positive neurons in the adult trigeminal and nodose ganglia (Vulchanova et al., 1998; Bradbury et al., 1998; Ramer et al., 2001; Boldogkoi et al., 2002; Ruan et al., 2004).

Cultured adult mouse DRG neurons showed no significant alteration in the levels of P2X3 mRNA expression over time in culture (figures 4.12 A, 4.19 A and 4.27A), suggesting that nerve injury does not affect the expression of this gene. However, peripheral nerve lesion has been shown to induce P2X3 mRNA and protein

expression in “spared”, predominantly small-diameter, neurons and decrease P2X3 mRNA and protein expression in damaged, ATF3-positive, neurons (Eriksson et al., 1998; Novakovic et al., 1999; Bradbury et al., 1998; Tsuzuki et al., 2001; Kage et al., 2002; Gardell et al., 2003). Since dissection and dissociation of DRG for culture emulates axotomy, an injury paradigm that damages all DRG neurons, it would be anticipated that P2X3 mRNA expression would increase over time in culture. P2X3 mRNA is unique in the context of this study, in that it is the only mRNA analysed that does not markedly change its levels of expression over time in control cultures to mirror the changes in gene expression that occur *in-vivo* following nerve lesion-induced neuronal damage. This would suggest that restricted access to target field-derived neurotrophic factors is not the major driving force behind the fall in expression of P2X3 mRNA in injured DRG neurons *in-vivo*. Rather, the data tends to suggest that lesion-induced production and release of an as yet unidentified neuroactive molecule/protein, that is not present in culture, is responsible for the injury-induced reduction in P2X3 expression.

Exogenous GDNF and artemin can both ameliorate the reduction in P2X3 expression in damaged adult DRG neurons (Bradbury et al., 1998; Wang et al., 2003; Gardell et al., 2003) and exogenous GDNF and NGF can induce P2X3 expression in adult DRG neurons in the absence of peripheral nerve injury (Ramer et al., 2001). This data raises the possibility that an increased availability of these three neurotrophic factors may induce the increase in P2X3 expression observed in “spared” neurons following peripheral nerve lesion. Curiously, NGF and artemin were unable to promote the expression of P2X3 mRNA in adult mouse DRG cultures (figures 4.12 A, 4.19 A and 4.27A), a rare example of the *in-vivo* regulation of gene expression within sensory neurons not being mirrored *in-vitro*. MSP was also ineffective in regulating the expression of P2X3 mRNA in cultured adult DRG neurons.

The addition of LIF to adult mouse DRG cultures did not significantly alter the expression of P2X3 mRNA, either in the presence or absence of the other three neurotrophic factors (figure 4.27A). This novel data strongly suggests that injury induced LIF is not responsible for the decrease in P2X3 expression that occurs in damaged, ATF3-positive, neurons following peripheral nerve lesion.

4.4.9. TTX-sensitive sodium channels

Nav1.6 mRNA expression levels increased between E16 and P5 in trigeminal and dorsal root ganglia, before dropping to slightly lower levels in the adult. In nodose ganglia, Nav1.6 mRNA expression peaked at P0 and there was a more substantial fall in the levels of Nav1.6 mRNA between neonatal and adult ages, so that the levels of Nav1.6 mRNA in the adult ganglia were similar to those at E16 (figure 4.2A). Nav1.6 is expressed at high levels at the nodes of Ranvier in mature myelinated sensory neurons where it is responsible for the high repetitive firing rates, lack of response to slow depolarising stimuli and resurgent sodium currents that are characteristic of myelinated sensory neurons (Cummins et al., 1998; Cummins et al., 2001; Herzog et al., 2003a; Cummins et al., 2005). Therefore, it is not surprising that Nav1.6 was expressed at high levels in adult neural-crest derived sensory ganglia and that its expression pattern is in accordance with the functional development of myelinated sensory neurons. Nav1.6 is also expressed at low levels in a uniform distribution along the axons of C-fibre non-myelinated sensory neurons (Black et al., 2002). The significantly lower levels of Nav1.6 mRNA in nodose ganglia compared to trigeminal and dorsal root ganglia may reflect a greater number of nociceptive (*unmyelinated*) C-fibres and a smaller number of low threshold, A- β fibre mechanoreceptors in this ganglion compared to neural-crest derived sensory ganglia.

Nav1.7 is found in both large and small sensory neurons of the developing rodent DRG, whereas Nav1.7 expression is predominantly localised to small-diameter, nociceptive sensory neurons in the *adult* DRG (Felts et al., 1997; Black et al., 1996; Gould et al., 2000; Djouhri et al., 2003). The increasingly restricted expression of Nav1.7 is reflected in the developmental expression pattern of its mRNA (figure 4.2B). Decreasing levels of Nav1.7 mRNA were observed in all three ganglia as development proceeds, with expression levels in the adult approximately 50% of those at E16.

The levels of both TTX-sensitive sodium channel mRNAs expressed within adult mouse DRG neurons fell significantly over 96 hours in culture, with the drop being 5 fold in most sets of cultures (figures 4.12A and B, 4.19B, 4.20A, 4.27B and 4.28A). The levels of Nav1.6 and Nav1.7 mRNAs expressed by adult L5 DRG neurons also falls following L5 spinal nerve ligation (Kim et al., 2002). Interestingly, the drop in both TTX-sensitive mRNAs was restricted to damaged neurons, as “spared” L4 DRG neurons showed no alteration in their expression of either mRNA (Kim et al., 2002). Therefore, my results, showing a fall in the levels of Nav1.6 and Nav1.7 mRNAs in “axotomised” cultured DRG neurons, agree with the previously published literature.

NGF, artemin and MSP were all ineffective at reproducibly attenuating the culture-induced decrease in Nav1.6 and Nav1.7 mRNA expression within DRG neurons in a statistically significant manner. Receptors for NGF and artemin are not expressed on the majority of myelinated neurons that show the highest levels of Nav1.6 mRNA expression *in-vivo* (Cummins et al., 1998; Averill et al., 1995; Molliver et al., 1997; Michael and Priestley, 1999; Orozco et al., 2001; Cummins et al., 2001 Black et al., 2002). If this *in-vivo* expression of TrkA, GFR- α 3 and Nav1.6 mRNAs is reflected in cultured DRG neurons, it would not be surprising that NGF and artemin were unable to regulate the *in-vitro* expression of Nav1.6 mRNA in a statistically significant way. Infact, the medium- to large-diameter myelinated neurons expressing high levels of Nav1.6 mRNA would be expected to respond to BDNF, NT-3, and to a certain extent GDNF, in culture, due to the neurotrophic factor receptors co-expressed on this subpopulation of neurons (Karchewski et al., 1999; Kashiba et al., 2003; Kashiba et al., 1998; Bennett et al., 2000). It is therefore possible that a reduced access to central and/or peripheral target field-derived BDNF, NT-3 and GDNF is the driving force behind the injury- and culture-induced decrease in Nav1.6 mRNA expression. Accordingly, the addition of these neurotrophic factors to cultures may ameliorate the decrease in Nav1.6 mRNA expression observed over time.

To date, no published data has emerged that describes the neurotrophic factor regulation of Nav1.6 mRNA or protein. In contrast, several publications have failed to demonstrate that NGF regulates Nav1.7 expression in sensory neurons. For example, transgenic mice over-expressing NGF specifically in the peripheral target fields of

DRG neurons do not show increased expression of Nav1.7 mRNA (Fjell et al., 1999b). Moreover, TTX-sensitive currents do not decrease in TrkA expressing nociceptive neurons (in which Nav1.7 is the main TTX-sensitive sodium channel in the non-lesioned rodent) in animals that have reduced levels of target field NGF as a result of immunization-induced anti-NGF antibody production (Fjell et al., 1999 (b)).

The addition of LIF to cultured adult mouse DRG neurons did not significantly alter the levels of Nav1.6 and Nav1.7 mRNA compared to control cultures (figures 4.27B and 4.28A). However, LIF was able to reduce the expression of Nav1.6 and Nav1.7 mRNAs in cultures containing MSP and NGF (although this only reached statistical significance in the case of Nav1.6 mRNA and MSP and Nav1.7 mRNA and NGF). This data raises the possibility that LIF may be at least partly responsible for the nerve injury- and culture-induced drop in the expression of these two sodium channel mRNAs in small-diameter DRG neurons expressing functional LIF receptors. The limited ability of exogenous LIF to reduce sodium channel mRNA expression raises the possibility that the expression of both mRNAs are almost maximally depressed by the low amounts of LIF released into the culture medium by Schwann cells contaminating DRG cultures.

Although the data presented in figures 4.27B and 4.28A suggest that LIF may play a role in orchestrating the culture- and injury-induced down-regulation of Nav1.6 and Nav1.7 mRNAs, the minimal efficacy of LIF raises the possibility that other mechanisms also act to decrease the expression of these mRNAs following neuronal damage. The inability of NGF, artemin and MSP to promote the expression of Nav1.6 and Nav1.7 mRNAs suggests that reduced access to peripheral target field-derived neurotrophic factors is not the primary reason behind the culture-induced down-regulation of TTX-sensitive sodium channel mRNA. This is especially true of Nav1.7 mRNA, since a large percentage of Nav1.7 mRNA-positive cells express receptors for NGF and artemin (and probably MSP, according to the data in this chapter), yet these neurotrophic factors are ineffective in maintaining Nav1.7 mRNA expression. *In-vivo* experiments have demonstrated that Nav1.7 plays a role in setting normal nociceptive thresholds to noxious mechanical stimuli. More importantly, Nav1.7 is implicated in the generation of inflammatory mechanical and thermal hyperalgesia (Nassar et al., 2004 and 2005; Black et al., 2004; Yeomans et al., 2005).

Therefore, determining the factors that regulate Nav1.7 mRNA expression, both following nerve lesion and in inflammatory conditions, is important and warrants further research.

4.5. Results in Brief

In chapter 4, the neurotrophic factor regulation of genes of interest in the adult mouse was studied using neuronal DRG cultures. The key results can be summarised as below:

α - and β -CGRP:

- Expression of both α - and β -CGRP mRNAs decreased over time in culture, reflecting effects observed in situations of nerve damage and axotomy.
- NGF, artemin and MSP were all able to significantly attenuate this culture-induced decrease in mRNA expression.
- Effects of NGF and artemin were additive, and may reflect culture induced alterations in receptors for NGF and artemin. A culture-induced decrease in TrkA mRNA has been observed and is accompanied by a similar increase in the levels of GFR- α 3 mRNA (S Wyatt unpublished observation – data not shown). The net result of these changes in receptor gene expression is likely to be a decrease in the proportion of DRG neurons expressing either both TrkA and GFR- α 3 together, or expressing TrkA alone. An increase in the number of neurons expressing GFR- α 3 in the absence of TrkA might also be anticipated. The partially additive effects of NGF and artemin in this experiment might be as a result of such changes.
- The addition of LIF to cultures had markedly different effects on α - and β -CGRP:
 - LIF had no effect on the culture-induced down regulation of α -CGRP or its regulation by NGF, artemin or MSP.
 - Addition of LIF significantly reduced the expression of β -CGRP mRNA compared to control cultures. Moreover it significantly inhibited the ability of MSP artemin and NGF to reduce the culture-induced decrease in mRNA expression.

SP:

- Expression of SP mRNA decreased over time in culture reflecting the injury-induced decrease observed previously in models of nerve injury.
- The presence of NGF, MSP or artemin significantly attenuated the culture-induced decrease in SP.
- Additive effects of NGF and artemin were observed and are likely to reflect the alterations in receptor expression in such culture situations (as mentioned above).
- Addition of LIF alone had no effect on the culture-induced down regulation of SP mRNA, however it did significantly attenuate the positive effects of NGF, artemin and MSP

VR1:

- Expression of VR1 mRNA decreased over time in culture.
- The addition of NGF could partially prevent this decrease, and interestingly, both artemin and MSP could also significantly attenuate this decrease.
- No additive effects of any of the three factors was observed suggesting that those adult DRG neurons that express VR1 mRNA in culture, also express receptors for all three neurotrophic factors.
- Effects of LIF were very similar to those effects on SP. LIF alone had no effect on the culture induced down-regulation of VR1 mRNA, however it could attenuate the ability of NGF, artemin and MSP to promote VR1 mRNA expression.

Nav1.8 and Nav1.9:

- A culture induced drop in both Nav1.8 and Nav1.9 mRNA expression was observed, mirroring the effects observed in peripheral nerve injury.
- NGF, artemin and MSP could all partially inhibit this downregulation, however levels were not returned to those at time 0.
- Additive effects of NGF and artemin were observed suggesting that there is a small proportion of TTX-R expressing neurons that express either TrkA in the absence of GFR α 3 or GFR α 3 in the absence of TrkA.
- LIF alone has no effect on the culture-induced downregulation of either TTX-R sodium channel, however it did appear to reduce the ability of NGF, artemin and MSP to promote the expression of Nav1.8 and Nav1.9.

Galanin:

- Galanin mRNA expression increased over time in culture in accordance with previous studies.
- This up-regulation was inhibited by the presence of NGF, artemin and/or MSP.
- Additive effects of MSP and artemin were observed suggesting a population of galanin expressing neurons express either RON or GFR α 3, but not both.
- The presence of LIF significantly enhanced the culture-induced up-regulation of galanin mRNA as well as inhibiting the ability of NGF, artemin and MSP to attenuate its expression. This might suggest that the increase in LIF observed

at sites of nerve injury may promote the subsequent alterations in gene expression that occur as a consequence of nerve damage.

PACAP:

- PACAP mRNA expression increased over 96 hours in culture.
- NGF could inhibit this upregulation, however the effect was small and not significant in all cultures. Effects of MSP and artemin were also apparent, but never reached statistical significance. These results reflect the idea that the upregulation in PACAP mRNA observed occurs predominantly in, TrkC expressing, medium to large diameter myelinated neurons that are not responsive to NGF.
- The additive effect of MSP and artemin observed suggests that most nociceptive DRG neurons expressing PACAP mRNA express either GFR α 3 or RON, but not both.
- Interestingly the presence of LIF appears to reverse the culture-induced upregulation in PACAP mRNA, and moreover enhances the limited ability of artemin and MSP (but not NGF) to attenuate the culture-induced upregulation of PACAP mRNA. This conflicts most of the previous data in which LIF appears to enhance the culture induced changes in gene expression.

ATF3 and DINE:

- Expression of both ATF3 and DINE mRNAs was markedly up-regulated over time in culture.
- NGF, artemin and MSP were all able to inhibit the culture-induced up-regulation in ATF3 mRNA, and also to some extent DINE mRNA (although results were not always significant for NGF).

- Artemin was consistently the most effective of the neurotrophic factors in reducing expression of both DINE and ATF3 mRNAs. This might be a reflection of the alteration in GFR α 3 and TrkA receptors that occurs over time in culture (as mentioned previously).
- LIF had no effect on ATF3 mRNA expression, however its presence partially prevented the culture-induced up-regulation of DINE mRNA observed in control cultures. Furthermore LIF appeared to enhance effects of artemin, NGF and MSP in attenuating the culture induced up-regulation in DINE mRNA.

P2X3:

- No alteration in P2X3 mRNA was observed over time in culture.
- NGF, artemin and MSP did not appear to regulate expression of P2X3 mRNA, and the addition of LIF also had no notable effects.

Nav1.6 and Nav1.7:

- Expression of both of these TTX-S sodium channels decreased over time in culture.
- No regulatory effects were observed for NGF, artemin or MSP.
- The presence of LIF alone had no effect on expression of Nav1.6 or Nav1.7 mRNAs, however in cultures containing MSP or NGF, the addition of LIF was able to reduce the expression of Nav1.6 and Nav1.7 mRNAs further. This result might suggest that the increase in LIF following nerve injury is partially

responsible for the associated alterations in the expression of these sodium channels.

Chapter 5

General Discussion

In the aims and objectives of this thesis I outlined the main objective of this project, - to gain an insight and expand knowledge of the regulation and patterns of expression of particular genes, which are required for a number of key functional properties of subsets of sensory neurons.

In this thesis I have indeed gained much knowledge of the neurotrophic factor regulation of such genes. I have identified the differences in expression of the selected genes at different ages, in different ganglia and in different subpopulations of sensory neurons. I have used both *in vitro* and *in vivo* methods and as well as enhancing and expanding upon previous research, I have in some cases found conflicting results. This is hardly surprising however, considering the number of variables involved – time, age, species and experimental approach to name just a few.

In chapter 3 of this thesis both *in vivo* and *in vitro* experiments were used, and on occasion, produced differing results. Likewise the *in vitro* cultures used in chapter 4, occasionally produced findings which conflicted with those from other published work in which *in vivo* approaches were utilised. This, however, is to be anticipated due to the different objectives underlying the two types of experiment. *In vitro* experiments tend to focus on organs, tissues, cells etc rather than the whole organism and are best suited at deducing the mechanism of action of a particular molecule. In *in vitro* experiments there are fewer variables, and reactions/effects are amplified making results more apparent. In contrast, experiments *in vivo* allow the observation of effects of an experiment on its living subject. As such, results from these two types of experiment can often be strikingly different, and should therefore be interpreted and compared with caution.

Key Findings

Two of the key main outcomes of this thesis were the discovery of the difference in regulation of α and β CGRP mRNAs, and the finding that MSP can regulate gene expression in the adult mouse.

α and β CGRP share high sequence homology, differing by just one amino acid in the rat (and three in the mouse). They are however the products of separate genes and show different expression profiles, and as such functionally distinct roles, and regulation might therefore be anticipated. Despite this, very few studies have discriminated between the two isoforms of CGRP, mainly due to experimental difficulties in trying to individually detect such highly homologous molecules.

Real time Q-PCR has enabled me to accurately detect the expression of both isoforms and interestingly my results do indeed show that although regulation may be similar in some circumstances, in other situations contrasting patterns of regulation are apparent.

In the embryonic mouse, results show that the α isoform of CGRP requires TrkA signalling for initial induction of expression within DRG, however β -CGRP shows no such requirement. By birth, NGF/TrkA signalling does seem to be important for the expression of both isoforms, since TrkA^{-/-}/Bax^{-/-} neonates showed a significant loss of α - and β - CGRP mRNAs in both trigeminal ganglia and DRG compared to Bax^{-/-} neonates. NGF/TrkA signalling also appeared to be required for full expression of both α - and β -CGRP mRNAs by nodose neurons.

In the adult mouse mRNAs of both isoforms were down-regulated, over time in culture and both could be positively regulated by the addition of NGF, MSP or artemin. Interestingly however LIF affected the expression of α and β CGRP in markedly different ways. LIF had no effect on the expression of α -CGRP mRNA, however its presence significantly reduced the expression of β -CGRP mRNA in

comparison to control cultures. Furthermore it inhibited effects of NGF, MSP and artemin, which all act to attenuate the culture induced decrease in β -CGRP

A second key finding of this thesis was the demonstration that MSP can regulate the expression of a number of functionally important sensory neuron mRNAs (α -CGRP, β -CGRP, SP, Nav1.8, Nav1.9, VR1, PACAP, galanin, DINE, ATF3) as outlined in chapter 4. This is an entirely novel result. To date effects of this factor on gene regulation has only been addressed in the embryonic mouse. This is of particular importance, since changes in the expression of the genes under test, that occur during inflammation and following nerve trauma, may be causally related to pathological pain conditions. A role for MSP and the onset of such pain states is thus indicated.

Further Research

Obviously, had time and cost not been a factor, the thesis could have been expanded further, with additional experiments to confirm or disprove many of the theories put forward throughout this study.

Experiments using temporally and spatially controlled knockouts of neurotrophic factors receptors would be useful as would avoid the pitfalls of aberrant differentiation and/or target field innervation affecting the results. However such experiments are costly and time-consuming and some strains of mice that would be useful do not yet exist.

Immuno-histochemical study of sensory ganglia from $TrkA^{-/-}/Bax^{-/-}$ and $NT-3^{-/-}/Bax^{-/-}$ alongside those from temporally controlled $TrkA^{-/-}$ and $NT-3^{-/-}$ would also prove useful to determine the subpopulations of neurons within such mice and how the loss of TrkA or NT-3 from birth affects the subpopulations of neurons in the sensory ganglia.

In vivo approaches involving injection of neurotrophic blocking reagents or neurotrophic factors would allow the study of effects of decreasing/increasing target field-derived neurotrophic factors on transcriptional regulation of the genes under test.

With the use of double null mutant adult mice I could explore *in vivo* effects in the adult, however this would be time consuming and costly and result in unnecessary animal wastage.

In summing up, this thesis provides further background to the role of neurotrophic factors within sensory neurons at different stages of development, providing insight into the regulation of some particularly important genes within different populations of sensory neurons of the mouse. Since many of the genes studied have roles both in determining normal nociceptive thresholds, and in the generation of inflammatory and neuropathic pain conditions, a thorough understanding of their expression and regulation is of importance for the therapeutic advancement of research in these fields.

Appendix I

Abbreviations

AC	Adenyl Cyclase
Ach	Acetylcholine
AChR	Acetylcholine receptor
AD	Alzheimer's disease
AM	Adrenomedullin
AMY	Amylin
ART	Artemin
ASO	Antisense oligonucleotide
ATF3	Activating transcription factor 3
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
CaP	Prostate cancer
CFA	Complete Freud's Adjuvant
CGRP	Calcitonin Gene Related Peptide
ChAT	Choline acetyltransferase
CH	Cyclohexamide
CHO cells	Chinese hamster ovarian cells
CLC	Cardiotrophin-like cytokine
CMF-HBSS	Calcium and Magnesium Free Hank's Balanced Salt Solution
CNS	Central Nervous System
CNTF	Ciliary neurotrophic factor
COX	Cyclooxygenase
CT	Calcitonin
CT-1	Cardiotrophin-1
DEPC	di-ethyl-pyrocabonate
DINE	Damage-induced neuronal endopeptidase
dNTPS	Deoxynucleotide triphosphate
DOPA	Dihydroxyphenylalanine

DRG	Dichlorobenzimidazole riboside
DRG	Dorsal root ganglion
ds	Double Stranded
ECE	Endothelin-converting enzyme
EDNRB	Endothelin receptor B
EGF	Epidermal growth factor
ERK	Expression-signal related kinase
EST	Expression sequence tag
ET-3	Endothelin-3
F12	Ham's nutrient mixture F12
F14	Ham's nutrient mixture F14
IFN γ	Interferon- γ
IR	Immunoreactivity
JNK	c-jun N-terminal kinase
GAPDH	Glyceraldehyde phosphate dehydrogenase
GDNF	Glial cell line derived neurotrophic factor
GFLs	GDNF family ligands
GFR α	GDNF family receptor α
GMAP	Galanin Message-Associated Peptide
gp130	glycoprotein 130
GPI	glycosyl-phosphatidylinositol
HBSS	Hanks balanced salt solution
HGF	Hepatocyte growth factor
HIHS	Heat-inactivated horse serum
IB4	Isolectin B4
IL-6	Interleukin-6
IP	Inositol Phosphate
JNK	c-Jun N-terminal kinases
kDA	Kilo dalton
L-15	Liebowitz-15 medium
LA	Local Anaesthetic
LC	Locus Coeruleus
LIF	Leukaemia inhibitory factor

LTP	Long term potentiation
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
MS	Multiple sclerosis
MSP	Macrophage stimulating protein
NCAM	Neural cell adhesion molecule
NEP	Neural endopeptidase
NGF	Nerve growth factor
NKA	Neurokinin A
NKB	Neurokinin B
NRIF	Neurotrophin receptor interacting factor
NTN	Neurturin
NTF	Neurotrophic factor
NT-3	Neurotrophin-3
OE	Over-expressing
OsM	Oncostatin M
6-OHDA	6-hydroxydopamine
PACAP	Pituitary adenylate cyclase-activating peptide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxy-kinase
PGE ₂	Prostaglandin E ₂
PHN	Post-herpetic neuralgia
PI3-K	Phosphatidylinositol 3-kinase
PLC	Phospholipase C
PNS	Peripheral nervous system
PPT	Preprotachykinin
PSP	Persephin
PTB	Phosphotyrosine binding domain
RH	Random hexanucleotides
RT	Reverse transcription
SCG	Superior cervical ganglion

SCN	Suprachiasmatic nucleus
SE	Status Epilepticus
SG	SYBR Green
SNC	Substantia nigra pars compacta
SNL	Spinal nerve ligation
SP	Substance P
SH2	Src homology domain-2
TAE	Tris-acetate-EDTA
Taq	Thermus aquaticus
TG	Trigeminal
TH	Tyrosine Hydroxylase
TTX	Tetrodotoxin
TNF	Tumour necrosis factor
Trk	Tropomyosin-related kinase
TRP	Transient receptor potential
VGSC	Voltage gated sodium channel
VRC	Vanadyl-ribonucleoside complex
VR1	Vanilloid Receptor 1
VRL	Vanilloid Receptor-like

Appendix II

Tissue culture reagents and media

Liebowitz-15 (L-15) (Dissecting Medium):

Stored at 4°C and made up every 3-4 weeks

1 pot of L15 powder (GIBCO, Invitrogen), 60mg penicillin (Sigma) and 100mg streptomycin (sigma) were dissolved in 1 litre deionised, distilled water. The pH was adjusted to 7.3 using HCl and NaOH and the media sterilised by filtering through a 0.22µm bell filter (PALL) in a laminar flow hood.

Ham's F12:

Stored at 4°C. After addition of HIHS should be kept for approx. 2 weeks

1 pot of L15 powder (GIBCO, Invitrogen), 60mg penicillin (Sigma) and 100mg streptomycin (sigma) were dissolved in 1 litre de-ionised, distilled water. The pH was adjusted to 7.3 using HCl and NaOH and the media sterilised by filtering through a 0.22µm bell filter in a laminar flow hood. For serum-supplemented media, 10% Heat inactivated horse serum (HIHS) was filtered in using a 50ml syringe and 0.22µm bell filter.

Ham's F14 (Final Growing Medium):

Stored at 4°C and made up fortnightly

10× stock was made up by dissolving a 5l unit of F-14 powder (Imperial) in 500ml of de-ionised, distilled water containing 500mg streptomycin (Sigma) and 300mg penicillin (Sigma). This 10× stock was stored as 25ml aliquots at -20°C until use.

1× F14 solution was made by diluting an aliquot of F14 (25ml) in 225ml de-ionised distilled dH₂O. 0.5g sodium hydrogen carbonate was added and pH adjusted to 7 by bubbling through CO₂. The media was filter sterilised by passing through a 0.22µm

bell filter. Whilst filtering, 2.5ml L-glutamine, 200mM (GIBCO, invitrogen) and 5.5ml Albumax I solution (see below) was added.

Albumax I solution:

Stored at -20°C

20g/ml albumax I (GIBCO, invitrogen) was dissolved in 100ml dH₂O and added to the solution below:

100ml dH₂O

160mg putrescine

1ml progesterone (0.625mg/ml in ethanol)

10ml L-thyroxine (0.4mg/ml in ethanol)

10ml sodium selenite (0.4mg/ml in PBS)

10ml tri-iodothyronine (0.34mg/ml in ethanol)

Laminin:

Murine Laminin (Sigma) was defrosted at 4°C and diluted in CMF-HBSS (Calcium and Magnesium free Hank's Balanced Salt Solution), GIBCO, Invitrogen to a concentration of 20µg/ml. 120µl of laminin was then pipetted into the centre of each 35mm dish and spread out using a pipette tip ensuring not to touch the sides of the dish. Laminated dishes were incubated at 37°C for a minimum of 4 hours before washing twice with F12 + HIHS and then adding 1ml final medium.

Poly-DL-ornithine:

Stored at 4°C and made up fortnightly

0.5mg/ml poly-DL-ornithine (Sigma) was prepared by dissolving**** in 0.15M borate buffer (4.6g boric acid (BDH) in 500ml de-ionised, distilled water, pH 8.4).

The solution was sterilised by passing through 0.22µm bell filter.

Trypsin:

1% stock solution was made by dissolving 50mg trypsin (Worthington) was added to 5ml CMF-HBSS) and sterilised with 0.22µm filter (Nalgene). 100µl aliquots were stored at -20°C until use.

0.05% working solution was made by diluting a 50µl aliquot in 950µl CMF-HBSS. Ganglia were incubated at 37°C in this solution to allow trypsinisation. Time varied according to age and type of ganglia.

Collagenase

100mg/ml stock solution was made up by dissolving 100mg in 1ml of HBSS supplemented with Calcium (0.097mg/ml) and Mg (0.185mg/ml). 20µl aliquots were stored at -20°C until use.

2mg/ml working solution was made by diluting a 20µl aliquot in 980µl HBSS. Adult ganglia were incubated in this solution prior to trypsinisation.

Appendix III

Real Time PCR

Reverse Transcription Mastermix:

REAGENT	VOLUME	MANUFACTURER
Buffer	4 μ l	Stratagene
Random Hexanucleotides	2 μ l	Pharmacia
dNTPS	2 μ l	Stratagene
Stratascript Enzyme	0.38 μ l	Stratagene
dH ₂ O	26.6 μ l	GIBCO, Invitrogen

Added to 5 μ l RNA

PCR Mastermix:

REAGENT	VOLUME	MANUFACTURER
10 \times buffer	2.5 μ l	Stratagene
MgCl ₂	1.5, 2 or 2.5 μ l to give final concn. of 3, 4 or 5mM resp.	Stratagene
dNTPs (20mM)	1 μ l	Stratagene
Rox (1/500)	0.4 μ l	Stratagene
Primers	0.5 μ l	MWG
Syber Green (1/4000)	0.25 μ l	Molecular Probes
dH ₂ O	15.8, 15.3, 14.8 μ l to give final concn. of 3, 4 or 5mM resp.	GIBCO, Invitrogen

Added to 2.5 μ l cDNA

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