Interaction between neurons, glia and target field cells in regulating the survival of cranial sensory neurons

A thesis submitted to the University of Edinburgh for the degree of Doctor of Philosophy (Ph.D.)

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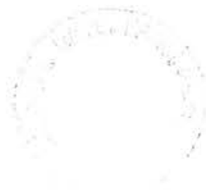

I dedicate this thesis to my parents and to Alison. I would not have got this far without them.

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ABBREVIATIONS

AC	Alternating Current
AIF	Apoptosis-Inducing Factor
Apaf-1	Apoptosis Protease Activating Factor-1
APS	Ammonium Persulphate
ATP	Adenosine 5'-Triphosphate
ART	Artemin
Ask-1	Apoptosis signal-regulating kinase 1
AVD	apoptotic volume decrease
BDNF	Brain-Derived Neurotrophic Factor
BH	Bcl-2 Homology
BMP	Bone Morphogenetic Protein
Bp	Base pairs
BSA	Bovine Serum Albumin
C. elegans	Caenorhabditis elegans
cDNA	Complementary DNA
CFV	Cresyl Fast Violet
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
CREB	cAMP response element-binding protein
CT-1	Cardiotrophin-1
DAB	3,3'-diaminobenzidine tetrahydrochloride
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DRG	Dorsal Root Ganglia
DTT	Dithiothreitol

E	Embryonic Day
E. Coli	Escherichia coli
EDTA	Ethylene Diamine Tetraacetic Acid
EMT	Epithelial-Mesenchymal Transition
ER	Endoplasmatic Reticulum
ERK	Extracellular-signal Regulated Kinase
ES	Embryonic stem
F14	Ham's Nutrient Mixture F-14
FADD	Fas-Associated Death Domain
FKHLR1	Forkhead transcription factor 1
FGF	Fibroblast Growth Factor
Gab	Grb-associated binder
GAPDH	Glyceraldehydes phosphate dehydrogenase
GDFs	Growth/Differentiation Factors
GDNF	Glial cell line-Derived Neurotrophic Factor
GFP	Green Fluorescent Protein
GFRα	GDNF Family Receptor alpha
GPI	Glycosyl-Phosphatidyl Inositol
GTP	Guanosine 5'-Triphosphate
HGF	Hepatocyte Growth Factor
HIHS	Heat Inactivated Horse Serum
IKK	Inhibitor of IκB kinase
IHC	Immunohistochemistry
IL-6	Interleukin-6
IRS	Ínsulin Receptor Substrate
JNK	Jun N-terminal kinase
KD	KiloDaltons
KOH	Potassium hydroxide

L15	Leibovitz's L15 nutrient mixture
LIF	Leukaemia Inhibitory Factor
LIFRβ	LIF Receptor beta
MAPK	Ras/mitogen-activated protein kinase
mRNA	Messenger RNA
MSP	Macrophage Stimulating Protein
NADE	p75NTR-associated cell death executor
NBF	Neutrally Buffered Formalin
NRAGE	Neurotrophin-receptor interacting melanoma-associated antigen
NGF	Nerve Growth Factor
NIK	NF-κB activating kinase
NRIF	neurotrophin receptor interacting factor
NT	Neurotrophin
NTN	Neurturin
NVI	Necrotic volume increase
OSM	Oncostatin M
P	Postnatal Day
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDK	3-phosphoinositide-dependent kinase 1
PI3K	Phosphatidylinositol 3-Kinase
PKB/AKT	Protein kinase B
PLC-γ1	Phospholipase C-gamma1
PNA	Peanut Agglutinin
PNS	Peripheral Nervous System
pRb	Retinoblastoma tumor suppressor protein
PSP	Persephin
PT	Permeability Transition

Ret	Rearranged During Transfection
RNA	Ribonucleic acid
RSK	Ribosomal S6 kinase
RT	Reverse Transcription
RT	Room Temperature
RTK	Receptor Tyrosine Kinase
SATO	Sato's Nutrient Mixture
SCG	Superior Cervical Ganglion
SDS	Sodium Dodecyl Sulphate
Shc	SH2-containing adaptor protein
Shh	Sonic Hedgehog
SHP-1	SH2-conteining tyrosine phosphatase-1
SOS	Son-Of-Sevenless
TAE	Tris-acetate
Taq	Thermus aquaticus
TEMED	Tetramethylethylene diamine
TGF-β	Transforming Growth Factor-beta
TNF	Tumour Necrosis Factor
TRAF6	TNF-associated factor 6
TRADD	TNF Receptor Associated Death Domain
Trk	Tropomyosin-Related Kinase
tRNA	Transfer RNA
UTR	Untranslated Region
UV	Ultraviolet
V	Volt
VRC	Vanadyl-Ribonucleside Complex
W	Watt

ABSTRACT

1. Aims: During embryonic development, most cutaneous sensory neurons depend for their survival on a supply of NGF synthesised in the skin. NGF promotes survival by binding to the trkA receptor tyrosine kinase whose signalling is modulated by the common neurotrophin receptor p75. trkA is expressed in trigeminal neurons shortly after axons reach their targets and NGF expression begins with the arrival of the earliest axons. This thesis was aimed at investigating interactions between neurons and targets using the trigeminal ganglion and its maxillary target field. Specifically, it assessed a. whether the induction and subsequent developmental changes in trkA mRNA seen in the ganglion in vivo are intrinsically regulated or dependent upon extrinsic signals, and whether b. regulation of NGF expression in the target field is influenced by the innervating ganglion. Further, it was aimed at c. assessing the importance of the trkA, trkB (BDNF-receptor) and full-length and truncated p75 neurotrophin receptors in promoting survival in the developing trigeminal ganglion, and d. determining the role of non target-related survival-mechanisms by Schwann cell precursors on trigeminal ganglion neurons and other cranial sensory ganglia, namely the nodose, dorsal root and superior cervical ganglia.

2. Methodology: a. and b. Ganglion-target interactions and their effect on trkA and NGF expression were assessed using cultures of trigeminal ganglia and its target fields alone or in combination. Complementary approaches used knockout mice that increased or decreased the neuronal population in the trigeminal ganglion in vivo. c. The role of the neurotrophin receptors trkA, trkB and p75 in trigeminal neuron survival was assessed using knockout mice, including double knockout mice for *trkA* and *trkB*. d. The role of Schwann cell precursors in the survival of different populations of cranial sensory neurons was assessed using *ErbB3* knockouts, which lack these cells.

3. Main Findings: a. Upregulation of trkA mRNA expression in the trigeminal ganglion appears to follow an intrinsic programme, with in vitro expression levels mimicking levels in vivo. However, extrinsic signals from the target-fields have a negative effect on trkA expression in vitro. b. Early target field NGF mRNA expression was positively influenced by

ganglion innervation in vitro, and was significantly lower in the early target fields of embryos lacking trigeminal neurons early in development in vivo. c. Double *trkA/trkB* knockouts displayed neuronal death in the trigeminal ganglion, in a pattern suggesting that during certain phases in development there are subsets of neurons, which can survive with either one or the other receptor, whereas at other developmental stages both receptors are required. Neuronal losses in different *p75* mutant embryos suggest a survival-promoting effect of p75 early in embryonic development, with truncated p75 having a role earlier in development than full-length p75. d. Neuronal deficiencies in *ErbB3-/-* embryos support the idea that populations of cranial sensory neurons differ in their survival-requirement for Schwann cell precursors early in development, with early trigeminal and dorsal root neurons being more dependent on this support than early nodose neurons.

4. Synthesis and conclusions: a. and b. The results suggest that in addition to intrinsic mechanisms of regulation, trkA and NGF expression are subject to complex reciprocal interactions between the trigeminal ganglion and its target fields early in development. The control of survival of neurons during development may thus involve more than the restricted supply of survival factor from the target field. c. Sequential dependence of sensory neurons on one or more survival factor probably serves to increase survival to maximize the 'choice' of the target field during naturally occurring cell death, and to establish heterogeneity in the ganglion. d. Differences in the sensitivity of cranial sensory neurons to trophic support by Schwann cell precursors during early development are presumably related to the distance different populations grow to their target fields. Thus, in addition to survival provided by the target field, neurons appear to depend on survival signals from surrounding cells between the ganglion and the target field.

Overall, these data support modifications to the way we should think about the way target derived signals regulate the survival of peripheral neurons. Rather than being a passive receipt of a restricted supply of NGF from the target field, it appears that complex target-ganglion interactions are involved, as well as input from other neurotrophic factors, either separately or in synergy with NGF, and input from non-target cells, such as Schwann cell precursors.

CHAPTER I

Introduction

In 1909 Shorey (Shorey, 1909) made an interesting discovery: she found that if the limb buds in chick embryos were removed, the numbers of spinal motor neurons and sensory ganglia neurons was subsequently greatly reduced. Transplantation of a supernumerary limb in contrast was found to lead to hyperplasia of the motor column and spinal ganglia supplying the limb (May, 1933). Further experiments by Hamburger *(1958)* found that removal of the 'target tissue' of motor neurons in chick embryos prior to innervation caused these to die. Hamburger (1975) even observed a 40 percent reduction in motor neurons after connection when the target field was present. This phenomenon was termed 'naturally occurring cell death'. These observations led to the hypothesis that these neurons are in competition for limited amounts of 'trophic factor' provided by the innervated target. According to this hypothesis, neurons are generated in excess, and due to the limited amount of trophic factor, neurons that do not get sufficient amounts die.

Since these discoveries, a wealth of evidence has emerged in support of the neurotrophic theory. The first neurotrophic factor to be identified was Nerve Growth Factor (NGF) (Cohen et al., 1954), which was found to support the survival of dorsal root sensory neurons (Levi-Montalcini and Hamburger, 1953). NGF is part of a family of trophic factors termed neurotrophins, which act through their tropomyosin-receptor-kinase (trk) and/or p75 receptors to promote survival. Other members of this family include Brain Derived Neurotrophic Factor (BDNF) (Barde et al., 1982), Neurotrophin-3 (Hohn et al., 1990), and Neurotrophin-4/5 (Hallböök et al., 1991).

As well as being supported by trophic factors from their target fields there is now also evidence of neurons being supported by other cells nearby, or indeed in an autocrine manner by themselves. As addition to their trophic function for neurons, some trophic

factors, such as the neurotrophins, have been found to fulfil important roles in supporting neuronal precursors, inducing neurogenesis, and in guiding neuronal processes to their target fields. The experiments presented in this thesis are aimed at clarifying the interactions between neurons, glia and target field cells in regulating the survival of cranial sensory neurons.

This introductory section is aimed at providing background information about the neuronal populations studied in these experiments, namely the trigeminal, nodose, dorsal root and superior cervical ganglia, and the actions exerted on them by growth factors belonging to the neurotrophin family. I will begin with an overview of the development of the nervous system, particularly the peripheral nervous system, from neural induction to the point that neurons extend axons to their target fields (1.1). I will then review evidence for the 'neurotrophic hypothesis', including a description of the discovery of neurotrophins and how they support neuronal survival when neuronal processes arrive in the vicinity of their target fields (1.2). The molecular biology of the neurotrophins, the main trophic factors of interest to this thesis, and their receptors, will then be discussed in more detail (1.3). The mechanism of programmed cell death taking place in neurons during development, 'apoptosis', will then be described, together with the intracellular events underlying this process, and the pathways through which neurotrophin signalling modulates these death/survival pathways (1.4). This will be followed by an overview of the role of neurotrophins in the developing peripheral nervous system and a detailed description of the experimental systems used, such as the trigeminal ganglion and other peripheral nervous system ganglia (1.5), before introducing the objectives of the experiments presented in this thesis (1.6).

1.1 Development of the vertebrate nervous system

This section will first describe the general origins of the nervous system, before going into more detail about the embryonic origins of the particular cell types of interest in this study, namely the neurons of the cranial ganglia. This will be followed by an overview of the cues that guide neuron progenitor cells to the sites of developing ganglia and stop them migrating, factors important for the differentiation of these cells, and the cues governing growth of axons to their target fields.

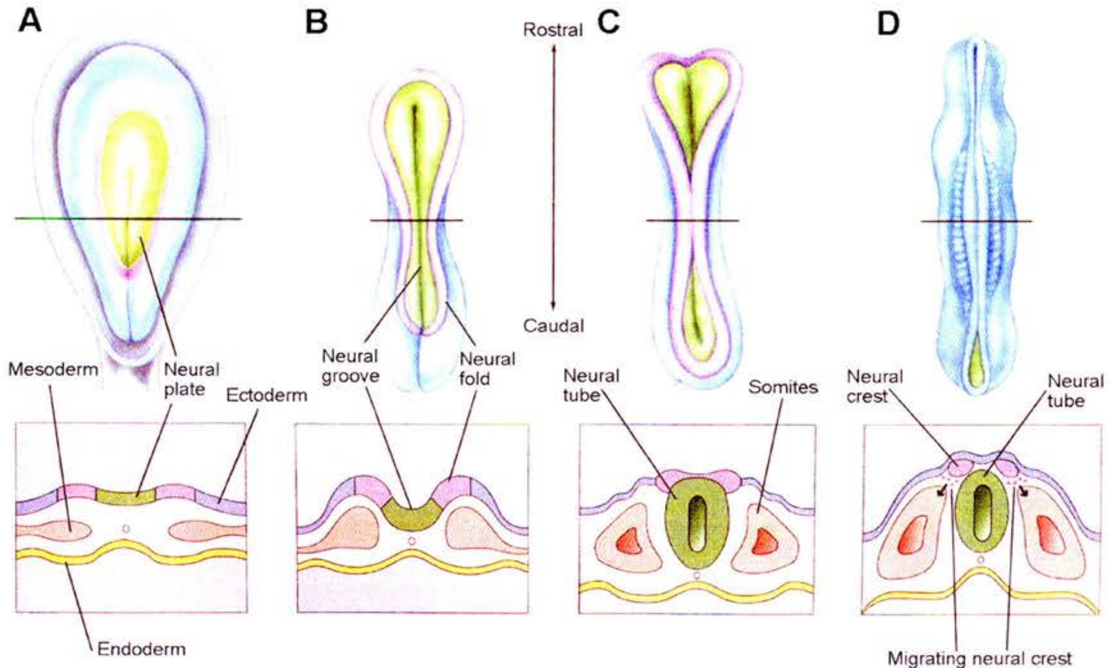
1.1.1 Origins of the nervous system

Christian Pander was the first to describe three germ layers created during early embryogenesis in the chick: the ectoderm, mesoderm and endoderm, which give rise to specific organ systems (Pander, 1817). Karl Ernst von Baer, having examined the fetal anatomy of numerous species, proposed that all animals have three germ layers, and that the organs derived from them are constant across species (von Baer, 1928) This has been found to be true for most species, although some invertebrate animals, like the jellyfish, lack a true mesoderm (Gilbert, 2003). The endoderm gives rise to epithelial lining of the digestive, respiratory, biliary and urogenital systems. The mesoderm gives rise to cells of the muscular, circulatory, lymphatic, reproductive and excretory systems, dermis of the skin and cells lining the body cavities. The ectoderm gives rise to the epidermis of the skin and its derivatives, the adrenal medulla, cornea and lens of the eye, epithelium of the pineal and pituitary glands and the neurons and glia (with the exception of microglia) of the nervous system (from

Gilbert et al., 2003). It is the fate of the cells in this last layer that the rest of this subchapter will describe.

The nervous system originates from ectodermal cells (neuroectodermal cells) located along the dorsal midline of the embryo at the gastrula stage, which form a thickened region of ectoderm termed the neural plate (figure 1.1A). During a process known as neurulation the neural plate invaginates ventrally and forms a neural groove. The walls of this neural groove are referred to as the neural folds (Figure 1.1B). These folds move together and join dorsally, forming the neural tube (Figure 1.1C). The central nervous system develops from the walls of the neural tube, with the caudal region giving rise to the spinal cord and the rostral region becoming the brain. While joining, some of the cells of the neural fold separate laterally from the others to form the neural crest between the neural plate and the surface non-neural ectoderm (Weston, 1963) (Figure 1.1D). The neural crest was first described in 1868 by Wilhelm His as *Zwischenstrang*. His also described this group of cells as the source of spinal and cranial ganglia (cited in Hall, 1999). This was confirmed by Harrison in 1904 (Harrison, 1904), in one of the first experiments to use microsurgery. Harrison destroyed segments of neural crest together with the dorsal half of the neural tube from frog embryos and obtained larvae lacking spinal ganglia. It has been shown that the induction of this neural crest is dependent on interactions between the neuronal and non-neuronal ectoderm (reviewed in LeDouarin and Kalcheim, 1999).

Figure 1.1: Formation of the neural tube and crest



Schematic representation of the origin of the nervous system. (A) shows ectodermal cells, along the dorsal midline of the embryo, forming the neural plate. (B) shows the process of neurulation – the neural plate invaginates ventrally and forms the neural grove. (C) shows the dorsal walls of the neural grove, the neural folds, subsequently joining together, forming the neural tube. (D) shows the formation of the neural crest from cells that separate from the neural fold as it joins (adapted from Bear et al., 1996).

1.1.2 Organisation and origin of the peripheral nervous system

The peripheral nervous system (PNS) can be subdivided into two divisions: the somatic PNS, and the visceral or autonomic PNS. The somatic PNS includes sensory and motor systems involved with voluntary control, whereas the autonomic PNS is involved with involuntary control of internal organs. The sensory neurons of the PNS are segregated into cranial and dorsal root ganglia, whereas the cell bodies of the motor neurons are located in the brainstem and spinal cord. The autonomic nervous system has three subdivisions: the sympathetic and parasympathetic, which consist of pre-ganglionic neurons located in the brainstem and spinal cord and post-ganglionic

neurons located in autonomic ganglia, and the enteric nervous system located in the gastrointestinal tract.

The ganglia in the peripheral nervous system have two distinct origins: the neural crest and the neurogenic placodes. Neural crest cells are a pluripotent, migratory population of cells that give rise to the facial skeleton, melanocytes, and most of the neurons and glial cells of the PNS (Weston, 1963). Placodes are paired ectodermal 'unthinnings' that form at the late gastrula stages. At early stages of development the embryonic ectoderm is generally thick, but it subsequently thins out, apart from regions giving rise to the placodes, hence 'unthinning' (Graham and Begbie, 2000). There are two distinct types of placodes: sensory placodes, which contribute to several sensory organs, and the neurogenic placodes, which supply neurons for the formation of certain cranial sensory ganglia (LeDouarin, 1982). The neurogenic placodes can be subdivided into groups that differ in the way they develop and in the structures they contribute to, namely, the dorsolateral and epibranchial placodes (Shimeld and Holland, 2000).

The dorsolateral placodes form in proximity of the CNS regions and include the olfactory placode, the opthalmic and maxillomandibular trigeminal placodes and the otic placode. The epibranchial placodes, forming at the clefts between the branchial arches, generate the neurons of the geniculate, petrosal and nodose ganglia (reviewed by Graham and Begbie, 2000). Like neural crest cells, placode cells are able to form cutaneous and visceral sensory neurons, neuroendocrine cells and glia (Baker and Bronner-Fraser, 2001). However, they are confined to the head region and cannot form autonomic or enteric neurons, melanocytes, smooth muscle and bone like neural crest cells can.

It was initially suggested that neurogenic placodes develop in response to migrating neural crest cells. Removal of the neural crest prior to migration and placode-specific markers has shown this not to be the case (Stone, 1922; Ma et al., 1998; Fode et al., 1998). Rather it seems that the pharyngeal endoderm has a role in inducing neurogenesis in the epibranchial ectoderm, an event mediated by bone morphogenetic protein-7 (BMP-7) (Begbie et al., 1999). The above-mentioned bone morphogenetic protein (BMP) family of growth and differentiation factors and its inhibitors, such as the secretory proteins Noggin and Chordin, perform important functions during the formation of the nervous system. Although these proteins, related to the transforming growth factor-β (TGF-β) superfamily, were first named for their ability to induce ectopic bone formation (Urist, 1965; Wozney et al., 1988), they have since been found to be important in developmental processes, such as the induction of the central nervous system (Sasai and De Robertis, 1997) and the formation of the neural crest as an example (Liem et al., 1997; Selleck et al., 1998; Streit and Stern, 1999; Garcia-Castro et al., 2002). The following sections will illustrate that the BMP family and its inhibitors are also important for other developmental processes, such as epithelial-mesenchymal transition and neurogenesis.

1.1.3 Epithelial-Mesenchymal Transition (EMT)

Both the neural crest and neurogenic placode populations of neurons migrate to their spatial position in the embryo head. The mechanisms governing the migration of neuronal precursors have mainly been studied in the migrating neural crest cells,

therefore I will concentrate on describing migratory mechanisms believed to act in this population, although much is still largely unknown.

Neural crest cells migrate along stereotypical pathways to their final destinations, with ventromedially migrating cells contributing to Schwann cells, chromaffin cells, and sensory and sympathetic ganglia (LeDouarin and Teillet, 1974). It seems that many factors necessary for neural crest induction are also necessary for migration of these cells. The first step of migration, the epithelial-mesenchymal transition (EMT), by which neural crest cells 'convert' to mesenchymal cells able to delaminate and migrate through the extracellular matrix (Duband et al., 1995) seems to be controlled by some of the crest inducing factors or homologues of them. Blocking of BMP in the chick spinal cord through Noggin, for example, has been shown to prevent neural crest delamination (Sela-Donenfeld and Kalcheim, 1999). Transgenic Noggin expression in the posterior hindbrain of mouse embryos greatly reduces the amount of crest cells in that region (Kanzler et al., 2000). BMP induced Slug has also been implicated in the initiation of EMT, with antisense oligonucleotides preventing migration of the crest in both chick (Nieto et al., 1994) and *Xenopus* embryos (Carl et al., 1999). Inducible *Xenopus* Slug mutants neural crest cells fail to migrate, and if the mutation is induced earlier fail to form a neural crest (LaBonne and Bronner-Fraser, 2000). Slug is believed to interact with the cadherin family of cell adhesion molecules, which has been implicated in the migration process of neural crest cells. The upregulation (Nakagawa and Takeichi, 1995; Takeichi et al., 2000), and downregulation (Akitaya and Bronner-Fraser, 1992; Nakagawa and Takeichi, 1995) of different members of the cadherins in neural crest cells suggests an important role in delamination and migration (Nieto, 2001). Indeed, it has been shown that a

relative of Slug, Snail, which is thought to mediate similar actions to Slug in mammals (Sefton et al., 1998), can trigger EMT by repressing E-cadherin (Cano et al., 2000).

The placode-derived neurons undergo a similar process of epithelial-mesenchymal transition as the neural crest cells before they migrate. Neurogenins 1 and 2 have been shown to play a role in this, as mutations in these neurogenin genes results in failure of the neuroblasts to delaminate, with mutations in neurogenin 1 influencing migration in trigeminal placodes (Ma et al., 1998), and neurogenin 2 mutations preventing migration in epibranchial placodes (Fode et al., 1998).

1.1.4 Migration

After delamination, neurons start migrating away from the dorsal neural tube towards their target areas, a migration regulated by attractive and repulsive signals obtained from the environment. Neurons migrate by the same mechanisms as other cells (reviewed by Lambert de Rouvroit and Goffinet, 2001): They extend their leading edges, preceded by filopodia and lamelipodia, followed by 'nucleokinesis', i.e. the nucleus moving into the leading processes, and retraction of the trailing process.

Early migration from the neural crest includes cells giving rise to Schwann cells and the neurons and satellite cells of dorsal root ganglia, autonomic ganglia, certain cranial sensory ganglia and the enteric nervous system. Neural crest cells specified as melanoblasts travel dorsolaterally in a later migration. This spatial and temporal pattern of migration is mediated by attractive and repulsive substances in the hindbrain, somites and surrounding structures (reviewed by Krull, 2001). Ephrins

and peanut agglutin (PNA) binding proteins are involved in the inhibitory forces preventing early migrating neural crest cells, giving rise to sensory and sympathetic neurons, from migrating dorsolaterally at the early stages of migration (Santiago and Erickson, 2002; Oakley et al., 1994). Interestingly, migration of late migrating melanoblasts is promoted by ephrins in the dorsolateral path, giving ephrins a dual repulsive/attractive role in sorting these two populations of neural crest cells (Santiago and Erickson, 2002).

Early migrating trunk neural crest cells that give rise to the sensory and sympathetic ganglia travel through the somatic mesoderm (see arrows figure 1.2D), shortly after this compartmentalises into an epithelial dermomyotome and a mesenchymal sclerotome (Guillory and Bronner-Fraser, 1986). Early migrating trunk crest cells enter the rostral half of the somatic sclerotome while avoiding the caudal half of the sclerotome, with the migration pattern becoming discontinuous and segmental (Keynes et al., 1990). The main repulsive cues identified for neural crest migration include repulsive cues in the caudal sclerotome, such as the Eph family of receptor tyrosine kinases (RTKs) and their ligands (Krull et al., 1997; Wang and Anderson, 1997), and PNA binding proteins, thought to play a role in generating the segmental pattern of migration of crest cells (Krull et al., 1995). Other proteins, such as F-spondin (Debby-Brafman et al., 1999), collagen IX (Ring et al., 1996), and Notch have also been proposed to play a role, with mutations of Notch and its receptor Delta-1 causing severe disruption in the segmental pattern of migration by neural crest cells, possibly due to modulation of the distribution pattern of the ephrins (De Bellard et al., 2002). A possible attractive cue for trunk neural crest cells has been identified in the form of thrombospondin (Tucker et al., 1999), which is found in the

rostral half-sclerotome that crest cells travel through in vivo and promotes neural crest migration and adhesion in vitro. The crest cells destined to form the dorsal root ganglion stop migrating and aggregate within the sclerotome, just outside the spinal cord, while some cells continue ventrally forming the sympathetic ganglia.

In the caudal region of vertebrates, neural crest cells migrate from the hindbrain in three main streams from rhombomeres 2, 4 and 6 into the branchial arches 1, 2 and 3 respectively, where they form cranial ganglia and some of the facial skeleton (Lumsden et al., 1991; Kontges and Lumsden, 1996). This migration in streams is thought to be crucial to patterning, committing neural crest cells to a specific anterior-posterior identity (Krumlauf, 1994), and has been suggested to be due to cell death, by apoptosis, of many of the neural crest cells in rhombomeres 3 and 5. This is induced by signals emanating from rhombomere 4, while cells not dying join the main streams of migrating cells in the rhombomeres next to them (Ellies et al., 2000; Birgbauer et al., 1995). There is some evidence that migrating crest cells are kept separate by ephrins, since blocking ephrins can lead to intermixing between the streams (Smith et al., 1997; Helbling et al., 1998). Some neural crest cells stop ventral migration in proximity of the neural tube and aggregate, forming cranial ganglia. Some of these aggregates are joined by placodal-derived cells to form ganglia of mixed origin, such as the trigeminal ganglion (D'Amico-Martel and Noden, 1983). The maxillary and mandibular processes in the embryo are derived from ectoderm and neural crest-derived mesenchyme that migrates into the first branchial arch (Cobourne and Sharpe, 2002). The satellite cells and most of the neurons of the trigeminal ganglion form from crest cells in the first branchial arch, the satellite cells of the vestibular and geniculate ganglia from crest cells in the

second branchial arch, and the satellite cells and neurons of the superior glossopharyngeal ganglion and the satellite cells of the inferior glossopharyngeal (petrosal) ganglion from cells in the third branchial arch (Lumsden et al., 1991).

The neural crest has been shown to be essential for the migration of epibranchial placode cells to their final positions (Begbie and Graham, 2001). Epibranchial placode-derived neurons are associated temporally and spatially with late streams of dorsal neural crest cells (Baker et al., 1999). When the migration of the crest cells is prevented by prior removal of the hindbrain using microsurgery, placode-derived neurons still form, but do not migrate from the placodal ectoderm any more and do not project axons in the way they do normally (Begbie and Graham, 2001). The same study found disruptions in the development of ganglia developing from dorsolateral placode-derived cells, such as the trigeminal ganglion, suggesting that there is a regulatory effect of the neural crest on migration and axon pathfinding of many placode-derived ganglia, although the molecular basis for this is not known thus far.

It has been suggested that neurotrophins such as Brain Derived Neurotrophic Factor (BDNF) and Neurotrophin-3 (NT-3) released from the neural tube can act to promote the survival of some migrating cells (Kalcheim, 1996), since some neural-crest progenitors die when the action of survival factors from the neural tube is prevented (Kalcheim and LeDouarin, 1986). BDNF, NT-3 and Fibroblast Growth Factor (FGF) were identified as factors able to support migrating neural crest cells in culture (Kalcheim et al., 1987, Kalcheim, 1989).

1.1.5 Arrest of migration, neurogenesis and gliogenesis of neural crest and neurogenic placode-derived cells

The signals involved in localising neural crest and placode-derived cells to their final destination are unclear. Physical barriers, changes in migratory ability and signals from surrounding tissues have been proposed (McKeown et al., 2003). A recent study has suggested that signals from the notochord and floor plate have a regulatory function in arresting migration of cells destined for the sensory ganglia (Fedtsova et al., 2003). A component of these signals was found to be Sonic hedgehog (Shh), a member of the Hedgehog family of secreted signalling proteins, which could arrest the migration of trigeminal precursors. This was confirmed by the phenotype of Shh null mutant mouse embryos, in which the cells destined for the trigeminal ganglion continued to grow towards the midline, eventually forming one single fused ganglion, instead of two symmetrical ganglia. Some effects on the arrest of migration of precursors of the DRG were also observed in these mice. Shh upregulates the expression of the hyaluronan-binding proteoglycan PG-M/versican which is naturally found in the extracellular matrix and belongs to a family known to have effects on the migratory properties of neural crest cells (Perris and Perrisinotto, 2000). Shh may act by restricting the migratory ability of sensory ganglia precursor cells through the extracellular matrix, setting an 'exclusion zone' for migration and determining the final morphology of the sensory ganglia at both cranial and trunk levels (Fedtsova et al., 2003).

BMP-4 and BMP-7 have been shown to induce neurogenesis of some sympathetic neurons (Varley and Maxwell, 1996; Reissmann et al., 1996; Schneider et al., 1999). This has been demonstrated in vivo by using ectopic expression of the BMP inhibitor

Noggin, which prevents formation of sympathetic neurons (Schneider et al., 1999). Mash1 (Shah et al., 1996; Schneider at al., 1999) and Phox2a (Hirsch et al., 1998; Stanke et al., 1999) have been shown to be downstream effectors of these BMPs.

The transcription factors neurogenin 1 and 2 have a role in neurogenesis of sensory neurons since their absence results in a reduction in sensory neuron subsets. In DRG, neurogenin 2 seems to be required for the formation of large-diameter sensory neurons, whereas neurogenin 1 is required for small-diameter neurons, as well as specifying large-diameter neuron precursors (Ma et al., 1999). Neurogenin1 has also been found to have a role in inhibiting gliogenesis, by binding the CBP/Smad1 or p300/Smad1 transcriptional co-activator and sequestering it away from the promoters of glial genes, preventing transcription (Sun et al., 2001). The same report suggests that BMPs can induce neurogenesis in the presence of neurogenin 1, whereas they induce gliogenesis in its absence. Another factor known to have an effect in the generation of sensory neuron subtypes, NeuroD, has been shown to act downstream of neurogenin (Anderson, 1999). Inhibition of Notch results in an increase of cells in the peripheral sensory ganglia expressing neuronal markers, suggesting an important role of Notch signalling in generating both neural cells and glial cells in the same aggregation of neural crest cells, since Notch expression has also been shown to lead to an increase of glial cells in peripheral ganglia (Morrison et al., 2000). Figure 1.2 summarises some of the factors involved in neuronal differentiation and prevention of glial differentiation.

There also seems to be a role for neurotrophins in neurogenesis (Kalcheim, 1996). In the chick embryo dorsal root ganglion, the maturation of some neurons is accelerated by BDNF and NT-3 (without effect on their survival). Antisense-BDNF

oligonucleotides greatly reduce the number of neurons undergoing this maturation in culture (Wright et al., 1992). BDNF and NT-3 also seem to promote maturation of early trigeminal ganglion cells (Buchman and Davies, 1993).

In more general terms, the differentiation of sensory neurons also appears to be linked to interactions between the neural crest and the neurogenic placodes, at least as far as the trigeminal ganglion is concerned. Neural crest cells, which make up the majority of cells in the ganglion, reach the position of the trigeminal ganglion first but don't differentiate. Placodal cells, which arrive later, actually differentiate first, before the neural crest derived cells do so (Covell and Noden, 1989).

Apart from Notch, gliogenesis from neural crest cells seems to be mediated by neuregulin. Neuronal derived neuregulins play a major role in controlling the development and survival of satellite and Schwann cell precursors. (Riethmacher et al., 1997; Hagedorn et al. 2000; Mirsky et al., 2002). Neuregulins signal through members of the ErbB family of receptor tyrosine kinases, namely ErbB 1, 2, 3 and 4 (Gassman and Lemke, 1997; Garratt et al., 2000). To induce gliogenesis, neuregulin acts via its ErbB3 receptor, which heterodimerises with the co-receptor ErbB2 to differentiate glia at the expense of neurons (Shah et al., 1994). Consequently ErbB3 knockout mice lack Schwann cells and their precursors (Reithmacher et al., 1997). (refer to chapters 2 and 6 for more detail on the ErbB3 knockout).

Figure 1.2: Interaction of BMP, Notch and Neurogenin in neuronal and glial differentiation

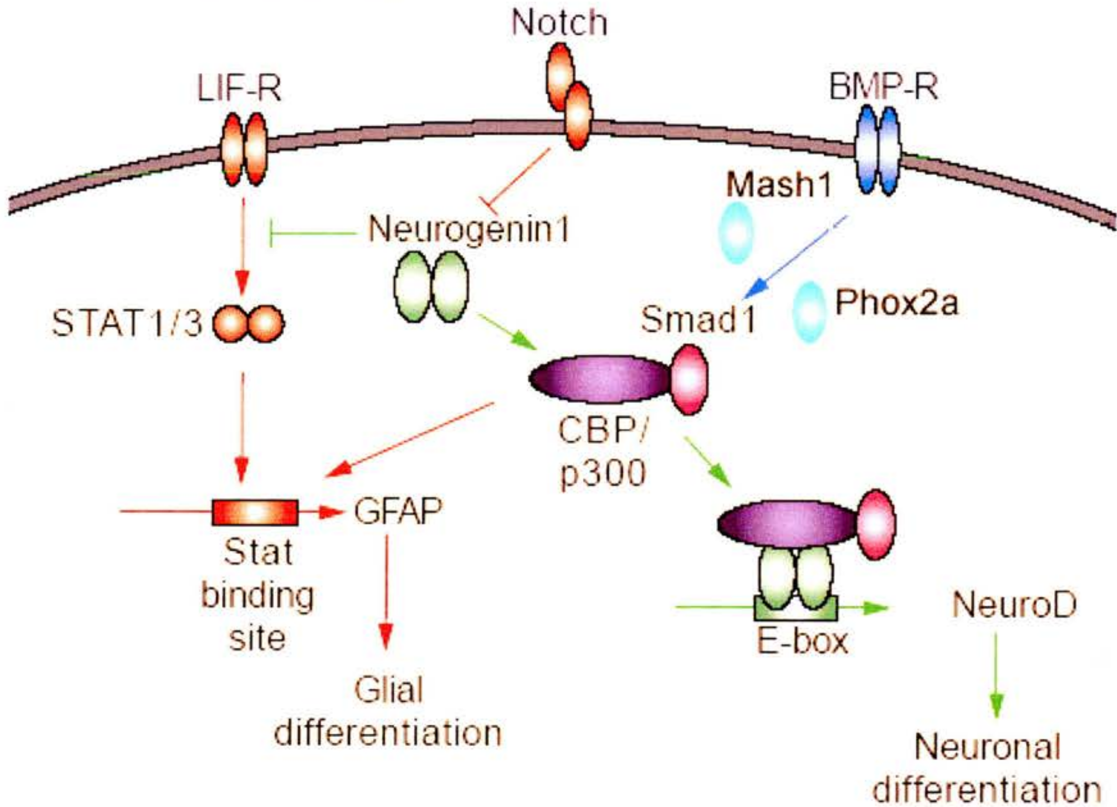


Figure showing some factors known to be involved in the control of neuronal and glial differentiation, and their interaction. The gliogenesis pathway is indicated with red arrows and the neurogenesis pathway with green arrows. Neurogenin1 inhibits gliogenesis by binding and sequestering the transcriptional co-activator CBP/p300/Smad1. In the absence of Neurogenin1, this transcriptional co-activator is recruited to the promoters of glial genes by binding to activated STAT1/3 (signal transducer and activator of transcription 1/3). This results in the expression of genes such as GFAP (glial fibrillary acidic protein) and glial differentiation. When present, Neurogenin1 binds the CBP/p300/Smad1 complex, recruiting it to the promoters of neuronal genes, and sequestering it from the promoters of glial genes. Neurogenin1 also inhibits the activation of STAT1/3. By these two mechanisms, Neurogenin1 not only promotes the expression of neuronal genes but inhibits the expression of glial genes. Consistent with this model, bone morphogenetic proteins promote neurogenesis in the presence of Neurogenin1 and gliogenesis in the absence of Neurogenin1 (Morrisson, 200. Notch activation may promote glial lineage determination by inhibiting the expression of proneural genes such as Neurogenin. LIF-R, leukemia inhibitory factor receptor; BMP-R, bone morphogenetic protein receptor; NeuroD, a neurogenic transcription factor downstream of Neurogenin1 (adapted from Morrisson, 2001).

1.1.6 Axonal guidance by diffusible substances in the peripheral nervous system

As a final step in their initial development, neurons extend processes to their target fields. Two types of guidance cues have been proposed to guide neuronal processes: local and target-derived factors. These factors can be either attractive or repulsive. Some embryonic structures known as 'choice points' serve as intermediate targets on the trajectory where the actions of guidance cues play a decisive role in directing nerve growth (Tessier-Lavigne and Goodman, 1996).

A key structure in the guidance of neuronal processes is the growth cone, an irregular, spiky enlargement at the tip of the growing fibre. It was first described in 1890 by Ramón y Cajal from sections of embryonic spinal cord stained with silver chromate (Ramón y Cajal, 1890). Described by Ramón y Cajal (from fixed samples) as being "endowed with amoeboid movements", the growth cone was indeed found to be a motile structure by Harrison (1910). In his classic experiments, Harrison isolated pieces of the neural tube of a frog embryo prior to outgrowth, and reared them in frog's lymph. He was then able to study individual nerve fibres and their growth cones under the microscope. Thus was invented tissue culture. This new technique also enabled Harrison to demonstrate that axons extend from a single cell body (Harrison, 1907, 1910), rather than being composed of individual sheath cells in segments, as had been previously claimed by Schwann, Hensen, Held and others (reviewed in Hamburger, 1980).

Following his discovery of the growth cone, Ramón y Cajal proposed that it was responsible for axonal pathfinding. He suggested 'chemotropism', a chemical attraction of the growth cone by substances produced by target structures, as a

mechanism by which neuronal processes may be guided (reviewed by Hamburger, 1980).

One of the early experiments studying the growth of neuronal processes and its dependence on guidance cues during development was done using co-cultures of the trigeminal ganglion and its targets, the maxillary and mandibular processes (Lumsden and Davies, 1983). Trigeminal ganglia were grown on a collagen matrix together with either one of their target fields, or with cutaneous targets innervated by other sensory neurons, such the hyoid process or the forelimb bud. The outgrowth of neurites from the ganglion was observed (Figure 1.3), and it was found that both the maxillary and the mandibular process elicited and attracted neurite outgrowth towards them at the stage when it is normally initiated in the trigeminal ganglion. The hyoid process or the forelimb bud in contrast did not elicit or attract axons growing from the trigeminal ganglion. It was thus hypothesised, that a diffusible factor emanated from the maxillary process, attracting outgrowth towards it. This trigeminal attractant was coined 'Maxillary Factor', thought to be emanating from the target field epithelium (Lumsden and Davies, 1986).

Some of the components of Maxillary Factor have recently been identified in the form of NT-3 and BDNF (O'Connor and Tessier-Lavigne, 1999). Anti-NT-3 and anti-BNDF were shown to reduce neurite outgrowth from the trigeminal ganglion, completely abolishing it when both were applied simultaneously. Studying ganglia of knockout mice for NT-3 and BDNF confirmed this inhibition in outgrowth, with NT-3 mutation having more effect than BDNF mutation. In vivo studies showed no defect in the growth of the trigeminal nerve in these two mutants however, raising the possibility that other factors may be involved in the initial growth of axons

(O'Connor and Tessier-Lavigne, 1999). Indeed it may be that these molecules have an effect on the outgrowth of secondary fibres, but not on pioneer axons, which would be guided by different mechanisms.

Neurotrophins have since been found to be involved in the guidance projections from other ganglia. As an example, using GFP-tagging to visualise outgrowth, it was found that neurotrophin-containing ectopically placed beads attracted sensory neurons growing in the mouse limb bud, whereas antibodies to the neurotrophins inhibited elongation of sensory and motor axons in the limb bud (Tucker et al., 2001).

In addition to neurotrophins, members of the GDNF family (glial cell-derived neurotrophic factor) may also play a role in guiding axons in the PNS (reviewed by Markus et al., 2002). It has recently been found that soluble GFRα1, a co-receptor for GDNF, is able to potentiate neurite outgrowth from nodose neurons in response to GDNF. Also, GFRα1 is downregulated in the target fields of the nodose ganglion (heart and carotid body) once these have been innervated. Together with other data, this raises the possibility that membrane-bound and soluble GFRα1 could act with GNDF as a long-range guidance cue for these axons (Ledda et al., 2002). Hepatocyte Growth Factor (HGF) acting through its receptor Met, and FGF signalling have also been implicated in axonal growth (Ebens et al., 1996; Maina et al., 1997; McFarlane, 1996).

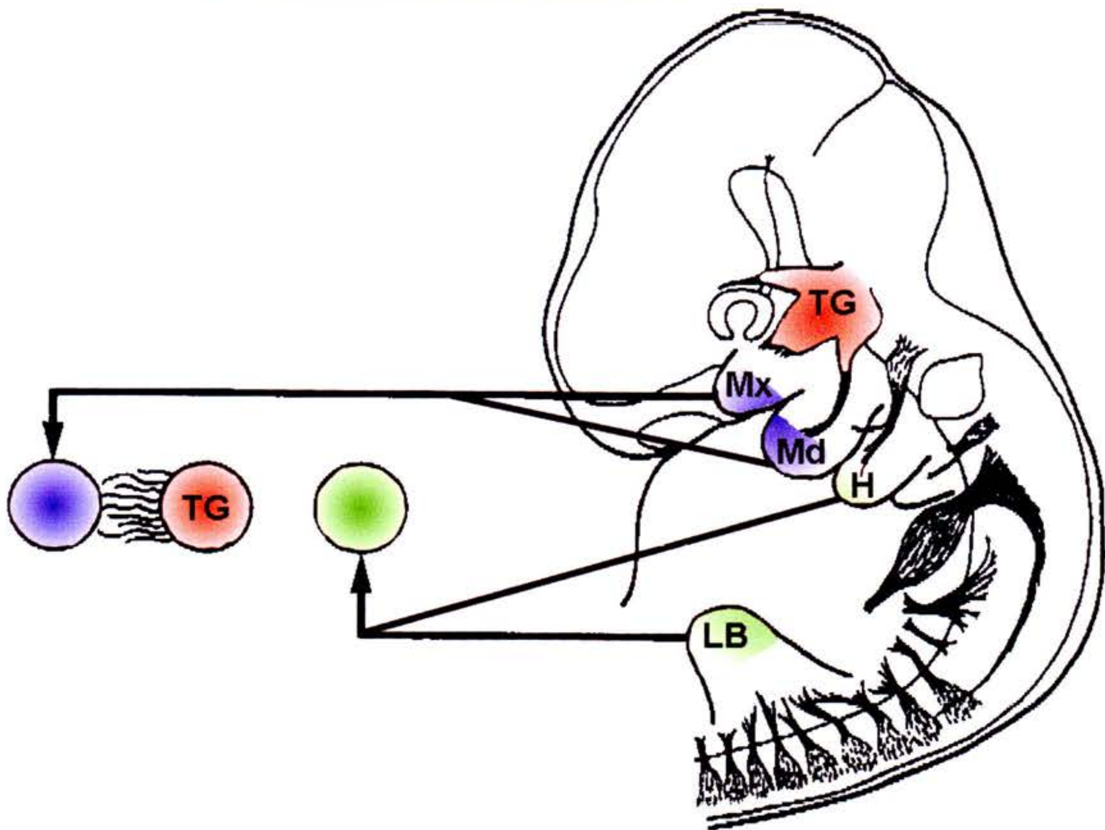
Other candidates for chemoattraction and chemorepulsion include the netrins. These proteins were originally shown in co-culture experiments to attract commissural neurons of the developing vertebrate spinal cord to their intermediate target, the floor plate (Tessier-Lavigne et al., 1988). Extracts of chicken brain were found to have

similar effects to that of the floorplate, and this led to the identification of netrin 1 and 2 as the active factors in the extracts. Netrin-1 mRNA is restricted to the floor plate during the period in development when commissural neurons grow toward it in vivo. Netrin-2 mRNA is expressed in the ventral 2/3 of the spinal cord, suggesting that it may function as an intermediate guidance factor. The netrins are homologous to UNC-6 in C.elegans, which can act as an attractant or a repellent for axons depending on the receptors they express. UNC-40 is the receptor mediating repellent action and UNC-5 is the receptor for attractive action (Hedgecock et al., 1990). The vertebrate homologue of UNC-5, DCC, is expressed on commissural neurons at the appropriate stage of development (Keino-Masu et al., 1996). Also, it was found that substances secreted in the floor plate could act as repulsive cues for some types of motor neurons (Guthrie and Pini, 1995). In vitro evidence suggests that Netrin-1, which acts as a chemoattractant for commissural axons (Tessier-Lavigne et al., 1988), is released from floorplate cells and repels trochlear motor axons (Calamarino and Tessier-Lavigne, 1995).

Another family of recently identified guidance cues are the semaphorins and their receptors, acting as chemorepellants for a variety of neurons in the CNS and PNS (reviewed by Raper, 2000), although it has recently been found that some semaphorins can act as chemoattractive guidance molecules too (Polleux et al., 2000). Knockouts of the chemorepellant semaphorin III/D for example have shown semaphorins are involved in pathfinding of projections from the trigeminal ganglia in the developing branchial arches (Kitsukawa et al., 1997; Taniguchi et al., 1997). Whisker follicle innervation appears normal however, suggesting that other factors are involved in trigeminal sensory fibre guidance (Ulupinar et al., 1999). Neuropilin-

2 (receptor for semaphorin III/F) is also required for the organisation and fasciculation of several PNS and CNS nerves, as has been demonstrated using knockouts (Chen et al., 2000; Giger et al., 2000). Other components associated with semaphoring signalling are the plexins, with PlexA3 knockouts showing that it is an essential component of the Semaphorin III/F receptor complex for guidance of SCG axons (Cheng et al., 2001).

Figure 1.3: Mouse embryo at the stage of trigeminal neurite outgrowth to its target fields and setup of co-cultures



Neurite outgrowth from the trigeminal ganglion in a collagen gel matrix is restricted to its cutaneous targets in vitro, whereas growth is not elicited by the presence of cutaneous targets of other ganglia. TG: Trigeminal ganglion, Mx: Maxillary process, Md: Mandibular process, H: Hyoid process, LB: Forelimb bud (Adapted from Davies and Lumsden, 1999).

The other well-studied family of guidance molecules in both the CNS and PNS are the ephrins and their receptors located on axonal growth cones (reviewed by Holder and Klein, 1999), which have been found to be able to influence neurite outgrowth from DRG for example (Lai et al., 1999). Slit-2, a factor so far mainly been found to mediate CNS axon guidance, has also been shown to induce axon branching in some sensory neurons. It stimulates the formation of axon collateral branches by NGF-responsive neurons of the DRG (Wang et al., 1999).

As well as acting as guidance cues themselves, it has been proposed that neurotrophins can modulate the action of some guidance cues. NGF, BDNF and NT-3 have all been shown to modulate the growth cone collapse induced by semaphorin (Dontchev and Letourneau, 2002; Ming et al., 1999).

1.2 The neurotrophic hypothesis, neurotrophic factors and modes of trophic support

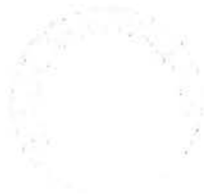
Some of the experimental observations leading to the formulation of the neurotrophic hypothesis have been mentioned in the introduction to this chapter. This section is aimed at providing more detail about the neurotrophic hypothesis, the main evidence in support of it, and the factors mediating trophic effects. Instances of target-derived trophic support and its initiation, and alternatives to target-derived trophic support will also be mentioned, showing how trophic factors can be provided by cells other than those of the target field (Davies, 2003). Finally, the interesting finding that trophic factors are able to mediate death in certain circumstances will be described.

1.2.1 The neurotrophic hypothesis

Neurons are initially generated in excess during neuronal development. Shortly after neurons begin to innervate their target tissue, large numbers die by apoptosis (reviewed by Oppenheim, 1991). The neurotrophic hypothesis states that the target fields regulate the final number of neurons innervating them by synthesising limited amounts of trophic factor(s) required by the neurons for their survival. Neurons that do not get sufficient amounts of this factor subsequently die, resulting in innervation appropriate to the requirements of the target field. This concept was confirmed by experiments in which the grafting of an additional limb bud near the original one reduced naturally occurring cell death of neurons, implying that two limb buds would release additional amounts of a putative survival factor that would result in the survival of more neurons (Hollyday and Hamburger, 1976). Also, the proportion of

neurons undergoing cell death was proportional to the amount of limb tissue removed (Hamburger, 1939a; b). Together with Rita Levi-Montalcini, Victor Hamburger demonstrated that DRG neurons are normally generated in great excess, with a subsequent reduction in number following the generation of these cells (Hamburger and Levi-Montalcini, 1949).

To examine the hypothesis that a trophic factor promotes neuronal survival sarcomas 180 and 37 were implanted in the hind-limb fields of embryonic chicks to increase the bulk of potential target tissue for innervation. These sarcomas were invaded by peripheral ganglia processes and certain ganglia grew in size up to six-fold their normal size (Bueker, 1948; Levi-Montalcini and Hamburger, 1951). This suggested that the sarcomas produced a factor that was conveyed along the nerve fibres to promote survival of the neurons. The factor in question was found to be diffusible, supporting neurons even when their processes did not invade the sarcomas (Levi-Montalcini and Hamburger, 1953). Levi-Montalcini decided to devise a tissue culture bioassay to help isolate this substance, by culturing small sympathetic and sensory ganglia in proximity of small fragments of the tumours. The result of these experiments was rather astonishing: after twelve hours in culture, nerve fibres had grown out from the entire periphery of sensory and sympathetic ganglia and had spread out radially around the explants (Levi-Montalcini et al., 1954). This "NGF (Nerve Growth Factor) halo" could then be used to assess the presence of this factor in any tissue or fluid. The term Nerve Growth Factor was coined when Stanley Cohen, Rita Levi-Montalcini and Viktor Hamburger identified the active part of the sarcomas in the microsomal fraction (Cohen et al., 1954). In an interesting coincidence, Cohen then attempted to use snake venom to further purify the tumoral



factor. As a source of phosphodiesterase, snake venom was meant to degrade the nucleic acids present in the active fraction. This was done to establish whether nucleic acids were an essential component of NGF or if it was the protein that was responsible for the observed effects. Surprisingly, addition of the snake venom potentiated the effect of the active fraction, an effect attributed to the presence of NGF in the snake venom, but at a much higher concentration than in the sarcomas (Cohen and Levi-Montalcini, 1956). Testing of the homologue of the venom gland in the mouse, the salivary glands, resulted in the identification of an even richer source of NGF, allowing Stanley Cohen to purify and characterize NGF further (Cohen, 1959; 1960) (see section 1.3.1 for a detailed description of the NGF protein).

Anti-NGF antibodies administrated during the phase of target field innervation reduced numbers of neurons in neural crest derived sensory and sympathetic ganglia (Levi-Montalcini & Angeletti, 1966, 1968) whereas administration of NGF reduced cell death in these ganglia (Hamburger et al., 1981). In mice with targeted null mutations of the NGF gene (Crowley et al., 1994) and its receptor trkA (Smeyne et al., 1994) the same populations of neurons were lost as a result of increased cell death.

Since the initial findings by Levi-Montalcini and her colleagues, a wealth of evidence has emerged in support of the neurotrophic hypothesis. NGF is retrogradely transported from the synaptic terminals to the cell bodies of sympathetic neurons (Hendry et al., 1974; Johnson et al., 1978). Smooth and cardiac muscle, the targets of sympathetic neurons, were found to be the source of this NGF (Korsching and Thoenen, 1983).

NGF does not affect the survival and proliferation of sympathetic neuroblasts

(Ernsberger et al., 1989), and sympathetic neurons become dependent on NGF close to the time they start innervating their targets (Korsching and Thoenen, 1988). Likewise, sensory neurons survive independently of neurotrophins when their axons are growing to their targets (Davies and Lumsden, 1984; Ernsberger and Rohrer, 1988; Davies, 1989), and upon reaching their targets, these neurons become dependent on neurotrophins for their survival (Buchman and Davies, 1993). It has been suggested that neurotrophins can promote the survival of some migrating cells destined to become sensory neurons, but this occurs before they differentiate (Kalcheim, 1996). Furthermore, the distance peripheral sensory axons need to grow to their targets is correlated with the duration of neurotrophic factor independence (Vogel and Davies, 1991). More details about this period of neurotrophin independence are described in chapter 6. Also, synthesis of NGF in target fields coincides with the time point of innervation by NGF-dependent neurons (Davies et al., 1987), and the final innervation density of target fields by neurons is proportional to the amount of NGF synthesised in them. Cutaneous regions with high innervation density have higher levels of NGF mRNA than regions with lower innervation density (Harper and Davies, 1990).

1.2.2 The neurotrophins

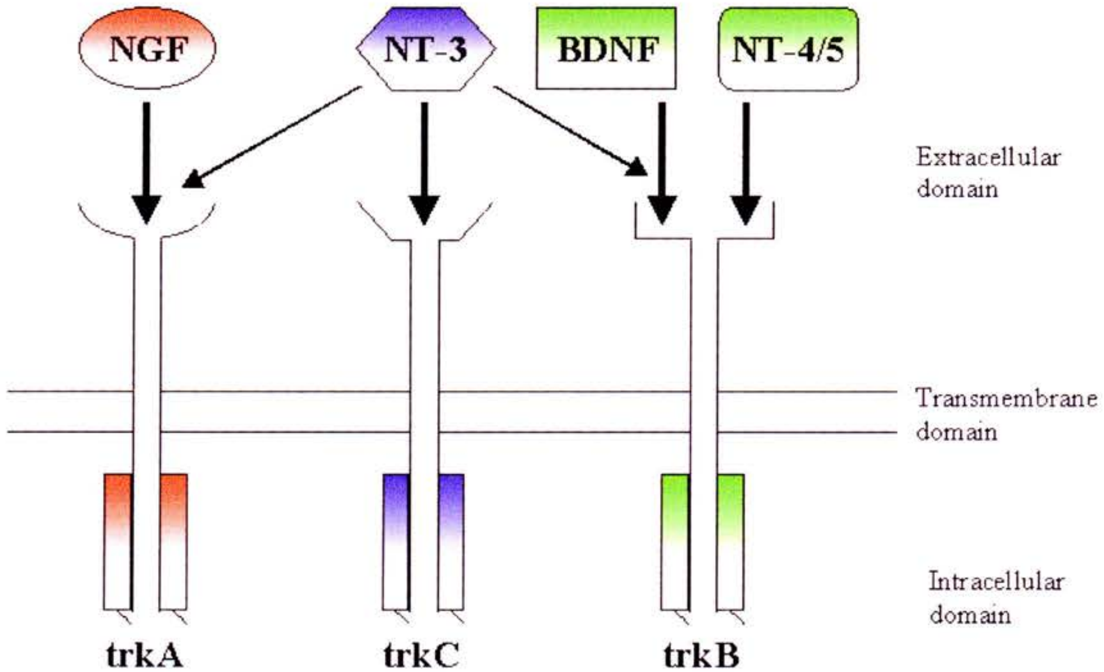
In 1982, Yves-Alain Barde purified Brain-Derived Growth Factor (BDNF) from pig brain, which bore a highly homologous primary structure to NGF. It promoted the survival of dorsal root ganglion embryonic sensory neurons in vitro, and administration in vivo prevented their death during the phase of naturally occurring

cell death (Hofer and Barde, 1988). Several additional related neurotrophins were subsequently identified: Neurotrophin-3 (NT-3) (Ernfors et al., 1990; Hohn et al., 1990; Jones and Reichardt, 1990; Rosenthal et al., 1990), Neurotrophin-4/5 (NT-4/5) (Berkemeier et al., 1991; Hallbröök et al., 1991; Ip et al., 1992), NT-6 (Götz et al., 1994) and NT-7 (Nilsson et al., 1998).

Several receptors have been identified for the neurotrophins: members of the trk (tropomyosin-receptor-kinase) family of receptor tyrosine kinases and the common neurotrophin receptor p75. TrkA predominantly binds NGF, trkB mainly binds BDNF and NT-4 and trkC binds NT-3, although NT-3 can also signal less efficiently via trkA and trkB (Barbacid et al., 1994). All neurotrophins bind to p75 with similar affinity (Rodriguez-Tebar et al., 1990, 1992). p75 has several functions depending upon the cells in which it is expressed. For example, it can enhance neurotrophin signalling in the presence of trkA (see sections 1.2.7, 1.4.9, 1.5.5), or promote cell death in the absence of trkA (see sections 1.2.7, 1.4.9).
The interactions between these neurotrophins and their receptors can be seen in figure 1.4. In addition to biochemical evidence for the above receptor/ligand interactions, histological studies of knockout mice have revealed corresponding neuronal deficiencies in *trkA, trkB,* and *trkC* knockout mice (Smeyne et al., 1994; Klein et al., 1993) and in *NGF*, *BDNF*, and *NT-3* knockout mice (Crowley et al., 1994; Ernfors et al., 1994; Liu et al., 1995; Fariñas et al., 1994). These studies also provided evidence that NT-3 signalling via TrkA and TrkB is physiologically relevant, since neuronal deficits in *NT-3* knockouts are more severe than in *trkC* knockout mice for certain neuronal populations. Further evidence for selective

receptor/ligand interactions was provided by the demonstration that transfection of BDNF-dependent neurons with a trkA expression plasmid conferred an NGF survival response (Allsopp et al. 1993) and vice versa (Ninkina et al., 1996).

Figure 1.4: Ligand-receptor specificities of the neurotrophin family and its trk receptors



Schematic diagram summarising the interaction of neurotrophins with members of the trk family of tyrosine kinase receptors. Large arrows indicate stronger interactions and weak arrows weaker interactions. NGF binds to trkA (Klein et al., 1991), BDNF and NT-4/5 bind to trkB (, and NT-3 binds predominantly to trkC (Lamballe et al., 1991), but also to a lesser extent to trkA and trkB (Berkemeier et al., 1991; Klein et al., 1991; Huang et al., 1999).

In addition to promoting neuronal survival, neurotrophins have been found to regulate neuroblast proliferation and differentiation, recovery after injury and synaptic plasticity in particular locations in the developing and mature nervous system. Dale Purves (1988) formulated the trophic theory of neural connections,

which extends the trophic actions of survival factors to neuronal connectivity, the regulation of axonal and dendritic branches. The pattern of neural connections would thus be regulated by factors in the target, allowing for adjustments during development and maturation of the animal. I shall not go into detail about other functions of neurotrophins, as this thesis is mainly concerned with the period in development relating to survival of neurons.

1.2.3 Other trophic factors

Although the neurotrophins and their receptors, described in detail in section 1.3, are the main factors of interest in the studies presented in this thesis, several other proteins have been identified that are able to support the survival of a range of neuronal populations during development. In this subsection I shall briefly mention the best characterised families of neurotrophic factors.

A The Neurotrophic Cytokines

The neurotrophic cytokines include ciliary neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF), oncostatin-M (OSM), cardiotrophin-1 (CT-1) and interleukin-6 (IL-6). They signal through a multicomponent receptor signalling system consisting of dimers of signal transducing β components, consisting of either gp130 homodimers (Il-6) or gp130/LIFRβ heterodimers (CNTF, LIF, CT-1). Receptor complexes for CNTF and IL-6 include an additional α component. Specific receptor subunits determine the specificity for each cytokine (Stahl and Yancopoulos, 1994).

CNTF was initially identified as a trophic factor for parasympathetic neurons of the

embryonic chicken ciliary ganglion (Adler et al., 1979; Barbin et al., 1984), and has since been demonstrated to be able to support various neuronal populations of both the CNS and PNS including motor, sympathetic and certain sensory neurons (Barbin et al., 1984; Manthorpe & Varon, 1985; Arakawa et al 1990; Oppenheim et al., 1991; Martinou et al., 1992; Burnham et al., 1994; Vejsada et al., 1995; Horton et al., 1998).

LIF promotes the survival of motor neurons, sympathetic neurons and certain sensory neurons (Li et al., 1995; Sendtner et al., 1996; Martinou et al., 1992; Murphy et al., 1993; Kotzbauer et al., 1994; Thaler et al., 1994; Horton et al., 1998).

OSM promotes the survival of a subset of late fetal DRG neurons in culture, as well as embryonic sensory neurons of the trigeminal and nodose ganglia (Ware et al., 1995, Horton et al., 1998).

CT-1 promotes the survival of cultured ciliary ganglion neurons (Pennica et al., 1995), midbrain dopaminergic neurons and motor neurons (Pennica et al., 1995, 1996) and embryonic and postnatal sensory neurons (Horton et al., 1998).

IL-6 has been shown to have a trophic action on a proportion of forebrain cholinergic and midbrain catecholaminergic neurons and embryonic sensory neurons (Hama et al., 1989; Kushima et al., 1992; Horton et al., 1998).

B The TGF-β superfamily

The Transforming Growth Factor- β (TGF-β) superfamily of neurotrophic factors includes a variety of subfamilies, such as TGF-β itself, Bone Morphogenetic Proteins (BMPs), Growth/Differentiation Factors (GDFs), and the glial cell line-derived neurotrophic factor (GDNF) family.

The GDNF family includes GDNF (Linet al., 1993), neurturin (NTN) (Kotzbauer et al., 1996), persephin (PSP) (Milbrandt et al., 1998), and artemin (ART) (Baloh et al., 1998b). Members of the GDNF family act through a multicomponent receptor signalling system comprising the transmembrane tyrosine kinase Ret plus one of four glycosyl-phosphatidyl inositol- (GPI-) linked proteins, the GDNF Family Receptors α1-4 (GFRα1-4) (Saarma and Sariola, 1999). Different members of the GDNF family preferentially activate Ret with one of these subunits, although some are able to use alternative heterodimer receptors in some circumstances (e.g. Buj-Bello et al., 1997; Baloh et al., 1997; Cacalano et al., 1998). The preferred receptors are GFRα1 for GDNF, GFRα2 for NTN, GFRα3 for Artemin, and GFRα4 for PSP. (Jing et al., 1996; Treanor et al., 1996; Baloh et al., 1997; Buj-Bello et al., 1997; Creedon et al., 1997; Klein et al., 1997; Sanicola et al., 1997; Baloh et al., 1998; Enokido et al., 1998; Thompson et al., 1998; Trupp et al., 1998; Forgie et al., 1999).

GDNF family members exert neurotrophic effects on both CNS and PNS neurons. In the CNS, GDNF, NTN and ART support the survival of midbrain dopaminergic neurons (Cacalano et al., 1998; Baloh et al., 1998) and GDNF, NTN and PSP promote the survival of some motor neurons (Henderson et al., 1994; Oppenheim et al., 1995; Moore et al., 1996; Sanchez et al., 1996; Heuckeroth et al., 1998; Milbrandt et al., 1998, Cacalano et al., 1998; Enomoto et al., 1998). In the PNS, GDNF, NTN and ART support sympathetic neurons and certain sensory neurons (Henderson et al., 1994; Kotzbauer et al., 1996; Buj-Bello et al., 1995; Moore et al., 1996; Sanchez et al., 1996; Pichel et al., 1996; Matheson et al., 1997; Molliver et al., 1997; Baloh et al., 1998; Millbrandt et al., 1998; Cacalano et al., 1998; Enomoto et al., 1998; Nishino et al., 1999; Enomoto et al., 2000; Baudet et al., 2000; Andres et

al., 2001).

TGFβ proteins appear to regulate the survival of some neurons by modulating their response to other neurotrophic factors (Krieglstein et al., 2002). For example, they increase the trophic potency of neurotrophins on DRG neurons (Krieglstein and Unsicker, 1996) and of CNTF and FGF on chick ciliary ganglion neurons (Krieglstein et al., 1998). TGFβs also synergise with GDNF in promoting the survival of certain PNS neurons and CNS dopaminergic neurons (Krieglstein et al., 1998).

C Hepatocyte Growth Factor (HGF) and Macrophage Stimulating protein (MSP)

Hepatocyte Growth Factor (HGF), which acts via the c-met receptor tyrosine kinase, is a pleiotropic factor with recently identified neurotrophic actions (reviewed by Maina and Klein, 1999). As well as being a chemoattractant and neurite growth promoter (Ebens et al., 1996; Maina et al., 1997; 1998; Yang et al., 1998; Davey et al., 2000), HGF promotes the in vitro survival of spinal motor neurons and sympathetic neuroblasts (Ebens et al., 1996; Wong et al., 1997; Yamamoto et al., 1997; Maina et al., 1998). It also enhances the neurotrophic actions of other trophic factors, such as CNTF, on spinal motor neurons, sensory neurons and parasympathetic neurons (Ebens et al., 1996, Wong et al., 1997; Davey et al., 2000). It is also needed for the survival of the full complement of DRG neurons in vivo, probably by synergising with NGF (Maina et al., 1997).

Macrophage Stimulating Protein (MSP) is a secreted protein that has a 45% sequence identity to HGF (Leonard and Skeel, 1978; Yoshimura et al., 1993) and signals

through the Ron receptor tyrosine kinase (Wang et al., 1994). It has recently been identified to act as a neurotrophic factor in the later embryonic and postnatal development of the trigeminal and dorsal root ganglia (Forgie et al., 2003), being able to support almost as many neurons as NGF in the postnatal period. MSP also promotes the survival early SCG neurons (Forgie et al., 2003).

1.2.4 Trophic support between ganglion and target field

As already stated, the first identified mode of action by neurotrophins was as factors released from the target field – an action that can be mediated by retrograde neurotrophin transport, with neurotrophins binding to their trk receptors and being transported to the cell body in a neurotrophin-receptor complex (Watson et al., 1999). Other modes of support have since been identified (Davies, 2003), including retrograde transport from non-target tissue, anterograde action from afferents and autocrine action.

It has been shown that some tissues lying on the pathway the axons grow towards their targets can provide intermediate trophic support to neurons (Davies, 1997). BDNF and NT-3 for example, which support the survival of early trigeminal ganglion neurons, are initially expressed in tissue that lies on the path trigeminal axons grow towards their peripheral targets (Buchman and Davies, 1993), and are likely to act as survival factors at this stage because substantial numbers die at this time in trkB and NT-3 knockout mice (Piñon et al., 1996; Wilkinson et al., 1996). NT-3 is also expressed in tissues along the course DRG axons grow to their targets (Fariñas et al., 1996; White et al., 1996; Liebl et al., 1997). In the central nervous

system there is some evidence that spinal commissural neurons also depend on substances released by an intermediate target, the floorplate (Wang et al., 1999).

Glial cells have also been implicated in the maintenance of neurons, since these have been found to synthesize trophic factors (Lindsay et al., 1982; Saarma and Sariola, 1999; Mirsky, 2002; Chapter 6). In embryos lacking Schwann cells and Schwann cell precursors due to mutations in neuregulin (the main trophic factor for early Schwann cells) or one of its ErbB receptors there is widespread loss of sensory and motor neurons during development (Meyer and Birchmeier, 1995; Gasmann et al., 1995; Lee et al., 1995; Riethmacher et al., 1997). It is possible that some of the trophic function of Schwann cells is mediated after the period of target field innervation, since the enhanced neuronal death observed in ErbB3 knockout embryos occurs later in development than the one observed in trkB and NT-3 knockouts (Davies, 2003). More evidence for intermediate trophic support by Schwann cells can be seen in chapter 6.

1.2.5 Anterograde trophic support

Another source of trophic factor for some neurons comes from their afferent inputs. The first evidence for this came from the observation that some populations of neurons undergo increased naturally occurring cell death upon deafferenation, and less upon hyperinnervation (Linden and Serfaty, 1985; Wright, 1987; Furber et al., 1987; Linden, 1994). Indeed, a number of instances have been identified, in which trophic factors are anterogradely transported. In some situations this may be due to a survival-unrelated function of the trophic factor at the synapse, but there is evidence

that some anterogradely transported factors do induce survival (Altar et al., 1997). For example, BDNF is anterogradely transported and released from some sensory neurons (Zhou and Rush, 1996; Michael et al., 1997) and is expressed during the period of naturally occurring cell death. When these NGF-dependent neurons are cultured in vitro they release BDNF, which is able to maintain co-cultured BDNF-dependent neurons (Robinson et al., 1996). Cortical axons have been shown to anterogradely transport BDNF to the striatum, and a subset of striatal neurons is missing in BDNF null mutant mice, suggesting that these neurons are reliant on anterogradely transported BDNF in vivo (Altar et al., 1997). Noradrenergic neurons overexpressing BDNF are neuroprotective to innervated motor neurons following axotomy, suggesting an anterograde support function (Fawcett et al., 1998). There is some evidence that anterogradely transported BDNF from retinal ganglion cells is involved in the survival of the superior colliculus (Caleo et al., 2000). NT-3 has also been shown to be anterogradely transported, by retinal ganglion cell axons (von Bartheld and Butowt, 2000).

Another mode of afferent support has been found to be dependent on electrical activity. Blocking electrical activity results in enhanced loss of neurons in the developing chick superior cervical ganglion (Madedrut et al., 1988). This trophic effect has been attributed to the afferents maintaining their target cells in a depolarised state (Bennet and White, 1979), leading to influx of calcium through voltage-dependent calcium channels and sustaining survival by intracellular mechanisms which are not fully understood (Franklin and Johnson, 1992).

1.2.6 Autocrine trophic support

Evidence that trophic factors can act by an autocrine mechanism comes from several sources. Many cortical neurons co-express BDNF and TrkB in vivo (Miranda et al., 1993; Kokaia et al., 1993; Ferrer et al., 1997; Schwartz et al., 1997; Pitts and Miller, 2000; Dieni and Rees, 2002), and the survival of these neurons in culture is reduced by BDNF antiserum (Ghosh et al., 1994). An early maturational change in embryonic chicken DRG neurons is accelerated by exogenous BDNF and reduced by antisense-BDNF oligonucleotides (Wright et al., 1992). No effects of BDNF on survival were found in this study or another recent study of early embryonic sensory neurons (Huber et al., 2000), but a subsequent study using antisense-BDNF in the adult DRG reported that a subset of neurons are supported by a BDNF autocrine loop (Acheson et al., 1995). However, since trkB appears to be expressed predominantly in NGF-dependent trkA-expressing neurons in the adult (Kashiba et al., 1997), an autocrine mechanism would appear to be ruled out in vivo (Lewin and Barde, 1996).

NGF has also recently been implicated in an autocrine loop in developing retinal horizontal cells (Karlsson et al., 2001). These neurons express both NGF and trkA, and can be killed with NGF antisense oligonucleotides. HGF appears to act by an autocrine loop mechanism in sympathetic neuroblasts, which express both HGF and Met, and many of these cells are killed by anti-HGF in culture (Maina et al., 1998). Further, neurotrophic factors promote the expression of second survival factors that act in an autocrine manner. There is evidence that the neurotrophic protein Reg-2, whose expression in motor neurons is activated by CNTF, mediates the CNTF survival effect on these neurons in an autocrine/paracrine manner. Blocking of Reg-2 expression abrogates the survival response of CNTF on cultured motor neurons

(Nishimune et al., 2000). TGF-β, which promotes the survival of some DRG neurons in combination with NT-3 and NT-4, does so in an autocrine loop manner. It is released by DRG neurons and promotes their survival (Krieglstein and Unsicker, 1996).

1.2.7 Trophic factors as pro-apoptotic factors

Although p75 has been shown to enhance the trkA-mediated survival effects of NGF (Davies et al., 1993; Horton et al., 1997; Ryden et al., 1997 and chapter 5), in oligodendrocytes p75 was shown to mediate a cytotoxic response to high levels of NGF (Casaccia-Bonnefil, 1996). The first indication for the involvement of p75 in promoting cell death came from the observation that a reduction in p75 expression by antisense oligonucleotides enhanced the in vitro survival of NGF-deprived postnatal DRG neurons (Barrett and Bartlett, 1994). Further evidence indicating that endogenous NGF acting via p75 promotes neuronal death came from studies of the chicken embryo retina (Frade et al., 1996). Anti-NGF antibodies or antibodies that block binding of NGF to p75 cause a marked reduction in the number of cells that undergo apoptosis in the E4 retina before retinal axons begin innervating the tectum. At this early stage of development NGF is expressed in the retina and the great majority of cells that normally undergo apoptosis express p75 but not TrkA. Further in vivo evidence includes the finding that basal forebrain cholinergic neurons, which express p75 under normal circumstances, are present in elevated numbers in p75-deficient mice (Van der Zee et al., 1996; Naumann et al., 2002).

BDNF may also have a detrimental effect by binding to p75, with BDNF increasing death in some sympathetic neurons (Bamji et al., 1998). Also, NT-4 appears to act through p75 to induce apoptosis in trigeminal neurons (Agerman et al., 2000).

Ontogenetic neuron death of ciliary, dorsal root and spinal motor neurons is largely prevented, and neuronal losses following limb bud ablation are greatly reduced following immuno-neutralisation of endogenous TGFβ in the chick embryo (Krieglstein et al., 2000). Further, TGFβ neutralisation has the same effect as NGF neutralisation in preventing neuronal death in the early developing retina (Dünker et al., 2001), suggesting that this trophic factor can signal to induce cell death as well.

The significance of the pro-apoptotic pathways activated by 'trophic' factors is not well understood as yet, with relatively few observations reported *in vivo*. In these instances, the observations have been made either during development or following ischaemic stress. Pro-apoptotic mechanisms may thus serve to ensure the efficient elimination of cells during developmental processes or in stress-related conditions.

1.3 Molecular biology of neurotrophins and neurotrophin receptors

This section describes the best-characterised neurotrophins. Emphasis will be on NGF, BDNF and NT-3 and their receptors, since these are relevant to the research presented in this thesis. In particular, this section focuses on the discovery, genetics and structure of these neurotrophins and their isoforms.

1.3.1 Nerve Growth Factor (NGF)

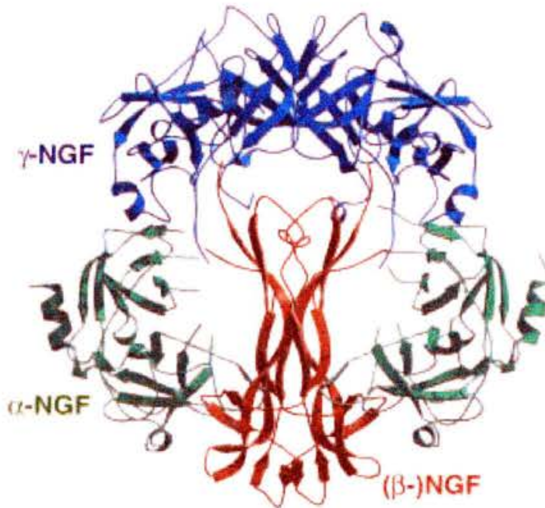
Analysis of the sarcomas 180 and 37 (see above) led to the initial isolation of Nerve Growth Factor (NGF) (Cohen et al., 1954). NGF was subsequently isolated and characterised from other sources, such as snake venom (Cohen et al., 1959) and the adult male mouse submandibular gland (Cohen, 1960).

The human NGF gene is composed of a single 3' exon that encodes the entire prepropeptide, and three upstream exons that give rise to 5' untranslated sequences (D'Mello and Heinrich, 1991). Promoter activity is associated with the first and third exon. The majority of transcripts contain exon I, with the other exons undergoing alternative splicing to give rise to various mRNAs with different 5'untranslated regions (5'UTR's), which may be responsible for stabilising the mRNA and facilitating translation (Metsis, 2001). Transcripts initiated from the promoter linked to the exon III encode this exon and the 4th coding exon. The mechanisms regulating the transcription of the NGF gene are not well understood, although it is known that transcription factors such as c-fos play a role (Hengerer et al., 1990).

Biologically active NGF (the β-subunit of NGF) extracted from the mouse submandibular gland (Angletti and Bradshaw, 1971) is expressed in a complex

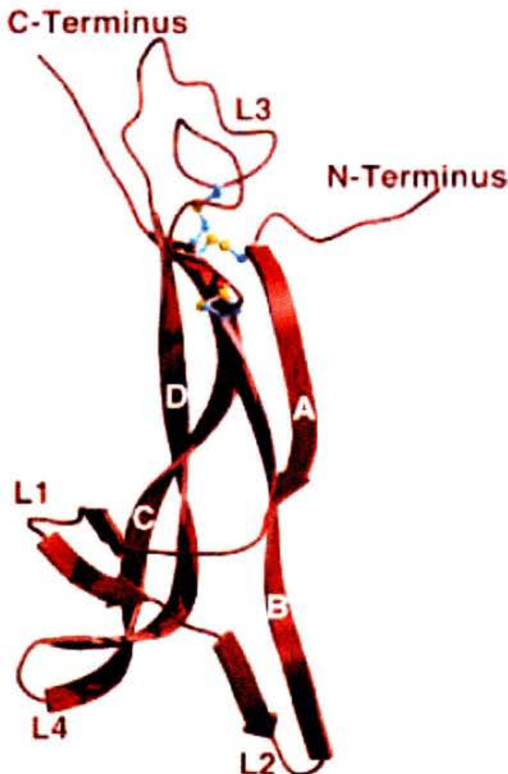
(termed 7S complex) with two other molecules termed α- and γ-NGF (Figure 1.5) (Bax et al., 1997). NGF is expressed as a prepropeptide comprising a signalling peptide and a propeptide. The γ subunit of the complex is a highly specific protease, and appears to be needed to process pre-NGF into its active mature form (Jongstra-Bile et al., 1989). The precise function of the α subunit of the complex is not known, although it has been suggested that it protects NGF from enzymatic degradation (Bax et al., 1997). Active NGF is a non-covalently held dimer of two subunits comprising 118 amino acids per monomer (Angletti and Bradshaw, 1971). Each monomer (figure 1.6) has at one end six cysteine residues forming three disulfide bridges. Two of these bridges and their residues form a ring structure. The third disulfide bridge passes through this ring, forming a 'cysteine knot' (McDonald and Hendrickson, 1993; Murray-Rust et al., 1993). Binding specificity is conferred by four hairpin loop regions, whose amino acids vary between neurotrophins (Ebendal et al., 1992). Braced by these two regions are two anti-parallel β strands, which form a flat surface where the two subunits associate, giving the molecule an elongated shape (McDonald et al., 1991; Holland et al., 1994). Hydrophobic forces stabilise the two protomers of the NGF dimer in a head-to-head conformation. This structural pattern is conserved in the other neurotrophins (Robinson et al., 1995), apart from the above mentioned hairpin loop regions.

Figure 1.5: Complex of β-, α- and γ-NGF



Ribbon diagram showing the structure of the 7S NGF complex (PDB access code I SGF). The β-NGF dimer is in red in the center, two copies of α-NGF are shown in green, and two copies of γ-NGF in blue (from Wiesmann and de Vos, 2000).

Figure 1.6: Structure of the NGF monomer



Ribbon diagram depicting the structure of the NGF monomer. Shown are the six cysteine-rich domains forming a cysteine knot (gray and yellow near top), the four hairpin loop regions (L1-L4), and the two pairs of twisted, anti-parallel β-strands (A-D) (from Wiesmann and de Vos, 2000). The structural pattern is conserved in other neurotrophins, with variations in the hairpin loop regions (Robinson et al., 1995).

1.3.2 Brain Derived Neurotrophic Factor (BDNF)

Like NGF, biologically active BDNF exists as a homodimer with each monomer being a 12.3 kDa 119 amino acid glycosylated protein. BDNF was first isolated from porcine brain (Barde et al., 1982) and has approximately 55% homology with the sequence of NGF. Like NGF, it conserves the six cysteine residues forming the three disulphide bridges (Leibroch et al., 1989), and exists as a homodimer (Radziejewski et al., 1992), giving it a very similar structure.

The rat BDNF gene contains four upstream noncoding exons in addition to the 3' coding exon. Each of the exons in the BDNF gene has its own promoter (Timmusk et al., 1994). Different promoters of the BDNF gene are used during development in different cell types, giving rise to mRNAs containing only one of the upstream noncoding exons spliced to the coding exon. BDNF mRNA containing exons I, II and III is mainly expressed in the brain, while transcripts containing exon IV predominate in the lung and heart (Timmusk et al., 1993; Metsis et al., 1993). Transcriptional up-regulation of the rat BDNF gene has been observed upon activation of glutamate receptors (Metsis et al., 1993). A neuron-restrictive silencing element-type sequence in the first intron of the gene is thought to modulate the levels of mRNA containing exons I and II in the glutamatergic system of the brain (Timmusk et al., 1999). It has been found that a 5kb region of promoter III is involved in gene induction after sciatic nerve transection (Timmusk et al., 1995), while regions flanking promoters I-III have been implicated as calcium-responsive regions (Bishop et al., 1997). Indeed, it was found that there were two regulatory sequences in the proximal promoter region involved in regulation of transcription in response to calcium influx. One, a calcium-response element, could regulate BNDF-

expression in both embryonic and postnatal cortical neurons (Shieh et al., 1998), the other, a cAMP response element (activated by CaM Kinase IV via CREB) appears to only regulate BDNF expression in postnatal neurons (Tao et a., 1998). Promoter IV is activated by CaM Kinase II (Takeuchi et al., 2000). c-fos also appears to regulate BDNF expression (Zhang et al., 2002).

1.3.3 Neurotrophin-3 (NT-3)

NT-3 was isolated by using degenerate primers with sequences based on the conserved domains of NGF and BDNF in the polymerase chain reaction (Hohn et al., 1990). It exists as a homodimer (Radziejewski et al., 1992) with each monomer being a 12.6 kDa 119 amino acid basic protein, and has a 50-60% amino acid identity with NGF and BDNF. Like these two proteins it possesses six cysteine residues.

The mouse NT-3 gene has two upstream non-coding exons that are flanked by promoters (Leingärtner and Lindholm, 1994). Transcription gives rise to mRNA containing only one of the upstream noncoding exons spliced to the 3' coding exon. There seem to be several transcription start sites in each of the upstream exons. Multiple NT-3 mRNA variants which differ slightly in length can be generated due to three different polyadenylation sites contained in the coding exon (Leingärtner & Lindholm, 1994).

1.3.4 NT-4/5

A phylogenetic analysis of the DNA sequences of members of the neurotrophin family in Xenopus ovary led to the discovery of NT-4/5 (Hallböök et al., 1991). NT-4 is a 13900 Dalton protein with a 50-60% amino acid homology with the previously discovered members of the neurotrophin family, and has a similar structure (Hallböök et al., 1991). Homologues of NT-4 were identified in mammals and termed NT-4 (Ip et al., 1992) or NT-5 (Berkemeier et al., 1991). It appears as if both proteins are identical and the mammalian equivalent of Xenopus NT-4 is often referred to as NT-4/5.

The rat NT-4 gene has two upstream non-coding exons (Salin et al., 1997). Transcription can be from exons I or II, giving rise to mRNA containing either both exons and the coding exon III, or just exons II and III.

1.3.5 NT-6 and NT-7

Attempts to clone the fish NGF gene in the platyfish *Xiphophorus maculatus* resulted in the discovery of NT-6 (Götz et al., 1994). NT-6 is a 16000 Dalton protein. It contains a hydrophobic domain at the N-terminus with the characteristics of a signal peptide, a pro-region containing basic motifs necessary for the proteolytic cleavage of the precursor protein, and the disulphide cysteine residues common to other neurotrophins. NT-6 is unique in that it has 22 amino acid residues inserted between the second and third conserved cysteine domains. NT-7 was identified from the carp *Cyprinus carpio* and zebrafish *Danio rerio* using a PCR-based approach (Nilsson et al., 1998; Ko et al., 1998). Amino acid sequence analysis of NT-7 has revealed a

high degree of homology with fish NGF and NT-6 (65% and 63% respectively) (Nilsson et al., 1998; Ko et al., 1998). NT-6 and NT-7 have only been found in fish.

1.3.6 Pro-neurotrophins

The importance of pro-neurotrophins was recently identified by Lee et al. (2001). Neurotrophins are initially produced as precursors, before being cleaved intracellulary by proteases, such as furin or the NGF subunit γ. Lee and colleagues discovered proNGF and proBDNF in a variety of tissues in vivo. Using a mutant NGF molecule with the 'pro' domain but resistance to furin cleavage, they performed a series of binding studies, and surprisingly found that the equilibrium-binding constant of proNGF to p75 was five fold higher than the one of mature NGF, whereas binding affinity to trkA was greatly reduced. Further, they found that proNGF could act as a potent cell-death inducer in sympathetic neurons. The balance between the availability of proNGF and mature NGF in cells expressing both trkA and p75 could thus represent the balance between death and survival in these cells. There may thus be a novel principle by which neurotrophins exert their actions through their two different receptors: in mature form, they would act as survival factors; and in proneurotrophin form, as a death signal. However, even using a mutant form of proNGF, isolation and further analysis proved difficult, since this was still sensitive to cleavage (Chao and Bothwell, 2002). So there would have to be mechanisms in vivo that protect the proNGF protein from cleavage.

1.3.7 The trk receptors

The *trk* proto-oncogene encodes a 140 kDa glycoprotein, and was initially identified in a human colon carcinoma (Martin-Zanca et al., 1986, 1989). The proto-oncogene encodes two tyrosine kinases of 790 and 796 amino acid residues known as trkA (Martin-Zanca et al., 1989). The two isoforms have a 32-amino acid long putative signal peptide, an NH2-terminal ligand binding extracellular domain (see below), a single transmembrane domain, and a cytoplasmic domain containing a catalytic tyrosine kinase domain. The 796 amino acid isoform is primarily expressed in neurons (Barker et al., 1993). Screening with the trk probe allowed the identification of two additional members of the trk family: trkB (Klein et al. 1989) and trkC (Lamballe et al. 1991). The tyrosine kinase domains of these two receptors share an 85% homology to trkA. The extracellular domain of all trk receptors includes two cysteine-rich domains (domains 1 and 3), 3 leucine-rich repeats (domain 2) and two immunoglobin (Ig)-like domains (domains 4+5) (Schneider and Schweiger, 1991). The extracellular domain shows 50-55% homology between the different trks (Lamballe et al., 1991).

Figure 1.7 shows the trk structures and some isoforms. Trk receptors bind their respective neurotrophins with high affinity (K_d ~10(-11) M; Godfrey and Shooter, 1986; Rodriguez-Tebar and Barde, 1988) and are capable of internalising them. Neurotrophin binding dimerises its receptor, inducing a series of autophosphorylations of the tyrosine kinases, which are connected to a second messenger system (Kaplan et al., 1991; Kaplan and Stephens, 1994). Domain 5 of the extracellular domain is the one most commonly associated with ligand binding, as it is sufficient for neurotrophin binding (Pérez et al., 1995; Ultsch et al., 1999).

Another component of the extracellular domain however, the leucine-rich domain 2, also appears to have a binding affinity for neurotrophins (Windisch et al., 1995; Haniu et al., 1997; Ninkina et al., 1997).

The extracellular domain of the trk receptors is encoded by nine exons in the case of trkA and C (Indo et al., 1997; Ichaso et al., 1998), and by 10 exons in the case of trkB (Strohmaier et al., 1996). Alternative splicing can give rise to isoforms of trk with different interaction specificities to its ligands (Strohmaier et al., 1996).

TrkA is highly expressed in NGF-dependent neurons of the mouse embryo (Martin-Zanca et al., 1990). Evidence for interaction between NGF and this receptor comes from ectopic expression in cells non-responsive to NGF (Allsopp et al. 1993). TrkB is the high affinity receptor for BDNF and NT-4 (Klein et al., 1991), and trkC binds NT-3 but not the other neurotrophins (Lamballe et al., 1993). Some signalling through trkA and trkB can occur via NT-3 (Klein et al., 1991), especially in the absence of p75 (Brennan et al., 1999).

Trk isoforms that lack the kinase domain and hence cannot activate the kinase domain associated signalling pathways are abundantly expressed in the mouse. Two truncated trkB receptor isoforms have been identified in the mouse, both containing a short cytoplasmic domain in place of the tyrosine kinase catalytic region (Klein et al., 1990; Middlemas et al., 1991). Other trkB variants containing deletions in domain 2 (leucine-rich) of the extracellular domain as a result of alternative splicing, have also been identified and shown to display impaired ligand binding. Both isoforms of trkB with normal and deleted domain 2, and normal or truncated kinase domain are found in neurons (Ninkina et al., 1997; Ninkina et al., 1996). TrkC has three isoforms with insertions in the kinase domain, and four lacking the kinase domain (Klein et al.,

1990; Middelmas et al., 1991; Lamballe et al., 1993; Tsoulfas et al., 1993; Valenzuela et al., 1993; Garner & Large, 1994). The exact function of these trk variants is unknown, but in the case of trkB it has been suggested that BDNF responsiveness in developing sensory neurons is modulated by the relative level of catalytic and non-catalytic trkB isoforms, as indicated by their relative expression levels in the trigeminal ganglion. Over-expressing truncated trkB isoforms decreases the sensitivity of these neurons to BDNF (Ninkina et al., 1996). Another proposed function of truncated forms of trkB is to present BDNF to full-length receptors, thereby positively regulating the BDNF response (Beck et al., 1993).

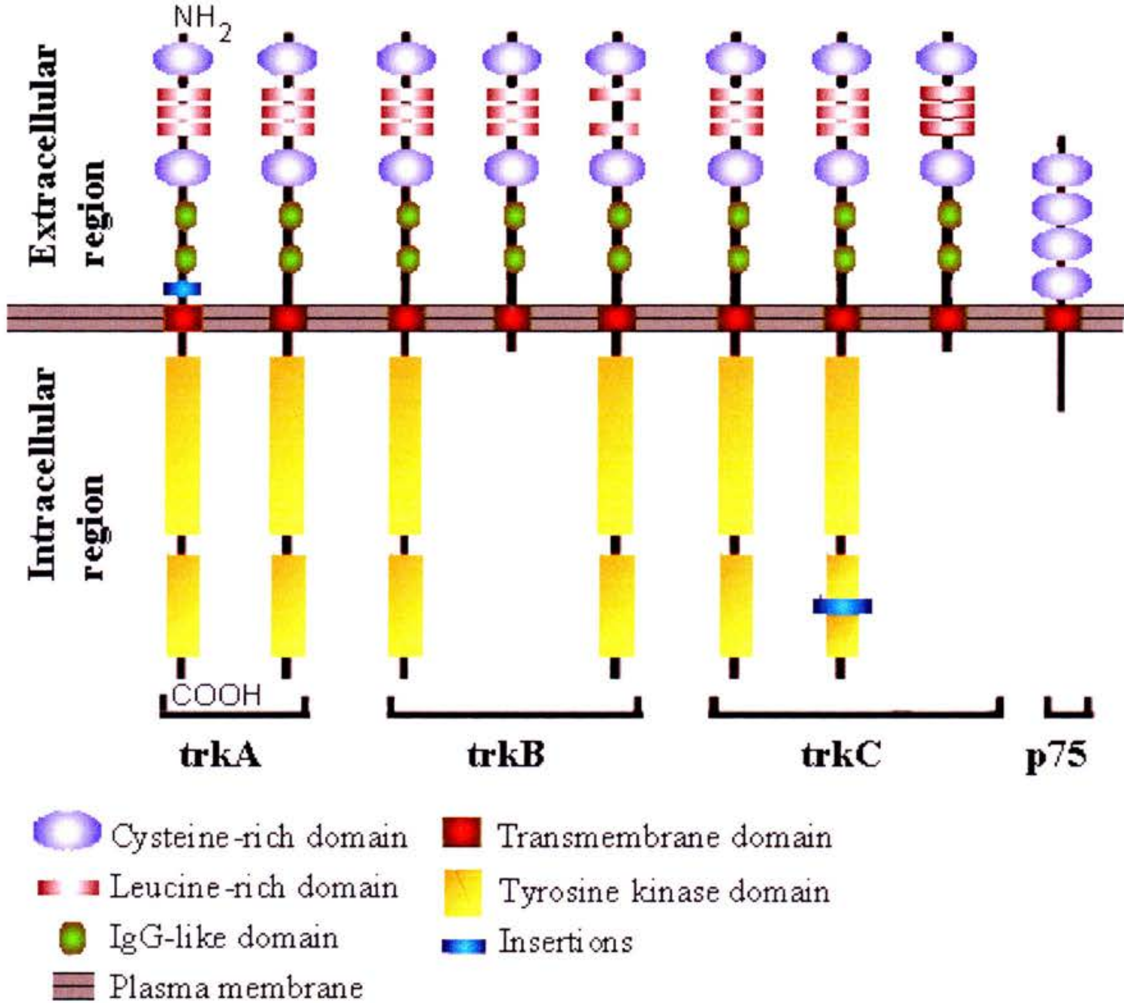
Although the mechanisms controlling trk receptor expression are still largely unknown, it has recently emerged that the transcription factor Brn3a plays an important role in regulating the expression of trks during development (Huang et al., 1999).

1.3.8 The low-affinity p75 receptor

The mature low affinity p75 receptor (K_d ~10(-9) M; Godfrey and Shooter, 1986; Rodriguez-Tebar and Barde, 1988) is a 75kD glycosylated 399 amino acid protein derived from a 427 amino acid polypeptide precursor (Radeke et al., 1987). It has a short cytoplasmic domain, a single transmembrane segment and four extracellular cysteine-rich domains, which are required for neurotrophin binding (see figure 1.7) (Welcher et al., 1991). The cytoplasmic domain incorporates a death domain similar to that of TNF receptors, although proteins such as TRADD or FADD cannot attach

(Wang et al., 2001). The domain consists of six α helices arranged in a compact globular domain typical for death receptors (Liepinsh et al., 1997).

<u>Figure 1.7: Neurotrophin receptors and their isoforms</u>



General structure of trk receptors, some of their isoforms, and p75. The trkA isoform on the left hand side with a 6 amino acid insertion is the one commonly expressed in neurons (Barker et al., 1993). Also shown is the trkB receptor, with its isoforms either lacking a kinase domain (Klein et al., 1990; Middlemas et al., 1991) or having deletions in the extracellular domain 2 (leucine-rich) (Ninkina et al., 1997), and the trkC receptor, which can exist with insertions in the kinase domain, or lacking the kinase domain (Tsoulfas et al., 1993; Valenzuela et al., 1993). The p75 receptor with its 4 cysteine-rich domains and short intracellular domain is also represented (Radeke et al., 1987).

A truncated form of p75 exists, which is composed of the extracellular domain (DiStefano and Johnson, 1988). This truncated form binds the neurotrophins with the same affinity as the full-length receptor and has been suggested to compete with the full-length receptor and thereby limit the activity of the neurotrophins. p75 has been shown to bind all neurotrophins with similar affinity, although the rate constants for the ligands are different: NGF was found to dissociate from the p75 receptor more rapidly than NT-3, which itself dissociates quicker than BDNF (Rodriguez-Tebar et al., 1992).

Weskamp and Reichhardt (1991) observed two distinct classes of high-affinity NGF receptors, one of which seemed to be dependent on p75 since it disappeared in the presence of p75 antibodies. This observation suggests that the high affinity NGF receptor can be composed of either two p75-trkA heterodimers or a trkA homodimer. By forming heterodimers with trkA, p75 could increase the number of available binding sites for NGF. Another possibility supported by experimental evidence (see below) is that p75 associated to trkA induces conformational changes in the latter, which leads to an increased binding affinity for NGF (Esposito et al., 2001). In p75 knockout mice there is a partial loss of sensory and sympathetic neurons, which would be explained by p75 enhancing trk activation (Lee et al., 1992).

1.4 Cellular mechanisms controlling death and survival and their interaction with neurotrophins

This section is aimed at examining the 'apoptotic' mode of cell death that neurons undergo during development. Once the components of the cells death/survival machinery have been described, I will give an overview of the major intracellular pathways, which connect the neurotrophins to these processes. This will include the second messenger pathways associated with trk autophosphorylation and p75 activation either in the presence or absence of trk signalling. The life/death mechanisms described below are not present in all neurons. Some of the mechanisms only exist in subpopulations of neurons. This explains why some populations can be insensitive to overexpression of a particular 'pro-apoptotic' molecule as an example, whereas others will undergo apoptosis at different rates in response to it. A summary of the mechanisms described in this section can be seen in figures 1.8 and 1.9.

1.4.1 Apoptosis versus necrosis

Cells undergoing cell death during development undergo a unique and distinct set of structural changes called 'Programmed cell death' or 'Apoptosis', that is a mode of gene-directed cellular self-destruction requiring RNA and protein synthesis (Cohen & Duke, 1984; Scott & Davies, 1990). Apoptosis was first identified in development and could be clearly distinguished from 'necrosis', the mode of cell death previously known. The term 'apoptosis' was not coined until 1972 however, when it was described in detail by Kerr, Wyllie and Currie as a means of distinguishing a

morphologically distinctive form of cell death that was associated with normal physiology (Kerr et al., 1972). Apoptosis during development is an active and widespread mechanism responsible for removing superfluous cells, or cells that have fulfilled their function and are not needed (Oppenheim et al., 1991; Williams, 1991). The 'naturally occurring cell death' described in section 1.2 occurs by apoptosis. Extrinsic or intrinsic 'induction signals' are followed by a cascade of physiological modifications in the cell, which lead to cell death. Typical signs of apoptosis involve separation of the cell from neighbouring cells and loss of membrane structures, such as microvilli and desmosomes. This is followed by plasma membrane blebbing and contortion. Apoptosis then enters its irreversible stage characterised by cytoplasmic condensation and increased cell density. The nuclear chromatin condenses to form dense granular caps, which is followed by DNA and cellular fragmentation, and phagocytosis by macrophages or surrounding cells (Wyllie et al., 1980).

Whereas early apoptosis tends to be associated with a shrinking of cells (apoptotic volume decrease – AVD), necrosis is characterised by cellular swelling (necrotic volume increase – NVI) and culminates in the rupture of plasma and internal membranes and leakage of cellular contents. Necrotic cell death is a response to major stresses to the cell and involves rapid lysis of cellular membranes without any requirement for protein synthesis. Necrosis is a far more dangerous route to follow for groups of cells, since the necrotic death of one cell and the intracellular spillage that ensues may cause stress to other cells, necessitating the activation of resident phagocytes and attraction of leukocytes (inflammation). In systems where the interaction between cells needs to be tightly regulated, such as in neuronal development, a more specific and controlled type of death is advantageous.

During neuronal development there are two modes by which apoptosis can be induced: firstly, there is a lack of neurotrophic factor support, suppressing the constant survival signal required by some neurons. Secondly, there is ligand-induced activation of 'death receptors', inducing intracellular apoptosis pathways.

1.4.2 Intracellular pathways associated with apoptosis

The first indications about the intracellular events required for apoptosis where gained from studying mutants of the nematode *Caenorhabditis elegans (C.elegans)* that were defective in the apoptotic pathway. *C.elegans* is particularly useful for studying apoptosis due to its low cell number, with a well-determined number of cells being born (1090 cells) and undergoing programmed cell death (131 cells) during development. Genetic analysis of mutants led to the identification of three genes needed for the life/death decision in somatic cells of *C.elegans*: *ced-3* and *ced-4* (Ellis & Horvitz, 1986), and *ced-9* (Hengartner et al., 1992). *ced-3* and *ced-4* activation is sufficient and necessary to induce cell death. *ced-9*, in contrast, is required for the survival of the cell.

Study of these genes revealed that *ced-3* encodes an aspartate-specific protease with a cysteine-containing active site, whose expression is required for all of the cell death in *C.elegans* (Xu et al. 1996). Related genes have been discovered in the mammalian genome, forming the caspase family (cysteine-containing asp-ase). ced-4 possesses an ATP-dependent hydrolytic activity thought to promote processing and activation of ced-3 by interacting with ced-3 in a 'casposome' (Hengartner, 1997). ced-9 represses activation of ced-4 and therefore ced-3 by sequestering ced-4 to the

mitochondrial membrane, preventing it from interacting with ced-3 (Chinnaiyan et al., 1997; Wu et al., 1997). A mammalian homologue of ced-4 exists in the form of Apaf-1 (Zou et al., 1997). Ced-9 has mammalian homologues in the form of bcl-2 and bcl-x_L, collectively called bcl-2-like proteins (Hengartner and Horvitz, 1994). The apoptotic machinery is extremely well conserved phylogenically. bcl-2 can act as a substitute in ced-9 nematode mutants for example (Hengartner and Horvitz, 1994). More recently another molecule known as EGL-1 was discovered in *C.elegans* (Conradt and Horvitz, 1998). EGL-1 binds to ced-9 via an amphipatic structure known as the BH3 domain (see below), inducing the release of ced-4. ced-4 can then translocate to the cytosolic side of the nuclear envelope where it forms the casposome with ced-3 and induces programmed cell death (Chen et al., 2000). EGL-1 has homologues in mammalian cells in the form of BH3-only proteins, such as Bik and Bad, which are thought to 'sense' apoptotic signals, and then 'mediate' these by releasing pro-apoptotic bcl-2 family members from intracellular structures they are sequestered to or from the anti-apoptotic bcl-2 family members they are bound to.

The main 'executors' of apoptosis are the caspases, cleaving selected intracellular proteins, essential for cell viability. So far ced-3 is the only caspase identified in *C.elegans*, whereas in mammalian cells there are 'initiator' and 'effector' caspases. 'Initiator' caspases, such as caspases-2, 8-10 and –12, may not have any direct role in cleaving intracellular proteins. Rather, they cleave and therefore activate 'effector' caspases, such as caspase-3, -6, and -7, which in turn execute apoptosis, creating a cascade of events (Chinnaiyan and Dixit, 1996). The constituent presence of caspases in cells necessitates the presence of powerful suppressors, to avoid inappropriate activation. The machinery for apoptosis is probably regulated by the levels/activity

of suppressor and executor present. Pro-apoptotic proteins, such as bax, are able to accelerate apoptosis, either directly (Xiang et al., 1996), or by blocking anti-apoptotic proteins such as bcl-2 (Oltvai et al., 1993).

Bax and bcl-2 are both members of the bcl-2-family of mammalian cell death regulators including pro-apoptotic proteins, such as Bax (Oltvai et al., 1993), Bad (Yang et al., 1995), and Bik (Boyd et al., 1995), and anti-apoptotic proteins, such as bcl-2 (Hockenbery et al., 1990) and bcl-x_L (Boise et al., 1993). The bcl-2 family can be defined by the presence of either some or all of four structural motifs in the protein sequence: BH1, BH2, BH3 and BH4. These motifs enable the proteins to hetero- or homodimerise, thereby interacting with each other to regulate apoptosis. Mutations in BH1 and BH2 of bcl-2 prevent it from binding to bax and lead to amplified apoptosis (Yin et al., 1994). The BH3-domain present in many pro-apoptotic proteins is essential for interaction with anti-apoptotic members of the family, such as bcl-2 and bcl-x_L (Wang et al., 1996; Zha et al., 1997). BH4 is needed for the protective effect of bcl-2 and bcl-x_L (Reed, 1997).

It has been proposed that the ratio of pro-apoptotic to anti-apoptotic proteins determines whether a cell survives or undergoes apoptosis by varying the amount of homodimers of the pro- or anti-apoptotic proteins present (rheostat model) (Korsmeyer et al., 1993). However, although interaction of bcl-2 family proteins is important in regulating apoptosis, they can each act independently of other family members (Knudson and Korsmeyer, 1997).

1.4.3 The role of Mitochondria in apoptosis

It is thought that a large proportion of caspase activation during apoptosis is due to the action of bcl-2 family members on mitochondria. Cytochrome c released from the inside of mitochondria strongly activates caspases. This, in conjunction with the finding that bcl-2 is primarily localized in mitochondrial plasma membranes (Hockenbery, 1990) led to the idea that the action of bcl-2 may be connected with the mitochondria. A collapse in the mitochondria's inner transmembrane potential occurs before DNA fragmentation (Vayssière et al., 1994), a phenomenon observed irrespective of the mode of apoptosis activation. This permeability transition (PT) is thought to be executed by mitochondrial megachannels (PT pores) (Petit et al., 1996), rendering the membrane permeable to ions and paving the way for an equilibration of ions across the membrane. This dissipates the H^+ gradient across the inner membrane, uncoupling the respiratory chain and ATP synthesis. Swelling of the matrix induced by this ion equilibration can lead to outer membrane disruption and release of cytochrome c and other important proteins, such as apoptosis-inducing factor (AIF) (Lorenzo et al., 1999). Products released from the mitochondria were found to activate nuclear endonucleases, as well as stimulating caspase-3 activation (Liu et al., 1996; Susin et al., 1997). Other pro-apoptotic molecules released from the mitochondria, such as Smac/DIABLO (Du et al., 2000) and the serine protease Htr2A/Omi (Hegde et al., 2002), can sequester or degrade anti-apoptotic substances and therefore potentiate the action of pro-apoptotic proteins. Cell-free studies have shown that mitochondrial products are necessary for many events associated with the induction of apoptosis in mammalian cells (Zamzami et al., 1996).

Bcl-2 and bcl-x_L inhibit or retard the mitochondrial changes observed in apoptotic cells (Susin et al., 1996; Kharbanda et al., 1997), an anti-apoptotic effect dependent on localization to mitochondrial membranes (Decaudin et al., 1997). Pro-apoptotic members of the bcl-2 family like bax increase mitochondrial membrane permeability. The structures of both pro- (bax) and anti-apoptotic (bcl-2, bcl-x_L) members of the bcl-2 family have similarities to some ion channel forming bacterial toxins (Muchmore et al., 1996). They are able to form ion-conducting channels in artificial lipid membranes, suggesting they may do so in vivo (Minn et al., 1997; Schendel et al., 1997; Schlesinger et al., 1997). It is thus possible, that some bcl-2 family proteins act by regulating an electrochemical gradient or by interacting with other components in the plasma membrane of mitochondria, such as the PT pore. This is especially significant since it was recently shown that the release of many apoptosis-inducing molecules, such as cytochrome c, from the mitochondria precedes the disruption of the mitochondrial membrane due to swelling (von Ahsen et al. 2000). This indicates that swelling and membrane disruption are by-products of the channels made by the pro-apoptotic molecules in the mitochondrial membrane, and that membrane disruption serves to potentiate the apoptotic processes further downstream. Bcl-2 and bcl-x_L can only form channels at a non-physiologically low pH of 4 however, and there are no measurements of these channels in vivo (Minn et al., 1997; Schendel et al., 1997). It is thus unlikely that channels formed by these two anti-apoptotic molecules are significant in vivo. Pro-apoptotic members of the bcl-2 family such as Bax, in contrast, form pores at physiological pH (Schlesinger et al., 1997). These pores are large enough to allow passage of fluorescent-labeled cytochrome c (Saito et al., 2001), and bax and bak are able to elicit cytochrome c

release from isolated mitochondria (Degenhardt et al., 2002). It is thought that anti-apoptotic bcl-2-family proteins prevent bax from undergoing the conformational change needed for these pores to form. Further to forming pores alone, bax can interact directly with components of the PT pore (reviewed in Zamzami and Kroemer, 2001). Blocking of the PT pore only slows the progression of apoptosis without being able to rescue the cell however (Desagher and Martinou, 2000). Also, the PT pore spans both inner and outer membranes of mitochondria. Most substances known to induce apoptosis lie between these membranes, making the PT pore an unlikely source for the release of substances from the intermembrane space (Borner, 2003).

Other organelles may also be involved in the actions of the bcl-2 family on mitochondrial permeability, such as the endoplasmatic reticulum (ER) and the perinuclear membrane, whose membranes bcl-2 is also localized to (Chen-Levy et al., 1989; Hockenbery et al., 1990; Jacobson et al., 1993). Bcl-2 bound to other membranes may be able to act as a scavenger molecule for pro-apoptotic molecules such as Bax, inhibiting their actions on mitochondria/caspases. ER-bound bcl-2, prevents Bax's translocation to mitochondria (Ferri and Kroemer, 2001). Anti-apoptotic members of the Bcl-2 family can prevent cytochrome c mediated apoptosis when overexpressed (Li et al., 1997; Rosse et al., 1998), suggesting actions downstream of their effects on mitochondria. Bcl-2 has been shown to regulate the activity of membrane associated pro-caspase-3 independently of cytochrome c (Krebs et al., 1999) and Bcl-x_L can bind directly to Apaf-1 preventing it from activating pro-caspase-9 (Hu et al., 1998; Pan et al., 1998). It remains to be determined whether this effect can occur at physiological concentrations of anti-

apoptotic bcl-2 proteins in vivo. Bcl-2 and Bcl-x_L have been found to bind to a variety of proteins non-specifically when overexpressed (Borner, 2003). Some of the above effects could thus be explained by non-specific binding of these proteins.

1.4.4 Receptor-mediated and other modes of apoptosis

There are other mechanisms of cell death that do not require mitochondria. The main identified mode is the mechanism of receptor-mediated apoptosis, by which extracellular ligands can induce direct activation of pro-apoptotic proteins such as caspases by interacting with so-called 'death-receptors', allowing them to bypass the mitochondrial apoptotic machinery. This pathway may at times use the mitochondrial pathway to amplify the death signals it induces. This is done after the point of no return is reached and is not essential to this pathway. This pathway is mainly induced by extracellular death-inducing ligands of the tumor necrosis factor (TNF) superfamily (reviewed by Locksley et al., 2001). These bind to specific receptors on the plasma membrane leading to their dimerisation. The 'death domain' of these receptors attracts adaptor proteins, such as TNF receptor associated death domain (TRADD) and Fas-associated death domain (FADD). These have a similar function as ced-4/Apaf-1, forming a casposome with the initiator caspase-8. The formation of this complex stimulates activation of caspase-8, which then in turn activates a variety of effector caspases leading to the execution of the 'death-signal'. Since FADD and TRADD have no homology to ced-4/Apaf-1 despite having a similar function, the bcl-2 family of proteins has no actions on this pathway (Huang et al., 1999). One of the actions by which caspase-8 can potentiate its apoptotic signal is by cleaving Bid,

allowing it to form pores in the mitochondrial membrane, a path likely to be used in vivo to enhance and accelerate the cells response to the death receptor.

There is evidence that other apoptosis pathways exist in addition to the mitochondrial and the receptor-mediated pathway. Apoptosis can be prevented in cells lacking functional mitochondria by overexpressing bcl-2 (Jacobson et al., 1993), suggesting some mitochondria-independent modes of action for the bcl-2 family. The ion pore forming capabilities of the bcl-2 family are not restricted to the mitochondrial plasma membrane, enabling it to make such pores or modulate existing ones in other organelles, such as the Endoplasmatic Reticulum (ER). Release of calcium from ER stores accompanies apoptosis in certain cells, with bcl-2 blocking this calcium release and apoptosis (Lam et al., 1994). Studies overexpressing bcl-2 found that it can have important effects on calcium handling by the ER, such as decreasing the calcium pool in the ER (Pinton et al., 2000). However, calcium release does not seem to be a prerequisite for apoptosis to occur, and bcl-2 can still prevent apoptosis associated with high cytosolic calcium levels (Zhong et al., 1993). Intracellular calcium may thus act as a potentiator rather than an initiator of apoptosis.

There appear to be apoptotic pathways involving the ER that are independent of the bcl-2 family and calcium release, such as over-activation of the ER processes involved in handling misfolded and aggregated proteins (Rao et al., 2002). Further, a casposome complex including caspase-8 was recently described recruited to the ER membrane (Breckenridge et al., 2002).

Another possible pathway for apoptosis is thought to occur via free radicals (Buttke and Sandstrom, 1994). Bcl-2 is found at sites of free radical generation in the cell, acting as an anti-oxidant, preventing lipid peroxidation as an example (Hockenbery

et al., 1993; Kane et al., 1993). Induction of cell death by free radicals is associated with necrotic cell death, illustrating the ability of bcl-2 family proteins to protect the cell from different components of the apoptotic machinery, and from different modes of cell death such as necrosis. It is likely that apoptosis in vivo uses more than one of the above mitochondria-dependent and independent mechanisms, with some triggers of apoptosis, such as ER stress, involving mitochondria in their apoptotic cascade (Elyaman et al., 2002).

1.4.5 Apoptosis in the nervous system

The first indication that neuronal cells undergo a programmed mode of cell death following neurotrophic factor withdrawal came from the discovery that the death of growth factor deprived cultured sensory and sympathetic neurons could be prevented using inhibitors of RNA and protein synthesis (Scott and Davies, 1990; Martin et al., 1988).

Subsequently, it was found that high levels of bcl-2 expression are found in neurons and supporting cells of developing peripheral sympathetic and sensory ganglia, and these levels were retained postnatally and into adulthood. In the CNS, levels of bcl-2 are initially high, but are downregulated after birth (Merry and Korsmeyer, 1996), whereas bcl-x_L levels continue increasing into adulthood (Gonzales-Garcia, 1995). Both the pro- and anti-apoptotic members of bcl-2-family protein are involved in the programmed cell death of neurons. Overexpression of bcl-2, for example, reduces the death of cultured sympathetic and sensory neurons deprived of survival factors (Garcia et al., 1992; Allsopp et al., 1993; Farlie et al., 1995). Overexpression of bcl-2

in vivo protects neurons from naturally occurring neuronal death and ischemia (Martinou et al., 1994). A reduction in the endogenous levels of bcl-2 reduces neuronal survival (Veis et al., 1993; Allsopp et al., 1995; Michaelidis et al., 1996; Piñon et al., 1997), and *bcl-*x_L deficiency produces massive neuronal loss (Motoyama et al., 1995). Cell death in *bcl-*x_L*-/-* mice occurs before target innervation and thus before the period of naturally occurring cell death and the main period of trophic requirement. Immature neurons are thus supported by bcl-x_L. Cell death in *bcl-2-/-* mice is increased during the period of naturally occurring cell death of neurotrophin-dependent sensory neuron populations, such as the trigeminal and nodose ganglia (Middleton et al., 2000).

Bax is widely expressed in the nervous system (Deckwerth et al., 1996). Overexpression results in an acceleration of neuronal death upon neurotrophin withdrawal (Vekrellis et al., 1997; Martinou et al., 1998), whereas a reduction of endogenous bax promotes the survival of some neurons, even without neurotrophic factors (Deckwerth et al., 1996; Gillardon et al., 1996; Miller et al., 1997; White et al., 1998; Middleton et al., 2000). Bim also plays an important role in the survival of neurons. Cultures of neurons from knockout embryos of the BH3 protein Bim are resistant to cytokine withdrawal (Putcha et al., 2001).

Apaf-1 (a homologue of ced-4) knockout embryos show a decreased apoptosis in many neuronal populations, with little effect on other cell types, suggesting that the Apaf-1/caspase-9 pathway is of particular importance to neuronal survival (Honarpour et al., 2000). Inhibition of caspases blocks neuronal cell death induced by neurotrophic factor withdrawal (Cryns and Yuan, 1998). Null mutations in *caspase-3* and *-9* (which activates caspase-3) produce similar effects on neural

progenitor cell populations in the brain to those observed in apaf-1 mutants, with ectopic cell masses appearing in the cortex, hippocampus and striatum of the mice (Kuida et al., 1996, 1998).

In addition to its role in apoptosis, bcl-2 is involved in axonal growth from trigeminal sensory neurons. Cultured *bcl-2-/-* neurons extend axons more slowly (44-60% shorter after 48h incubation of E11 trigeminal neurons), independently of bcl-2's influence on survival (Hilton et al., 1997). bcl-2 also appears to be involved in the early maturation of this neuronal population (Middleton et al., 1998).

Receptor-mediated death has recently been identified as important in mediating the death of sensory and sympathetic neurons upon NGF withdrawal (Barker et al., 2001). Antibodies against tumor necrosis factor α (TNFα) and TNF-receptor 1 rescue neurons deprived of NGF, and fewer neurons die during the phase of naturally occurring cell death in *TNF*-deficient embryos.

NF-κB, a protein that can suppress receptor-mediated apoptosis (Van Antwerp et al., 1996), is induced by NGF in Schwann cells (Carter et al., 1996). Activation of NF-κB following NGF binding to p75 also promotes the survival of developing trigeminal neurons (Hamanoue et al., 1999). Furthermore, NF-κB is able to mediate the survival response of developing nodose neurons to cytokines (Middleton et al., 2000).

1.4.6 Second messenger pathways activated by trks

More and more evidence has emerged dissecting the intracellular pathways that neurotrophins and their receptors utilise to influence the above described

death/survival mechanisms. The first step is the activation of second messenger pathways through receptor binding. Ligand-induced dimerisation and trans-phosphorylation of the trk receptor leads to the recruitment and phosphorylation of proteins, such as phospholipase C-γ1 (PLC- γ1) (Ohmichi et al., 1991; Belia et al., 1991), the Suc1-associated neurotrophic factor target (SHP-1), the SH2-containing adaptor protein (Shc) (Stephens et al., 1994; Nakamura et al., 1996), and the SH2-containing tyrosine phosphatase-1 (SHP-1) (Vambutas et al., 1995).

Phosphorylated PLC-γ1 mediates the phosphorylation and activation of protein kinase C and induces mechanisms that play a part in calcium homeostasis (Guiton et al., 1994).

To instigate the Ras/mitogen-activated protein kinase (MAPK) pathway that is implicated in neuronal survival, phosphorylated Shc recruits the adaptor protein Grb2, which in turn leads to activation of the guanine nucleotide exchange factor son of sevenless (SOS) that subsequently activates Ras. Downstream of Ras lie Raf, ERK-1, 2 and 5, as well as p38MAPK (Grewal et al., 1999; Ballif and Blenis, 2001; Stephens et al., 1994; Xing et al., 1996; Meakin et al., 1999; Atwal et al., 2000). The ERKs lead to the activation of p90 ribosomal S6 kinase (RSK), which in turn phosphorylates the cAMP response element-binding protein (CREB) (Xing et al., 1996; Watson et al, 2001), whereas p38MAPK can directly activate CREB transcription factor (Riccio et al., 1999).

The Shc/Grb2 complex is also involved in the activation of the PI3K pathway. The complex activates insulin receptor substrates (IRS)-1 and –2 and Grb-associated binder-1 (Gab-1), which induces PI3K causing recruitment of 3-phosphoinositide-dependent kinase 1 (PDK) and its substrate protein kinase B (AKT/PKB) (Holgado-

Madruga et al., 1997; Yamada et al., 1997; Vanhaesebroeck and Alessi, 2000). In addition to the above pathway, PI3K can also be activated directly through Ras, a pathway essential for the maintenance of some sensory neurons (Klesse and Parada, 1998). It has recently been demonstrated that inhibition of the PI3-Kinase pathway induces cell death in adult SCG neurons and nodose neurons (Orike et al., 2001; Alonzi et al., 2001).

1.4.7 Transcriptional induction of survival/death

Ligand-induced dimerisation and autophosphorylation of trks leads to the activation of CREB by the Ras/MAPK pathway. CREB absence in some sympathetic and sensory neurons triggers Bax-dependent apoptosis (Lonze et al., 2002). One of the actions of CREB is to up-regulate bcl-2 upon growth factor stimulation (Pugazhenthi et al., 1999, 2000). AKT, activated by the PI3K pathway can inhibit the forkhead transcription factor 1 (FKHLR1), which controls the expression of apoptosis-inducing gene products, such as FasL (Brunet et al., 1999), which promotes apoptosis by binding to the Fas receptor (Le-Nicolescu et al., 1999). ERK5 has been shown to activate the MAF-2 transcription factor thought to be involved in neuronal survival (Kato et al., 1997; Mao et al., 1999).

De novo synthesis of proteins is required for most modes of apoptosis, and it is essential for apoptosis upon NGF-withdrawal (Martin et al., 1988). An inducible transcription factor, which coordinates the expression of other genes, is thus likely to be activated by neurotrophin withdrawal. A possible candidate for this is the c-Jun proto-oncogene that is activated by Jun N-terminal kinase (JNK). In sympathetic

neurons, anti-c-Jun antibodies and dominant-negative c-Jun have been found to block cell death (Deshmuk and Johnson, 1998; Ham et al., 1995). Also, specific antibodies blocking JNK signal transduction inhibit neurotrophin-withdrawal mediated cell death. Downstream signalling in response to neurotrophic factor withdrawal/p75 activation leads to an increase in the levels of the tumor suppressor p53. Apoptosis in these cells can be partially blocked by inhibiting the increase in p53. p53 activation is downstream of JNK and upstream of Bax activation (Aloyz et al., 1998). Other signalling molecules have been identified, which are induced upon neurotrophic factor withdrawal and are upstream of JNK activation, such as the small GTP-binding proteins cdc42/Rac1 (Bazenet et al., 1998), Apoptosis signal-regulating kinase 1 (Ask-1) (Kanamoto et al., 2000), and MAPKKKs (Aloyz et al., 1998; Xu et al., 2001). MAPKKKs can phosphorylate and activate MKK 4/7 (Xu et al., 2001), which can activate JNK isoforms (Bruckner et al., 2001). p53 induces the BH3-only proteins PUMA/Bbc3 and Noxa (Han et al., 2001; Nakano and Vousden, 2001). Although the precise role of these proteins in apoptosis is not known yet, they may act like other BH3-only proteins by releasing pro-apoptotic members of the bcl-2 family. The JNK-p53-Bax apoptotic pathway that is initiated upon neurotrophin withdrawal can be induced by p75 activation alone, suggesting that p75 takes a major role in mediating this effect (Aloyz et al., 1998). Interestingly, trkA activation in sympathetic neurons can prevent death by silencing JNK and the connected activation of p53. This occurs through activation of Ras (Mazzoni et al., 1999).

Another pathway, not requiring the activation of JNK, can operate upon neurotrophic factor withdrawal. It involves the activation of the cell cycle regulatory molecules CDK4/6 (Park et al., 1997), which phosphorylate and activate the retinoblastoma

tumor suppressor protein (pRb) (Park et al., 2000). pRb in turn is a potential activator of p53.

A further example of transcriptional control by neurotrophins involves the BH3-only proteins Hrk/DP5 and Bim. Hrk/DP5 is upregulated upon NGF withdrawal from neurons via c-jun, as is Bim (Harris and Johnson, 2001, Whitfield et al., 2001). Hrk/DP5 protein is particularly interesting since its levels reach their peak simultaneously with the peak of programmed cell death in neurons (Imaizumi et al., 1997), and activation of Hrk/DP5 induces cell death in cerebellar granule neurons in a Bax-dependent manner (Harris and Johnson, 2001). The potent pro-apoptotic action of Bim is described below.

1.4.8 Modulation of BH3-only proteins by trk signalling

One of the major ways through which neurotrophin receptors signalling activates survival mechanisms is by inactivating BH3-only proteins. The BH3-only protein Bad for example is sequestered by phosphorylations to 14-3-3 scaffold proteins in the cytoplasm, preventing its activation of Bax and other pro-apoptotic bcl-2 family proteins. AKT/PKB, a transducer of growth factor survival signals via the PI3K pathway, is one of the kinases known to phosphorylate Bad (Datta et al., 1997; del Peso et al., 1997). Raf, which links growth factor receptors to the Ras/mitogen-activated protein kinases (MAPK) cascade can also phosphorylate Bad (Wang et al., 1996). In the absence of growth factors, the Bad 'BH3-only sensor' is dephosphorylated and released from the 14-3-3 scaffold proteins where it is free to interact with members of the bcl-2 family and activate the apoptotic machinery.

Other BH3-only proteins, such as Bim and Bif, are also involved in apoptotic pathways during development, with knockouts leading to embryonic lethality by E9.5 (Buillet et al., 1999). Like Bad, they are normally sequestered in an inactive state. Bim is sequestered to the dynein light chain LC8, a component of the microtubules (Puthalakath et al., 1999). Withdrawal of NGF can induce expression of Bim in cultured sympathetic neurons, and neurons from *Bim-/-* mice are transiently protected against apoptosis in response to NGF withdrawal. This suggests that Bim is involved in the induction of death upon trophic factor withdrawal (Putcha et al., 2001). Indeed, NGF-induced activation of the MEK/MAPK pathway, leads to down-regulation and phosphorylation of Bim, thereby inactivating it (Biswas and Greene, 2002). JNK-mediated Bim phosphorylation in contrast has been shown to lead to Bax activation and apoptosis upon trophic factor deprivation (Putcha et al., 2003). One of the downstream targets of Bim appears to be bcl-2, since the phenotype of *bcl-2* knockouts can be prevented by knocking out *Bim* (Buillet et al., 2001). Bim may thus act by binding to bcl-2/bcl-x_L and releasing Bax or Bak to carry out their apoptosis-inducing functions. This is reinforced by the discovery that Bim, Bad and Bid are unable to induce apoptosis in Bax/Bak double knockout cells (Cheng et al., 2001). NGF can thus function both by inhibiting the transcription of pro-apoptotic molecules, and inactivating the proteins already present in the cell.

1.4.9 Survival/death pathways associated with the p75 receptor

p75 has been found to suppress receptor-mediated apoptosis by activating NF-κB (Van Antwerp et al., 1996; Hamanoue et al., 1999). p75 is believed to activate NF-

κB through its adaptor protein TNF-associated factor 6 (TRAF6) (Khursigara et al., 1999), which has been shown to activate 'NF-κB activating kinase' (NIK), which phosphorylates 'inhibitor of IκB kinase' (IKK). Phosphorylation of IκB by IKK results in the release and nuclear translocation of NF-κB (Arch et al., 1998). NF-κB activates genes involved in neuronal survival (reviewed by Wang et al., 2002). p75 has been suggested to act as a 'switch' in neurons, activating the JNK/p53 pathway in the absence of trk signalling, leading to the death of the neuron, whereas in the presence of trk signalling it would activate the NF-κB pathway, collaborating with trk survival signals to maintain the neuron. However, p75 may also signal for survival in the absence of trkA or ligand-binding to p75, by activating the PI3K/AKT-pathway (reviewed by Roux et al., 2002).

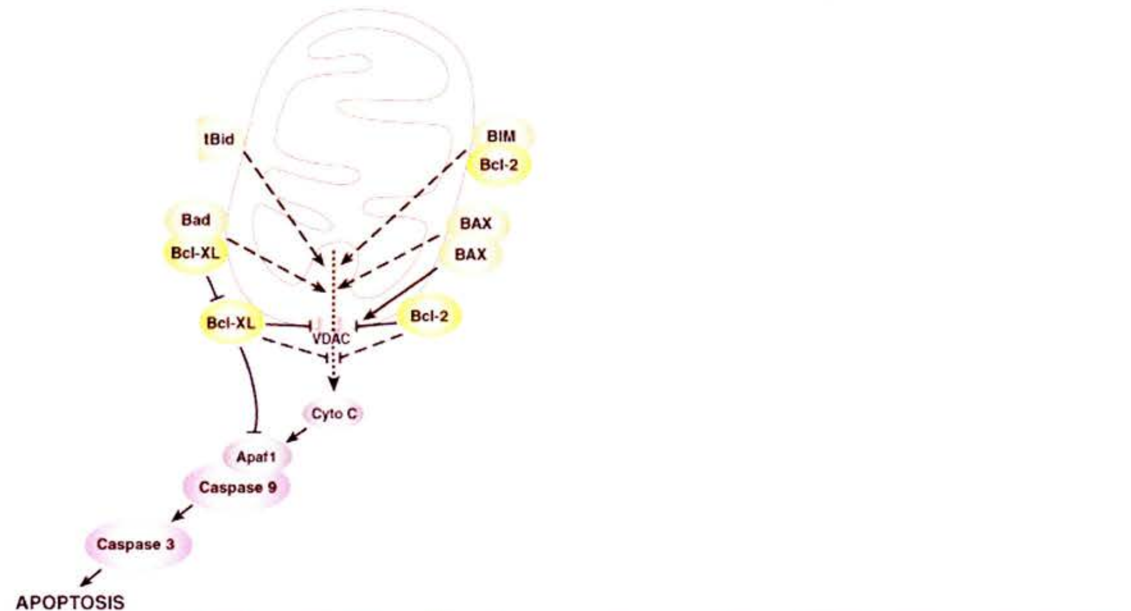
Ceramide is generated upon p75 ligand binding (Dobrowsky et al., 1995) and it has been suggested that it inactivates Ras-dependent pathways (Müller et al., 1998) and PI3K-dependent pathways (Zundel et al., 2000), thereby promoting cell death.

Another pathway via which p75 could induce apoptosis in the absence of NGF/trk signalling, is through proteins which have recently been described to interact with it: neurotrophin receptor interacting factor (NRIF) (Casademut et al., 1999), neurotrophin-receptor interacting melanoma-associated antigen (NRAGE) (Salehi et al., 2000) and p75NTR-associated cell death executor (NADE) (Mukai et al., 2000). TRAF family members also interact with p75 and induce apoptosis (Ye et al., 1999). It is not known however, whether these proteins are regulated by neurotrophins.

In addition to neurotrophins, p75 can bind several other proteins with nanomolar affinities, such as the neurotoxic prion protein fragment PrP and the Aβ amyloid peptide. p75 is also a co-receptor for myelin-derived axon growth inhibitory proteins

(Dechant and Barde, 2002; Hempstead et al., 2002). Signalling pathways activated by binding of these alternative ligands have yet to be determined.

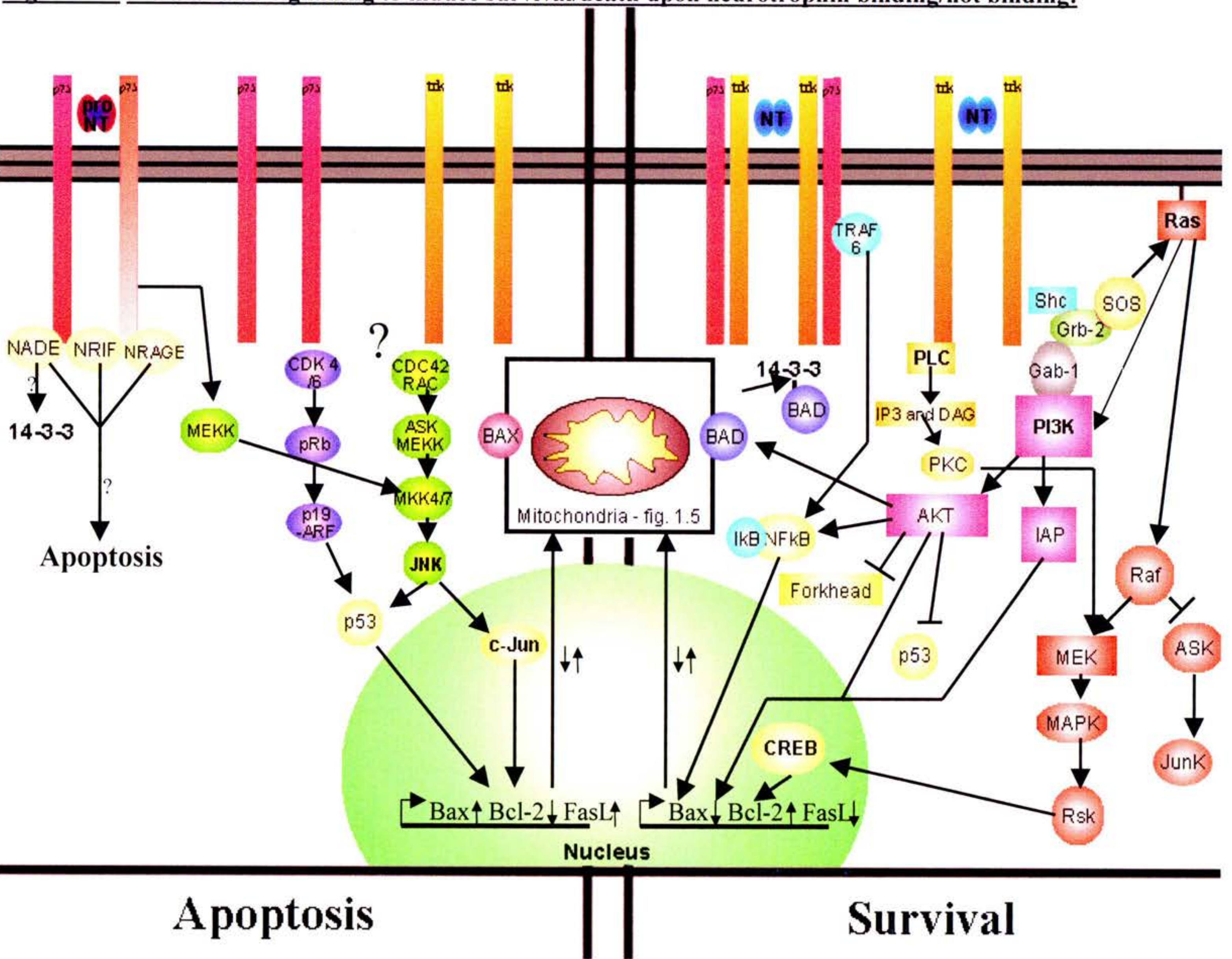
Figure 1.8: Bcl-2 proteins and their interaction with mitochondria



Interaction of the bcl-2 family with mitochondria. Bax, Bid, Bim and Bad promote apoptosis by interacting with mitochondria and making the mitochondrial outer membrane permeable to cytochrome c (Cyto C) and other pro-apoptotic proteins. These in turn activate the apoptosome, which initiates a cascade of caspases that execute cell death. Anti-apoptotic proteins, such as bcl-2 and bcl-XL prevent the actions of the pro-apoptotic members of the family.

Figure 1.9 legend: Figure showing the intracellular signalling mechanisms leading to death or survival upon neurotrophins binding or not binding. The left side shows the intracellular apoptosis pathways thought to occur when neurotrophins bind to p75 alone, or when p75 and/or trk have no ligand. The right side shows the intracellular pathways thought to mediate survival upon neurotrophin binding to its trk receptor or to a high affinity receptor created through an association of trk and p75. NT: Neurotrophin; pro NT: Proneurotrophin.

Figure 1.9: Intracellular signalling to induce survival/death upon neurotrophin binding/not binding:



1.5 The PNS – model of neurotrophin action

The experimental systems that form the basis of this thesis are the ganglia of the mouse peripheral nervous system. In this section I shall briefly describe the importance of neurotrophins and their receptors in the developing PNS as determined by expression studies, tissue culture and knockout studies. I shall then describe the ganglia studied in the experiments presented in this thesis in more detail and illustrate their suitability for the types of study conducted and their dependence on neurotrophins. Peripheral sensory ganglia are ideal for studies of neuronal numbers through development, as their numbers tend to be rather constant at determined stages of development, and unlike neuronal populations in the CNS, sensory ganglion neurons reside in discrete ganglia throughout development. This enables quantitative determination of the effects of trophic/death factors and cell-cell interactions during development (Kirstein and Fariñas, 2002).

1.5.1 Function of NGF in PNS development

NGF-/- mice have an impaired nociceptive system and thus do not respond to noxious mechanical stimuli such as pinching of their tail (Crowley et al., 1994). Histological analysis of these knockouts showed reduced numbers of neurons in the trigeminal, dorsal root (restricted to neurons conveying nociceptive and thermoceptive information) and sympathetic ganglia (Crowley et al., 1994). The sympathetic chain of knockout mice is non-existent 2 weeks after birth, due to the excessive death of differentiated sympathetic neurons at the period when they undergo naturally occurring cell death (Crowley et al., 1994). Further, small

nociceptive neurons in the DRG continually express trkA, whereas thermoceptive neurons do so only prenatally. Both are lost in trkA knockouts (Smeyne et al., 1994), revealing the importance of NGF during the phase of naturally occurring cell death (Silos-Santiago et al., 1995). *trkA* deficient mice exhibit similar deficits to *NGF* knockouts, such as insensitivity to pain, temperature and noxious olfactory stimuli. Also, extensive neuronal cell loss occurs in trigeminal, sympathetic and dorsal root ganglia of *trkA* deficient mice (Crowley et al., 1994).

NGF overexpression in the mouse results in increased neuronal survival in certain sensory and sympathetic ganglia, indicating a reduction of programmed or naturally occurring cell death during development (Albers et al., 1994). These mice also display hyperalgesia to noxious stimuli.

In vitro experiments have demonstrated that most sensory neurons that respond to NGF are of neural crest origin (Davies and Lindsay, 1995), whereas neurogenic placode-derived neurons seem unresponsive to NGF (Davies, 1989). Recently however, it was demonstrated that a subset of the placode-derived nodose neurons depends on trophic support by NGF during development (Forgie et al., 2000).

1.5.2 Function of BDNF in PNS development

The central nervous system is the predominant site of BDNF mRNA expression, especially the cerebellum, cerebral cortex and the hippocampus, where it is confined to a subset of pyramidal and granular neurons (Liebrock et al., 1989; Hofer et al., 1990; Maisonpierre et al., 1990a, Yan et al., 1997). It is expressed at much lower levels in the peripheral nervous system in specific neuronal populations and central

and peripheral target fields (see below). trkB is widely expressed in both the CNS and PNS (Klein et al., 1990a,b).

BDNF promotes the survival of DRG, trigeminal, geniculate, vestibular and nodose neurons in culture (Lindsey et al., 1985; Davies et al., 1986; Davies et al., 1987; Hofer & Barde 1988, Ernfors et al., 1994; Jones et al., 1994). Central target fields (i.e. spinal cord and hindbrain) of BDNF-dependent neurons express BDNF mRNA (Ernfors et al., 1990a; Ernfors et al., 1990b; Hofer et al., 1990; Philips et al., 1990). BDNF mRNA is also detected in DRG, trigeminal, jugular ganglia and sympathetic ganglia themselves (Ernfors et al., 1988; Schecterson and Bothwell, 1992; Wright et al., 1992; Robinson et al; 1996a), which suggests paracrine or autocrine survival mechanisms for some BDNF-dependent neurons as described above (see 1.2.6).

BDNF-/- mice develop symptoms of nervous system dysfunction and display substantially reduced numbers of cranial and dorsal root sensory ganglion neurons (Ernfors et al., 1994, Jones et al., 1994). The majority of *BDNF-/-* mice die within 2 days of birth. Major neuronal deficiencies in sensory ganglia, including the vestibular ganglia, result in deficiencies in coordination, balance and respiration. Some CNS populations of neurons, such as cortical neurons that respond to BDNF in culture (Ghosh et al., 1994), appear unaffected by the mutation, raising the possibility of some compensatory effect by other neurotrophins. For example, motor neurons are supported by BDNF, NT-3 and NT-4 in vitro (Henderson et al., 1993). It may thus be that some of the other factors compensate for the gap left by BDNF in vivo, especially since NT-4 binds to trkB with high affinity. *trkB-/-* mice are viable at birth but are unable to feed and die within the first 48hrs of birth (Klein et al., 1993). These knockouts display neuronal loss in the trigeminal, nodose and dorsal root

ganglia (Klein et al., 1993). *trkB* knockouts also display some neuronal loss in the facial motor nucleus and in spinal cord motor neuron populations at lumbar levels L2-L5 (Klein et al., 1993).

1.5.3 Function of NT-3 in PNS development

NT-3 mRNA is found in a variety of tissues during development and adulthood. In the peripheral nervous system NT-3 is widely expressed, including in target tissues of sensory and sympathetic ganglia (Ernfors et al., 1992; Pirvola et al., 1992; Hallböök et al., 1993; Henderson et al., 1993), with the spatio-temporal pattern of expression indicating an action as a trophic factor for neurons. Consequently, trkC has been detected within cranial and DRG sensory and sympathetic ganglia (Tessarollo et al., 1993; Lamballe et al., 1994). Early neurons of the trigeminal ganglion depend on NT-3 for survival as well as BDNF (Buchman and Davies, 1993; Wilkerson et al., 1996), and NT-3 expression in the whisker pad dermis and epidermis increases towards the time point that trigeminal ganglion neurons reach this target field. It reaches its maximum during the early stages of innervation before rapidly declining following the loss of responsiveness of trigeminal neurons to BDNF and NT-3 (Buchman and Davies, 1993).

Further, NT-3 promotes the survival of other sensory and sympathetic neurons such as neurons of the lumbar and cervical DRG (Hory-Lee et al., 1993), nodose neurons (Gaese et al., 1994; Ockel et al., 1996), neurons of the spiral ganglia (Gao et al., 1995; Ernfors et al., 1995), and a subset of sympathetic neurons that also require NGF (Wyatt et al., 1997; Francis et al., 1999). Localised expression studies show

that NT-3 is able to rescue proprioceptive neurons in NT-3 knockout mice, suggesting a trophic role for NT-3 on this population (Wright et al., 1997). Cutaneous overexpression of NT-3 increases the number of sensory and sympathetic neurons in peripheral ganglia, enlarges touch dome mechanoreceptor units and increases hair follicle innervation (Albers et al., 1996).

NT-3-/- mice lack the main components of the proprioceptive system, such as the large diameter myelinated DRG sensory neurons, explaining the severe defect in limb movement observed after birth (Ernfors et al., 1994b; Farinas et al., 1994; Tessarollo et al., 1994). These mutants die shortly after birth due to absent food intake, and display neuronal loss in other neuronal populations, such as the nodose, trigeminal, superior cervical and spiral ganglia (Ernfors et al., 1994b; Tessarollo et al., 1994; Bovolenta et al., 1996; Farinas et al., 1996; Fritzsch et al., 1997; Wyatt et al., 1997). Like *NT-3-/-* mice, *trkC-/-* mice have deficiencies within the peripheral nervous system, such as reductions of neuronal numbers in vestibular neurons and some cochlear neurons innervating inner hair cells (Schimmang et al., 1995). Interestingly, lumbar DRGs of *trkC-/-* embryos loose 18% of their neurons (Klein et al., 1994; Minichiello et al., 1995), whereas *NT-3-/-* mice loose 66% at birth (Ernfors et al., 1994a; Farinas et al 1994). Neurons in spiral ganglia, display 51% (*trkC-/-*) and 86% (*NT-3-/-*) reductions (Schimmang et al., 1995). This indicates that NT-3 can signal via other receptors to promote survival. This is most likely to occur through trkA and trkB, as trigeminal and nodose neurons from *trkC-/-* embryos can be supported by NT-3 in vitro, whereas neurons from *trkA* and *trkB* knockouts cannot (Davies et al., 1995).

Further to its role as a neurotrophic factor, NT-3 promotes the differentiation and maturation of PNS sensory neurons before dependence on other neurotrophins (Wright et al., 1992; Buchmann & Davies, 1993; Elshamy and Ernfors, 1996). It also promotes neurite outgrowth from embryonic sensory neurons and supports the development of distinct axon morphologies (Dijkhuizen et al., 1997; Lentz et al., 1999).

1.5.4 Function of NT-4/5 in PNS development

Peak expression of NT-4/5 within peripheral targets coincides with the peak of naturally occurring neuronal cell death in peripheral ganglia, suggesting a role for NT-4/5 as a target-derived trophic factor. NT-4/5 can support mouse NGF-dependent trigeminal and jugular neurons at early stages of target field innervation, and mouse BDNF-dependent nodose neurons during the phase of naturally occurring cell death (Davies et al., 1993; Ibañez et al., 1993). It also supports the in vitro survival of developing DRG and paravertebral sympathetic neurons (Hallböök et al., 1991), and postnatal spiral ganglion and vestibular neurons (Zheng et al., 1995a; Zheng et al., 1995b).

NT-4/5-/- mice display a marked loss of sensory neurons in the nodose-petrosal and geniculate ganglia.

1.5.5 Function of p75 in PNS development

p75 is expressed in the PNS and CNS of mice (Ernfors et al., 1988; Yan & Johnson, 1989). During development it is expressed in NGF-dependent spinal and cranial sensory neurons, as well as sympathetic neurons (Heuer et al., 1990; Hallböök et al., 1990). The dose-response curve to NGF in trigeminal, DRG and SCG ganglia of p75-/- mice is shifted to higher NGF concentrations (Davies et al., 1993; Lee et al., 1993; 1994). This indicates that p75 enhances the sensitivity of NGF-dependent neurons to NGF. In accordance with this, mutant NGF which can solely bind to trkA but not to p75 is less efficient at promoting the survival of embryonic trigeminal sensory and postnatal SCG neurons (Horton et al., 1997). It has been shown that overexpressing trkA or trkB with p75 alters the binding affinity of trkA for NGF and trkB for BDNF, NT-3 and NT4/5 (Makadeo et al., 1994; Bibel et al., 1999). The sensitising effect appears to be restricted to NGF though, since *p75* deficient neurons do not show a decreased sensitivity to other members of the neurotrophin family (Davies et al., 1993b; Lee et al., 1994).

Recent evidence shows that in some neuronal populations, such as in the early retina, p75 expressed without trkA may induce cell death upon NGF-binding (Frade et al., 1996; see 1.2.7). BDNF activation of p75 in the absence of trkB may also contribute to developmental cell death of sympathetic neurons (Bamji et al., 1998).

p75-/- mice are viable and fertile (Lee et al., 1992). However, they display ulceration of the extremities, and a decrease in response to thermal stimuli (Lee et al., 1992). Neural loss is apparent in embryonic and postnatal trigeminal, dorsal root, and postnatal SCG (Lee et al., 1994). This observation supports the *in vitro* observation

that p75 enhances the sensitivity of NGF-responsive neurons to NGF (Davies et al., 1993; Lee et al., 1993; 1994).

Apart from its survival-associated effects, p75 has recently been found to promote peripheral nerve development by regulating axonal growth and arborisation, with peripheral nerves being severely stunted in *p75-/-* mice (Bentley and Lee, 2000). Further, it plays an important role in Schwann cell migration in the PNS, with reduced migration observed in vitro (Anton et al., 19994; Bentley and Lee, 2000).

1.5.6 The Trigeminal ganglion

Cutaneous sensory neurons in the trigeminal ganglion, derived mainly from the neural crest, but also from the dorsolateral placodes (D'Amico-Martel and Noden, 1983), transmit information on touch, pain and temperature from the face and jaws (Dubner et al., 1978; Davies, 1988). The main peripheral sensory projections from the ganglia are to the maxillary and mandibular processes, and an opthalmic projection. In vertebrates, the maxillary nerve innervates amongst other things the upper lip, lateral portions of the nose, part of the oral cavity, maxillary sinus and the upper dental arch. The mandibular nerve mainly provides innervation to the lower lip, the chin, the lower teeth gingival and the anterior two thirds of the tongue. The opthalmic nerve principally innervates the forehead, upper eyelid and cornea (refer to Lazarov, 2002 for exhaustive listing). Central projecting axons of trigeminal neurons terminate on several groups of second order sensory neurons in the brainstem, which convey the information to the somatosensory cortex via the thalamus (Kruger and Young, 1981; Pfaller and Arvidsson, 1988). The mouse trigeminal ganglion is highly

advantageous to study sensory neurons at various stages of development (Davies, 1997), as has become clear by the wealth of studies reviewed in this Introduction thus far. Substantial knowledge has been gained about the neurotrophins and their actions by studying this neuronal population. It is the largest of the sensory ganglia in the embryo and one of the first neuronal elements to differentiate in utero (Stainier and Gilbert, 1991). It is thus easily identifiable for dissection, being visible from E9 (Stainier and Gilbert, 1991), and enough neurons can be isolated from it at very early stages of development for assessment of survival and gene expression in culture. Its size makes the ganglion readily identifiable very early in development using histological techniques. Further, the peripheral (maxillary and mandibular) and central (hindbrain) target fields of the trigeminal ganglion are clearly defined structures, which enables their dissection during early stages of development. This enables the study of the way the trigeminal ganglion and its target fields interact in vitro and allows quantitative studies of both receptor expression in the trigeminal ganglion and trophic factor expression in the targets (reviewed by Lindsay, 1996; Davies, 1998).

Trigeminal ganglion neurons in the rodent belong to three distinct groups: small-sized cells, related to cutaneous branches, medium-sized cells related to corneal afferents, and large-sized cells related to oral and perioral branches, including the tooth pulp (reviewed in Lazarov, 2002). The neurons in the ganglion are pseudounipolar with a single axon, which divides into a peripheral and a central branch. Although glutamate appears to be the principal neurotransmitter in the trigeminal sensory system, GABA and a variety of peptides also play an important role (reviewed by Lazarov, 2002).

During development of the mouse embryo, neurons in the ganglion are first distinguishable at about E9 (Stainier and Gilbert, 1991). The trigeminal ganglion sends fibres to its peripheral targets from E9.5 to E13.5 (Davies, 1988). The number of postmitotic neurons in the trigeminal ganglion increases between E9 and E13, before decreasing due to apoptosis and reaching a stable number by birth (Davies and Lumsden, 1984; Piñon et al., 1996). The peak in apoptosis occurs between E13 and E15 with hardly any apoptotic cells visible prior to birth (Piñon et al., 1996).

Initially, before the phase of axonal outgrowth and target field innervation, trigeminal neurons survive independently of survival factors. Cultured mouse neurons at E10 are independent. This is the stage at which the first neurons start growing towards their peripheral targets in vivo. 24 to 48hrs into the culture, at the time they begin contacting the target field in vivo (~E11-E12), these neurons become dependent on BDNF and NT-3, but not NGF, for their survival (Buchman and Davies, 1993; Paul and Davies, 1995). This is supported by in vivo studies of *trkB*-/- mice, which are missing this early population of neurons, an effect not seen in *trk*A-/- mice (Piñon et al., 1996). When cultures are established later in development, NGF promotes the survival of an increasing proportion of neurons (Buchman and Davies, 1993), an observation confirmed by a later loss of trigeminal neurons in *trkA*-/- mice (Piñon et al., 1996; Wilkinson et al., 1996). This switch in neurotrophin dependence from BDNF/NT-3 to NGF during development will be described in more detail in chapter 5. Later in development, there is a further change in the survival requirements of the trigeminal ganglion neurons, maintaining their dependence on NGF, but also becoming dependent on MSP (Forgie et al., 2003). The sequential nature of this response by trigeminal neurons to trophic factors is matched by the

sequential nature of the expression of their receptors (Buchman and Davies, 1993; Ninkina et al., 1996; Forgie et al., 2003). During the late fetal period, CNTF, LIF, OSM and CT-1 promote the survival of a subset of NGF-responsive neurons (Horton et al., 1998). The discoveries of neurotrophin-independent survival, neurotrophin switching and neurotrophin co-operativity were mainly made from work on the trigeminal ganglion, illustrating the importance of this ganglion for studying developmental neurobiology. In the adult, trigeminal primary sensory neurons mature and become less dependent on classic neurotrophic factors for their survival, as it seems that they become more dependent on neurotransmitters and neuropeptides (Lazarov, 2002).

1.5.7 The nodose ganglion

Nodose neurons, derived from the epibranchial placodes, act as baroreceptors and chemoreceptors, transmitting information about heart rate, blood pressure, bronchial irritation and visceral distension for example (reviewed by Lindsay, 1996; Davies, 1998). Nodose neurons are first discernible at ~E11 and increase in number up to ~E13, before decreasing due to programmed cell death. Cell death, observed as pyknotic nuclei, reaches its peak at E13 (see chapter 6). Multiple neurotrophins control the survival of neurons from the nodose-petrosal sensory ganglion complex (NPC), so-called because it is difficult to distinguish between these two neuronal populations in the mouse. Approximately half of all neurons in this complex are lost in null mutants for *BDNF*, *NT-3* or *NT4/5* and *GDNF* (Ernfors et al., 1994; Liu et al., 1995; Conover et al., 1995; Erickson et al., 2001), indicating that many NPC neurons

must require more than one factor to promote their survival during development. Further knockout studies indicate that BDNF and NT-4/5 promote the survival of predominantly different subpopulations of these neurons by signalling through the trkB receptor (Conover et al., 1995; Erickson et al., 1996). BDNF is required for the survival of functionally distinct populations of dopaminergic vascular afferents innervating the carotid body. This suggests that trophic requirements of NPC neurons are not modality-specific but may instead be associated with innervation of particular organ systems (Brady et al, 1999). BDNF/GDNF double knockouts have shown that petrosal ganglion innervation, but not nodose ganglion innervation of the carotid body by dopaminergic neurons of the NPC complex require both BDNF and GDNF (Erickson et al., 2001). GDNF and BDNF are transiently expressed in the targets of these afferents from the onset of sensory innervation up to the time at which fetal NPC neurons become BDNF-dependent in vitro (Brady et al., 1999; Erickson et al., 2001). GDNF along with BDNF may play a role in regulating neurite growth, branching and terminal arborisation from neonatal and adult nodose neurons (Niwa et al., 2002).

Throughout their development, nodose neurons are supported by CNTF, LIF, OSM and CT-1, and display an additional survival response to IL-6 in the late fetal period (Harper et al., 1998).

1.5.8 The dorsal root ganglion (DRG)

Neural crest derived dorsal root ganglia (DRG) perform a similar sensory function to the trigeminal ganglion in the trunk, acting as nociceptors, mechanoceptors and

proprioceptors to the skin, muscle and viscera. The neuronal population of the DRG displays variations in cell soma size and axon diameter, reflecting differences in function, although there is some overlap (Scott, 1992). DRG neurons are first discernible at ~E10. Neuronal numbers in the DRG increase up to ~E13, before decreasing (Lawson and Biscoe, 1973; Fariñas et al., 1996). The main phase of cell death occurs between E11 and E12, before the axons have reached their target fields (Fariñas et al., 1996; ElShamy and Ernfors, 1996).

Survival of early embryonic DRG neurons in culture is promoted by NGF, BDNF and NT-3, whereas neonatal neurons mainly respond to NGF alone (Memberg and Hall, 1996; Lefcort et al., 1996). This suggests a dependence on multiple factors early in development before dependence on predominantly NGF at later stages, a hypothesis supported by DRG phenotypes in single and double mutant mice for *trkA* and *trkB*, and for *NT-3* and *trkC* (Minichiello et al., 1995; White et al., 1996; Fariñas et al., 1997). This indicates neurotrophin switching, as observed in the trigeminal ganglion. Just after birth, some of the trkA/NGF-dependent neurons switch to GDNF and FGF responsiveness for survival (Molliver et al., 1997; Acosta et al., 2001). As DRG neurons mature, they become less dependent on neurotrophins for survival, with adult DRG neurons surviving for long periods in vitro in the absence of NGF (Dodge et al., 2002).

NT-3 is also involved in the formation of neurons from precursor cells in the DRG. Premature differentiation of precursor cells occurs in the absence of NT-3, reducing the generation of post-mitotic neurons between E12 and E13 (Fariñas et al., 1997).

1.5.9 The superior cervical sympathetic ganglion (SCG)

The SCG consists of postganglionic cells, which receive preganglionic innervation from the spinal cord and project to structures in the head. Their axons travel along branches of the carotid arteries to their targets in the head, such as the eyes and the lacrimal and salivary glands (Kandel et al., 2000). The SCG is first distinguishable at approximately E13 in the mouse, with most neurogenesis completed by E15.5 and apoptotic bodies observed between E15.5 and P0 (Fagan et al., 1996).

During development, sympathetic neurons express both trkA and trkC, but no trkB. trkC expression precedes trkA expression, but decreases to very low levels by birth (Birren et al., 1993; DiCicco-Bloom et al., 1993; Wyatt and Davies, 1995; Fagan et al., 1995; Wyatt et al., 1996). Postnatally, the SCG is virtually absent in *NGF-/-* and *trkA-/-* mice (Crowley et al., 1994; Smeyne et al., 1994; Fagan et al., 1996). In *NT-3-/-*mice the SCG is reduced by half postnatally (Ernfors et al., 1994; Fariñas et al., 1994; Tessarolo et al., 1996). The absence of such a strong effect in *trkC-/-* mice (Fagan et al., 1996; Tessarolo et al., 1997) suggests that NT-3 mediates its trophic action through trkA.

Contrary to evidence that the survival of early sympathetic neuroblasts is enhanced by NT-3 and that this may be the cause for the reduced neuronal numbers seen postnatally in NT-3 deficient mice (Dechant et al., 1993; Birren and Anderson, 1993; ElShamy et al., 1996), it was found that the main phase of increased cell death in NT-3-deficient embryos in the SCG occurs between E16 and E18, thus post-neurogenesis (Wyatt et al., 1997). Postnatally, a large proportion of neurons still respond to NT-3 withdrawal by apoptosis (Zhou and Rush, 1995). In *NGF-/-, NT-3-/-* double knockout mice cell death is not significantly increased compared to *NGF-/-*

mice, suggesting that these neurotrophins control the survival of the same neurons (Francis et al., 1999). Knockout studies have revealed that GDNF, artemin and the c-ret receptor are also important to SCG neuron survival (Moore et al., 1996; Durbec et al., 1996; Nishino et al., 1999).

1.6 Research objectives

The general aim of the experiments presented in this thesis was to examine factors involved in the neuronal survival of sensory neurons during embryonic development. This included assessing the regulation of survival factors, such as NGF, BDNF and NT-3 in the target fields, and the factors influencing neurotrophin receptor expression by neurons. In addition, the effect of mutations in receptors/survival factors on the survival/apoptosis rates in the developing ganglia was assessed using mice with null mutations in specific genes. The specific aims were:

I To examine the regulation of trkA receptor expression in the trigeminal ganglion during the time period that axons from the ganglion reach their target fields, an event associated with an increase in trkA expression. This included investigations on whether expression requires extrinsic or intrinsic signals, and whether growth factors affect trkA expression in vitro. The influence of the peripheral and central target fields of the trigeminal ganglion was also assessed (Chapter 3).

II To assess the role of the trigeminal ganglion in regulating NGF, BDNF and NT-3 expression in the maxillary process, its main peripheral target field, during the period of target field innervation (Chapter 4).

III To assess the role trkA and trkB play in the development of the trigeminal ganglion using double mutants of these two receptors, with the aim of identifying whether there are periods during development where neurons

critically require neurotrophin signalling through either alone or both. The importance of p75 during the development of the trigeminal ganglion was also studied using p75-deficient mice, to assess when in development p75 begins to play a role in regulating neuron survival. The role of a recently discovered truncated p75 isoform in regulating neuronal survival was also assessed. This was done by comparing a newly generated null mutant lacking this isoform with a partial p75 mutant in which it is still functional (Chapter 5).

IV To assess the role of neuroglial cells in the survival of different populations of cranial sensory neurons. This was done by assessing cell survival and death in the trigeminal, dorsal root, nodose and superior cervical ganglia of ErbB3 deficient embryos, which lack Schwann cells and their precursors (Chapter 6).

CHAPTER II

Materials and Methods

2.1 Retrieval and dissection of embryos

This section aims describe the methods employed to make the tungsten needles used in dissections, the way embryos were retrieved from the pregnant mothers and how embryos were subsequently dissected for use in either histology, RT-PCR or in tissue culture.

2.1.1 Preparation of tungsten needles

Tungsten needles were made from 0.5mm tungsten wire cut to 3 to 5 cm lengths using a grind wheel. The end 1 cm was bend by an angle of approximately 60° and immersed horizontally in 1M KOH solution. A current of 3 to 12V AC was passed through the wire and another electrode immersed in the solution. The tungsten was etched away, until it formed a taper from the bend to the tip of the needle. The bent portion was then briefly placed vertically in the solution until a sharp point was formed at the tip, and was then washed in water to remove the solution.

2.1.2 Preparation of dissection and culture media

All dissections and preparations of neuronal cultures were carried out in a laminar flow hood using standard aseptic techniques. Dissections involved using straight and curved fine watchmaker's forceps, fine scissors and tungsten needles. For dissection of ganglia a Nikon stereomicroscope with zoom lens was used, which was illuminated by a fibre optic light source. All dissections were carried out in sterile

Petri dishes containing L15 medium without sodium bicarbonate that had previously been heated to 37°C.

Dissection Media:

PBS was produced by adding 10 tablets of PBS (OXOID) to 1l of distilled water. Once the tablets had dissolved the solution was autoclaved at 115°C for 10 minutes. To make L-15 a 1l unit of L-15 powder (GibcoBRL) was dissolved in 1l of double distilled water. 100 mg streptomycin (Sigma) and 60 mg penicillin (Sigma) were added and the pH set to 7.3. The solution was then filter sterilised through a 0.22μm filter (Gelman Sc.).

Culture media:

Powdered F14 (a special formulation from Imperial labs) was made up with highly purified water that was sequentially passed through a charcoal filter, reverse osmosis system and a Milli-Q system before being double distilled. F14 medium was made from frozen 10x concentrate. To prepare the 10x concentrate a 5L unit of F14 powder (Imperial Labs) was added to 1L of double distilled water containing 500 mg streptomycin (Sigma) and 300 mg penicillin (Sigma) and the solution frozen in 50ml aliquots. A 50ml aliquot of 10xF14 was diluted in 450ml of distilled water followed by the addition of 1g of sodium bicarbonate (BDH). CO_2 was passed through the medium until the pH reached between pH6.5 and pH7 changing the colour of the mixture to brownish-yellow. Following addition of supplements (see below), the medium was then filter sterilised through a 0.22 μm filter, and stored at 4°C.

Two supplements were use in these studies: Sato and Albumax® I.

Sato is made up from 2 mM L-glutamine, 0.35% bovine serum albumin (Pathocyte-4, ICN), 60 ng/ml progesterone, 16 μg/ml putrescine, 400 ng/ml L-thyroxine, 38

ng/ml sodium selenite and 340 ng/ml tri-iodo-thyronine. The use of the Sato supplement was employed, as it is conducive for the growth of neuronal but not non-neuronal cells.

Albumax® I is a lipid-rich bovine serum for cell culture provided by Gibco. It is chromatographically purified, isolating naturally occurring lipids with purified albumin, leading to excellent growth promoting characteristics. Albumax® I promotes the survival of neurons but can also support the survival of some non-neuronal cells. Albumax® I was used at approximately 2mg/ml.

These media were used with and without neurotrophins in the medium. When neurotrophins were added they were used at 10 ng/ml (concentration recommended by Prof. Alun Davies, EC50 varies with age and neurotrophin used between 2 and 60pg/ml (Buchman and Davies, 1993)). The neurotrophins employed in these experiments were NGF (Genentech), BDNF (Regeron Pharm.), and NT-3 (Regeron Pharm.). For storage neurotrophins were kept at –80°C in F-14 with 10% HIHS at pH5.5.

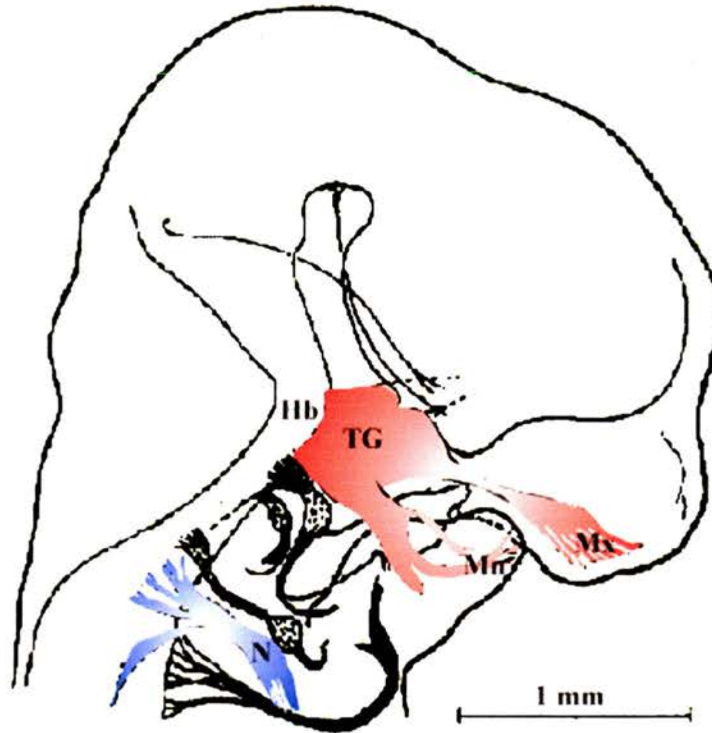
Explant studies were conducted in BD Matrigel™ (BD Biosciences). This is a solubilised basement membrane preparation extracted from the Engelbreth Holm Swarm mouse sarcoma rich in extracellular proteins, mainly laminin and collagen IV, as well as some growth factors occurring naturally in the tumour. At room temperature the Matrigel Matrix polymerises, producing biologically active matrix material resembling the mammalian cellular basement membrane.

2.1.3 Retrieval of embryos

The pregnant female was killed by cervical dislocation at the required stage of gestation. After washing the female's abdomen with 70% alcohol, an incision was made in the skin on the front of the abdomen. The skin was pulled away from the incision by grasping the skin above and below between the index finger and the thumb of each hand. A small incision was made in the anterior abdominal muscle using a pair of fine scissors, while holding the muscle with a pair of toothed forceps. This allowed air to enter the peritoneal cavity and hence enabled the incision to be extended without risk of cutting the intestines (which could contaminate the dissection with bacteria). The uterine horns were removed by holding them with toothed forceps and cutting them free with fine scissors. The uterine horns were then transferred to a Petri dish with PBS for histology or L15 for further dissection and tissue culture.

For young embryos (E10.5 to E12.5) a small hole was made in the musculature on the anti-mesometrial border of the uterine horn, causing the embryo to be extruded within its membranes by contraction of the remaining uterine muscles. The membranes could be removed using watchmaker forceps after detaching the embryo from the uterine horn. Older embryos (> E13) could be removed from the uterine horn by making a continuous incision along the antimesometrial border of the uterine horn, exposing them with their membranes. Embryos were staged according to the criteria set by Theiler (1970). Figure 2.1 shows an E11 mouse head indicating the main structures of interest in these experiments, giving a foundation for the description of the dissections.

Figure 2.1: Medial aspect of the head of an E11 mouse embryo



Drawing showing the medial aspect of the head of an E11 mouse embryo. The location of the trigeminal (red - TG) and nodose (blue - N) ganglia is marked, as well as the Maxillary process (Mx), Mandibular process (Mn) and hindbrain (Hb) (adapted from Davies and Lumsden, 1987).

2.1.4 Dissection and processing of embryos for histology

After removal of the membranes the embryos were transferred to a new Petri dish with PBS and washed a minimum of 3 times in fresh changes of PBS. In knockout studies, the embryos were washed separately in at least 2 fresh changes of PBS before dissection to minimize contamination by the mothers or siblings blood. For the dissection, the tools were sterilized with alcohol between each embryo. The embryos heads were separated from their bodies using a tungsten needle for embryos of E13 and under or scissors for older embryos. The cut was made just above the arms to be able to incorporate the nodose, cranial DRG and the SCG ganglia in block

for the histological analysis. The embryo's bodies were retained for genotyping as described before. The heads were transferred to the chosen fixative for the required amount of time.

2.1.5 Dissection of the maxillary process, hindbrain and trigeminal ganglion

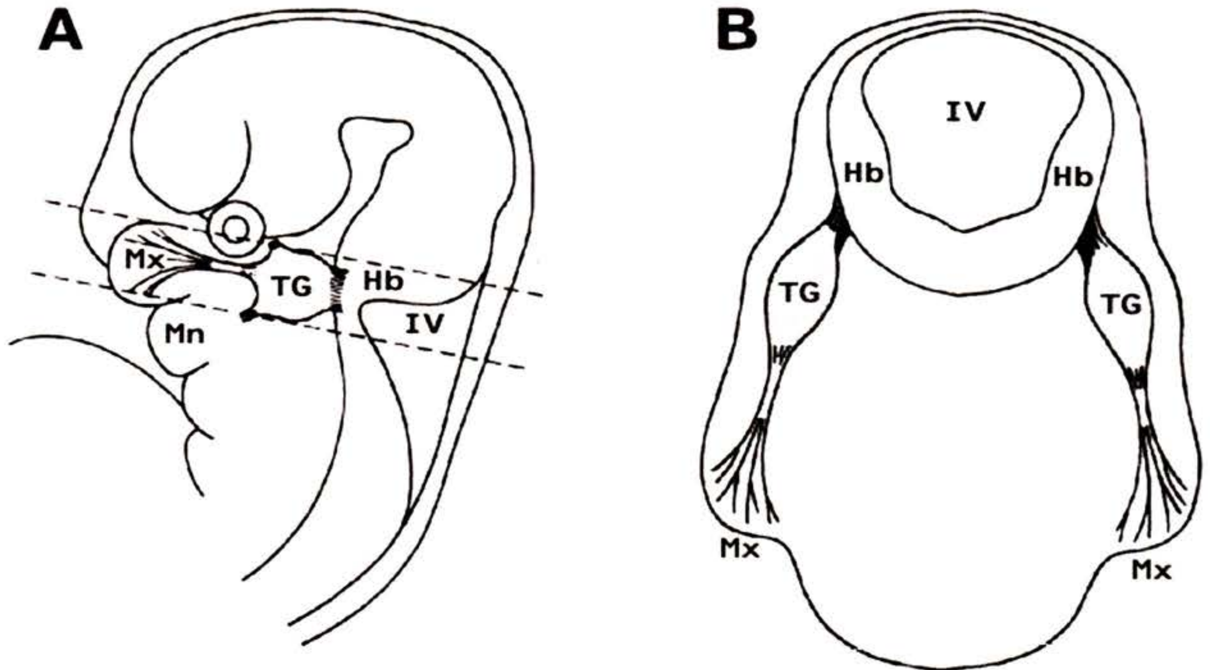
After removal of the chorion and amnion using watchmaker's forceps, the embryos were transferred to a new Petri dish with L-15 and washed a minimum of 3 times in fresh changes of L-15. In knockout studies, the embryos were then washed as described above to avoid contamination with DNA from other embryos.

To dissect the trigeminal ganglion and its target-fields different techniques were employed depending on the age of the embryo to be dissected. For embryos younger than E13 two coronal incisions were made through the head using tungsten needles, one above each eye, the other between the maxillary and mandibular processes of the first branchial arch (see figure 2.2A).

In the slice obtained (figure 2.2B) the trigeminal ganglion, the maxillary process and the hindbrain were easily recognisable due to slightly opaque colorations (trigeminal and hindbrain) or the physical position of the maxillary, slightly sticking out from the slice. The observed structures can be removed from the tissue slice and freed of any adherent tissue using tungsten needles. When the structures were needed with their neural connections intact, these could also be distinguished due to their opaque colour, and the dissection performed so as to not damage them. To free the trigeminal ganglion of mesenchymal tissue, one needle was used to steady the

ganglion and the other to pinch off the adherent tissue against the bottom of the Petri dish. The hindbrain and maxillary process were freed in a similar fashion. The dissected trigeminal ganglion, maxillary process and hindbrain were then transferred to a Petri dish containing pre-heated L15 medium using a siliconised Pasteur pipette.

Figure 2.2: Dissection of the trigeminal ganglion, maxillary process and hindbrain from mouse embryos younger than E13



A: Lateral aspect of an E11 head indicating the location of the transverse incisions used to obtain the slice of tissue containing the trigeminal ganglion (TG), maxillary process (Mx) and Hindbrain (Hb). B: Rostral aspect of the slice showing the position of these structures. Also shown are the mandibular process (Mn) and fourth ventricle (IV) (Adapted from Davies and Lumsden, 1984).

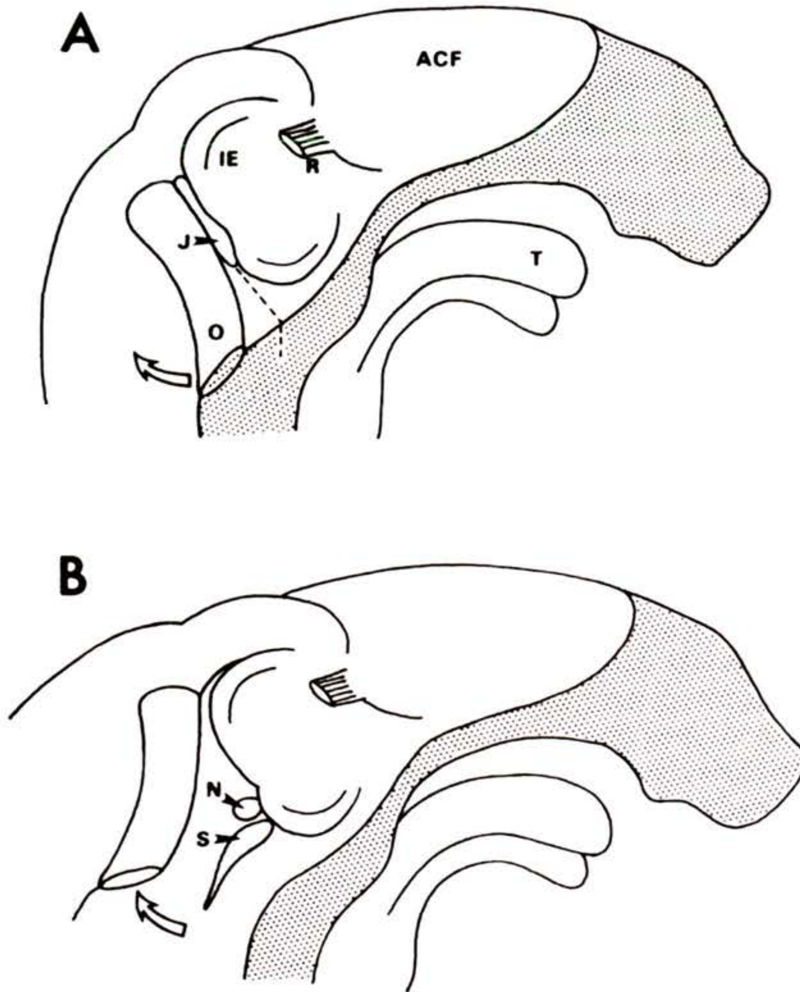
For the dissection of trigeminal ganglia of embryos E13 or older, a fine pair of scissors was used to cut through the cartilage and bone of the developing head. Using a pair of forceps to steady the embryo, the top of the skull was removed in a plane just above the eyes and whisker pads. This was followed by a second cut parallel to the first, passing through the mouth. The tissue slices were transferred to a fresh Petri dish containing L15 medium and two further cuts were made with tungsten needles

in front and behind the trigeminal ganglia. The tissue in front of the ganglia contained the maxillary process (easily recognised by its five rows of vibrissae), and the tissue behind the ganglia contained the hindbrain. Tungsten needles were used to free the ganglia, maxillary processes and hindbrains from the surrounding tissues and remove adherent connective tissues.

2.1.6 Dissection of the nodose ganglion

These dissections were carried out at E12.5. Using the same plane of section described for the first incision of the trigeminal dissection above, the top of the skull and underlying forebrain were removed from the embryo. The embryos were decapitated and the head was cut in half along the saggital plane. The nodose ganglion (also known as the inferior vagal ganglion) is situated close to the base of the skull. Tungsten needles were used to remove the hindbrain from each bisected head. The slit-like jugular foramen was then opened to the midline by inserting one tungsten needle into the jugular foramen so that it lay beneath the base of the skull, medial to the foramen, and bringing a second needle into apposition with the first, so as to cut through the intervening tissues (Figure 2.3). The 'mouths' of the jugular foramen were opened using a pair of tungsten needles to reveal the SCG and the nodose ganglia lying at the base of this foramen. The nodose ganglion was identified due to its spherical structure and the prominent vagus nerve attached to its distal aspect, differentiating it from the SCG, which is an elongated structure that is attached caudally to the sympathetic chain, a nerve that is much thinner than the vagus nerve and is situated anterior to the internal carotid artery.

Figure 2.3: Successive stages in the dissection of the nodose ganglion



This figure illustrates successive stages in the dissection of the nodose ganglia (E14 in this example). The stippled areas show the bisected midline structures lying along and in front of the cranial base. Part A shows the incision from the jugular foramen (J) to the midline (interrupted line), and the arrow indicates the direction in which the large ossified part of the occipital bone (O) should be reflected to subsequently open up the jugular foramen. T, Tongue; ACF: anterior cranial fossa; R: root of the trigeminal nerve; IE: inner ear. Part B shows the position and shape of the nodose (N) and SCG (S) ganglia following these steps (from Davies, 1988).

2.2 Culture of trigeminal ganglion, maxillary process and hindbrain

2.1.1 Cultures of trigeminal ganglia with/without the maxillary process or hindbrain

All incubations took place in a humidified CO_2 incubator at 37°C with 4% CO_2. Trigeminal ganglia were dissected with or without their central and peripheral target-fields as described and incubated in 500µl L14 + Sato (preheated to 37°C) in Nunclon™ four well dishes for 24hrs. For E11 ganglia this was performed in Matrigel with NGF, BNDF and NT-3 (10ng/ml). For experiments where the target was kept separately to the ganglion, Matrigel™ was employed. A test showed this to have no significant effect on the observations. Ganglia were cultured either by themselves, attached to their target-fields, separated and put back together, or left at a slight distance. The way this was done is represented in Figure 2.4. Matrigel was kept on ice during use, as this is the temperature required for it to remain in its liquid form. Ganglia and targets were placed into the culture dish using siliconised pipettes before ~40µl of matrigel was added, and the ganglia and target-fields were arranged in the desired configuration using tungsten needles. The matrigel was then left to set for about 5 minutes, before F14 and Sato were added.

After 24hrs the attached ganglia were removed from the medium into a petri dish with L-15. They were then detached from any target field they had been in contact with and freed from matrigel, before being processed for assessment of mRNA expression.

Experiments using E10, E12, E13, E14, and E15 ganglia and target-fields were also carried out in matrigel in medium enriched with NGF, BDNF and NT-3.

Figure 2.4: Variations in culture of the trigeminal ganglion with its central and peripheral target-fields

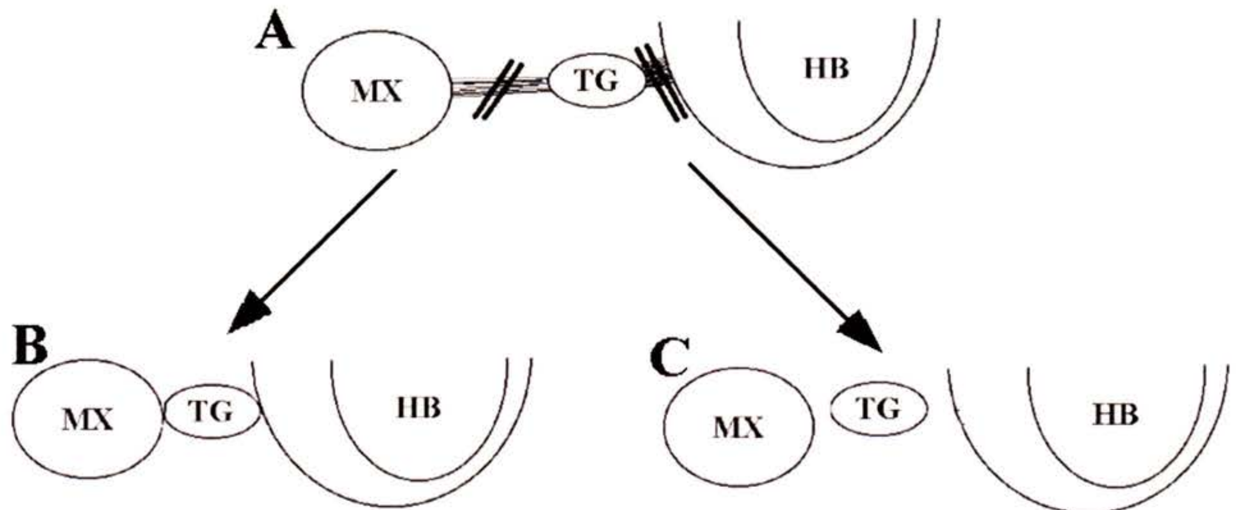


Figure showing the subdissection of the ganglion and its target fields (A) and the final position of these structures when put back together after severing the axons connecting the structures (B). (C) shows the setup when the structures were left separated by the matrigel matrix. To ascertain that the matrigel has no effect on the results, the trigeminal by itself or left attached to its target fields were also cultured in matrix, and the results compared to the ones in absence of matrigel. The comparison suggested that matrigel had no significant effect on expression. MX: Maxillary; TG: Trigeminal ganglion; HB: Hindbrain.

2.2.2 Culture of the trigeminal ganglion in maxillary process or hindbrain homogenate

E11 maxillary processes and hindbrains were collected and 26 of each placed into 500μl of F14 + Sato with proteinase inhibitor. The tissues were then homogenised and the solution transferred to a fresh 1.5ml Eppendorf tube, before being centrifuged at 2°C for 2 hours. The liquid phase was then transferred to a fresh Eppendorf tube and 50% and 10% dilutions from this stock were made in fresh F14

+ Sato. 50μl of either the 100%, 50% or 10% stock were then used to incubate E11 trigeminal ganglia as before.

2.2.3 Culture of the maxillary process with/without the trigeminal ganglion attached

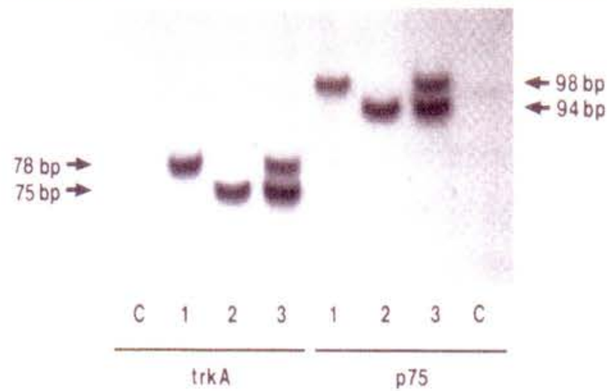
E10.5 and E11 maxillary ganglia were cultured with or without the trigeminal ganglion left attached to them for 48 or 60hrs in 500μl F14 + Albumax in 4-well Nunclon dishes in Matrigel. Matrigel was used since it has some effect in suppressing the dissociation of the E10.5 ganglion that may take place when the ganglion is grown in F14 + Albumax alone.

2.3 RNA extraction and RT-PCR

2.3.1 Background

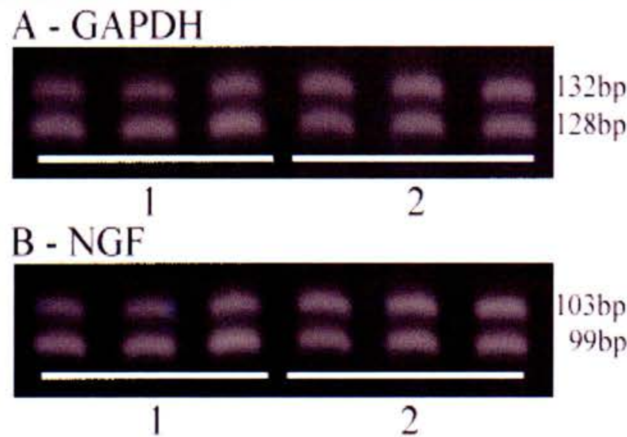
Of the techniques currently available for mRNA detection and quantification, reverse transcription-polymerase chain reaction (RT-PCR) is the most sensitive. RT-PCR involves the reverse transcription of RNA to cDNA, followed by amplification of a specific cDNA using the PCR technique. The particular variety of RT-PCR used in these experiments is known as competitive RT-PCR. This enables measurement of the absolute amount of the mRNA of interest by using known amounts of synthetic RNA (competitors). These have slightly longer or shorter sequences and are co-transcribed and co-amplified with the target mRNA. Competitors are designed to amplify by the same primers in the same reaction, nullifying variables affecting amplification efficiency. This has been confirmed by carrying out reactions with known quantities of target and competitor RNA transcripts. The ratio between the reaction products during the log-phase of the PCR reaction was identical to the initial ratio between target and competitor RNAs irrespective of the starting levels of these RNAs (Wyatt and Davies, 1993). Being slightly lighter or heavier enables the separation of the competitor from the amplified cDNA of interest by gel electrophoresis. Comparison of the amplified cDNA of the gene of interest with the closely resembling, known amount, of cDNA from the synthetic RNA enables quantification of the amount of mRNA of interest present. The sensitivity and specificity of this RT-PCR technique, as demonstrated by Wyatt and Davies (1993) using trkA and p75 competitors, can be seen in Figure 2.5, while Figure 2.6 shows the reliability of this method, demonstrated using GAPDH and NGF competitors.

Figure 2.5: Sensitivity and specificity of the competitive RT-PCR technique



Autoradiograph showing the products of RT-PCR reactions amplified with either trkA-specific primers or p75-specific primers. (1) Reactions containing 1 fg of either the trkA or p75 competitor RNA templates, showing the 78bp and 98bp amplification products, respectively. (2) Reactions containing total RNA from ten E12 trigeminal neurons, showing the 75bp trkA mRNA and the 94bp p75 mRNA amplification products, respectively. (3) Reactions containing 1fg of the respective competitor RNA template plus total RNA from ten E12 trigeminal neurons, showing both sets of the respective amplification products. (C) No product resulted from control reactions that contained the respective competitor RNA template plus total RNA from ten E12 trigeminal neurons, but from which the reverse transcription step was omitted (from Wyatt and Davies, 1993).

Figure 2.6: Reliability of the competitive RT-PCR technique



	Sample 1	Sample 2
Mean	11.51	11.68
SE	0.36	0.67

SYBR®Gold (Molecular Probes) stained gels showing the products (in triplicate) of two RT-PCR reactions amplified with either GAPDH-specific primers (A) or NGF-specific primers (B). (A) Reactions containing 25pg of the GAPDH competitor RNA (132bp) plus total RNA from two E16 maxillary processes ((1) and (2)) (128bp), showing both sets of the respective amplification products. (B) Reactions containing 250fg of the NGF competitor RNA (103bp) plus total RNA from the same E16 maxillary processes ((1) and (2)) as used for the GAPDH reaction (99bp). The table below shows the mean expression of NGF in fg per pg of GAPDH for the two triplicate samples and the standard error, at 3.2% and 5.7% of the mean.

Detailed comparison of competitive RT-PCR with quantitative Northern blotting has also demonstrated its accuracy and reproducibility over a wide range of concentrations of target mRNA (Wyatt and Davies, 1993), and a recent study comparing RT-PCR techniques found mRNA profile trends of competitive RT-PCR to produce comparable results to real-time RT-PCR (Wall and Edwards, 2001).

Competitor RNA is usually produced from a cDNA template with a deletion or insertion for distinguishing from endogenous RNA. RNA polymerase is used to *in vitro* transcribe the cDNA template into RNA.

Competitors in these experiments were designed by insertion of a small amount of extra base pairs into cDNA clones, and thus could be identified by being heavier using gel electrophoresis. All competitors employed in these experiments were designed and generated by Sean Wyatt and colleagues (references below): Using primers the specific sequence of cDNA of the gene of interest was amplified using RT-PCR. RNA for the reverse transcription was extracted from the species that gene expression was to be studied in (mouse in this case). The size of amplified cDNA of interest was identified by running the cDNA with a DNA ladder on an agarose gel, and then removed and purified. The cDNA fragment generated from the PCR reaction was then cloned into a transcription plasmid vector used to transform competent cells, which were then plated and grown on agarose. Once colonies were visible DNA was extracted from several of them using a mini-prep system, and it was identified which of them had been transformed successfully. This was done by digestion of plasmid DNA using restriction enzymes. Electrophoresis was used to identify which constructs contained the correct insert. Once the correct plasmid DNA had been identified, it was purified using a miniprep plasmid purification kit.

This was followed by modification of the cDNA insert to distinguish it from the native template: Plasmid DNA was digested with a restriction enzyme producing X bp 5' overhangs. These were filled in with Klenow polymerase and the blunt ends re-ligated, producing an X bp larger cDNA than the native template (creating a recombinant plasmid).

The recombinant plasmid was then digested using the same restriction enzyme, which had been used to produce the 5' overhang previously. This was done because it would cut any plasmids not containing the filled-in sites, making them linear and thus greatly reducing the transfection efficiency of these now unwanted fragments. The plasmids were then transformed into competent bacteria, which were grown on agar plates. As before, once the colonies were visible, the presence of the correct insert in colonies was asserted using plasmid DNA restriction enzymes and electrophoresis. PCR and acrylamide gel electrophoresis against an unfilled plasmid was used to assert that the correct amount of base pairs had been filled in.

The plasmid DNA was then linearised and cut to the required size using a restriction enzyme. The plasmid was then run on an agarose gel with a DNA ladder and the band containing the correct linearised DNA excised and purified. The RNA competitor was then created using *in vitro* transcription, and any plasmid DNA eliminated using DNAse. An aliquot of this RNA stock was then used to determine the RNA concentration spectrophotometrically. The remaining RNA was then ethanol precipitated and diluted to a concentration of 1ng/μl, before being stored at – 20°C until required.

Competitors used in these experiments were for the endogenous RNA of glyceraldehyde phosphate dehydrogenase (GAPDH), trkA, full-length trkB, full-

length trkC, p75, NGF, BDNF, and NT-3. Details of the competitors used and their deviation from endogenous RNA are given in Table 2.1, as well as the reference describing competitor generation.

Table 2.1: Details of size differences between used competitors and endogenous RNA

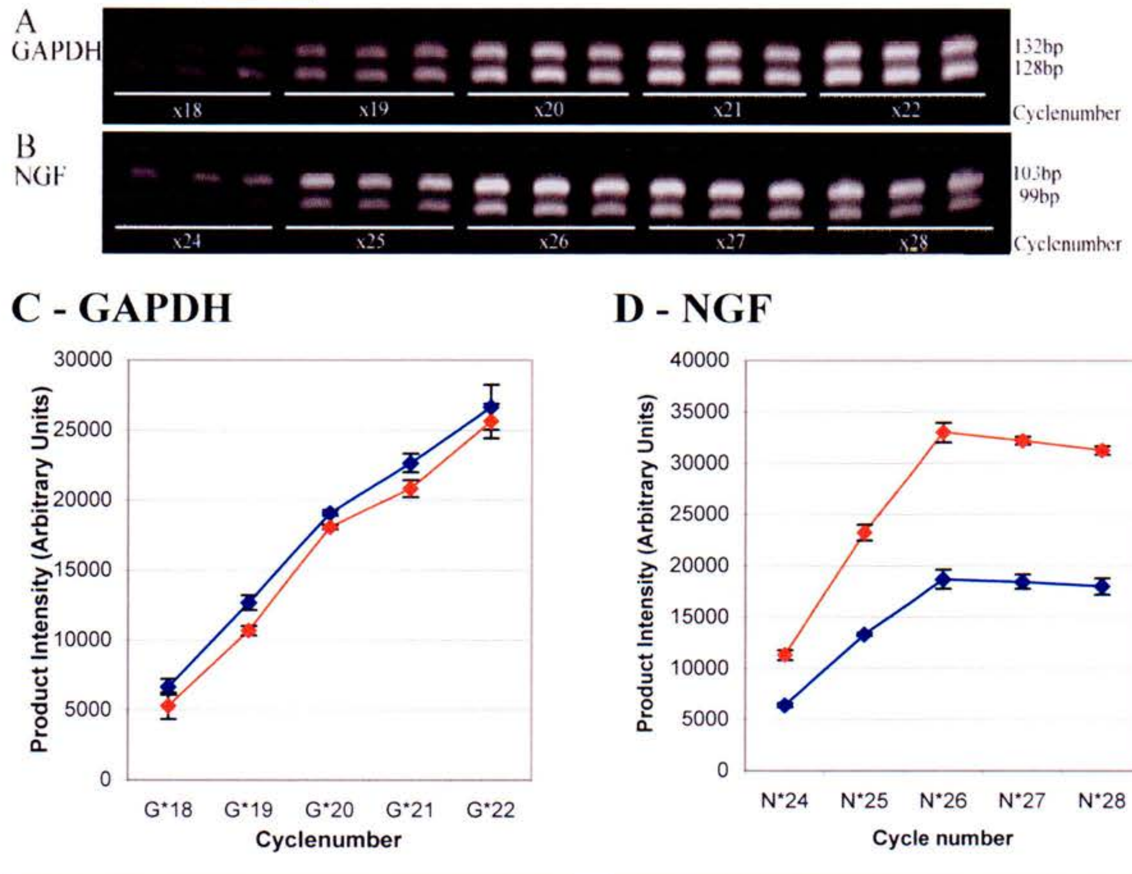
Competitor	Difference with endogenous RNA	Reference
GAPDH	4 base pairs	Wyatt, S. et al. 1997.
trkA	3 base pairs	Wyatt, S. and Davies, A. 1993.
trkB	4 base pairs	Huber, K. et al. 2000.
trkC	4 base pairs	Wyatt, S. et al. 1997.
p75	4 base pairs	Wyatt, S. and Davies, A. 1993.
NGF	4 base pairs	Produced by S. Wyatt
BDNF	4 base pairs	Produced by S. Wyatt
NT-3	4 base pairs	Produced by S. Wyatt

GAPDH is a ubiquitous, constitutively expressed housekeeping gene, whose expression was assessed together with each of the other RNAs of interest. This allowed the expression to be quantified as expression/amount of GAPDH mRNA. This gives a control for differences occurring due to differences in size of the assessed pieces of tissue, and for differences in concentration due to slight variations in the extraction procedure. It is important to include the competitor for GAPDH in the same transcription reaction as the RNA competitor of interest, thus ensuring that they both get compared to the same amount of sample added to thc tube.

Anothcr factor of importance was to ensure that the PCR reaction remained in the log phase. If the samples were allowed to reach the plateau phase, this induces the formation of heteroduplex DNA products, seen as a third band becoming visible in

electrophoresis. Heteroduplex DNA produced in the plateau phase interferes with the ratio of the homoduplex products of interest, thus leading to inaccuracies. Figure 2.7 shows the example of NGF and GAPDH target and competitor mRNA amplified for different cycle numbers, illustrating the consistency of competitive RT-PCR over different cycle numbers. No significant differences were found between the ratios of sample over competitor at any of the assessed cycle numbers (GAPDH: ANOVA, $p>0.05$; NGF: ANOVA, $p>0.05$). Graph D shows the amplification of NGF reaching the plateau phase at cycle 26. However, the ratio of sample over competitor does not seem to be significantly affected, illustrating that competitive RT-PCR can remain accurate even after the exponential phase of the PCR, as claimed previously (Siebert and Larrick, 1992). Nevertheless we ensured that measurements taken in this study were in the log phase. Experiments in which heteroduplex DNA was evident, indicating that sample amplification had reached the plateau phase, were repeated with lower cycle numbers until no heteroduplex DNA was observed.

Figure 2.7: Amplification of sample and competitor – relation to cycle number



Picture: SYBR®Gold (Molecular Probes) stained gels showing the products (in triplicate) of RT-PCR reactions amplified with either GAPDH-specific primers (A) or NGF-specific primers (B). (A) Reactions containing 20pg of the GAPDH competitor RNA (132bp) plus total RNA from an E13 maxillary process (128bp), showing both sets of the respective amplification products over 18 to 22 cycles. (B) Reactions containing 600fg of the NGF competitor RNA (103bp) plus total RNA from the same E13 maxillary process as used for the GAPDH reaction (99bp), showing both sets of the respective amplification products over 24 to 28 cycles. The graphs below (C and D) show the product intensity as measured by Phoretix Analyzer, over the number of amplification cycles. The product intensity for the competitor (red) and the target (blue) are shown. (C) The GAPDH competitor and sample cDNA amplify at approximately the same rate during the log-phase of amplification, as shown by the similar increase in product intensity of the two bands (Pearson correlation r^2=0.9985; $p<0.001$). Measurements for this sample were done after cycle 19, thus in the log phase. (D) The NGF competitor and sample cDNA amplify at approximately the same rate, as shown by the similar increase in product intensity of the two bands (Pearson correlation r^2=0.9998; $p<0.001$). The plateau phase is clearly reached at cycle 26, as there is no further increase in product intensity in Phoretix Analyzer. Measurements for this sample were done after cycle 25.

2.3.2 RNA extraction and purification

RNA extractions were carried out using the protocol by Chomczynski and Sacchi (1987). Following the final dissection the tissues of interest were placed in a 1.5ml eppendorf using siliconised pipettes before addition of 500μl of solution D (250g guanidinium thiocyanate (Sigma), 293ml DEPC-treated Water (Ambion), 17.5ml 0.75M sodium citrate (BDH) pH7, and 24.6ml 10% N-laryl sarcosyl (BDH)). 360μl of 2-mercaptoethanol (Sigma) was added per 50ml of Solution D before use. The tissue in the solution D was then lysed by passing the solution through a 25 gauge needle fitted to a 1ml syringe. This was followed by addition of 1μl of 10mg/ml *E. coli* tRNA (Sigma), 50μl of 2M sodium acetate (BDH) pH4.4, 500μl water-saturated acidic phenol (Sigma), and 150μl 25/1 chloroform/isoamyl alcohol (Sigma). The solutions were thoroughly mixed and then incubated on ice for 5 minutes, before being centrifuged at 15000 rpm for 5 minutes. The aqueous supernatant (~450μl) was then transferred to a fresh 1.5ml Eppendorf and the RNA precipitated by adding at least two volumes of ethanol (~1ml), before being transferred to a –20°C freezer overnight. The RNA was then recuperated by centrifuging the ethanol mixture at 15000 rpm for a minimum of 30 minutes. This led to the RNA forming a pellet at the base of the eppendorf. The absolute ethanol on top was removed and the pellet washed using 70% ethanol, which was then removed in the same manner before leaving the pellet to air dry in order to remove any traces of ethanol.

The pellet was then resuspended in a 50μl mixture of 40mM Tris (Sigma) pH7.5, 6mM $MgCl_2$ (Biogene) 20mM ribonucleoside vanadyl complex (VRC) (Sigma), and 5μl of 10000 units/ml DNase I (Anachem). This solution, made to remove DNA

(Dnase I) while protecting RNA from RNases (VRC), was incubated at 37°C for 2-4 hrs.

Purification of the RNA was performed using an RNA binding matrix based technique (RNaid Kit by Bio 101). This was done by adding 200µl of RNA binding salt (made from 3M $NaClO_3$) to create the right conditions for RNA binding matrix (RNAMATRIX®) addition. The amount of RNAMATRIX® added was dependent upon the approximate amount of RNA in the tissue being extracted (approx. 1-2µl of RNAMATRIX®/µg RNA). Because in these experiments a whole ganglion or target-field was examined, 30µl of RNAMATRIX® was used, although less is used for extraction of RNA from cultures as an example.

The solution with the RNA binding salt and RNAMATRIX® was mixed thoroughly and incubated at room temperature for 30 minutes to 2 hours, while mixing regularly. RNA was absorbed by the matrix at this stage. After incubation the solution was centrifuged for 1 minute at 13000 rpm and the liquid phase removed. The solid phase was then resuspended and washed twice in 600µl fresh RNA wash solution. After removal of the last traces of RNA wash solution by centrifugation, 100µl of DEPC-treated water was added to resuspend the matrix, followed by incubation at 60°C for 2-3 minutes, causing the RNA to separate from the matrix. The solution was then separated from the matrix by centrifugation of the sample at 15000 rpm for 1-2 minutes. The liquid phase was then carefully transferred to a new 0.5µl tube before storage at –80°C until needed.

2.3.3 Reverse Transcription

Competitor aliquots of various concentrations were prepared by shaking the precipitated 1ng/μl stock and taking a 50μl aliquot from it. These were spun at 15000rpm for a minimum of 15 minutes before removing the ethanol solution and resuspension with 100μl DEPC-treated water (Ambion). This gave a 500pg/μl solution of the competitor. This was diluted step by step by taking 10μl out and diluting it with 90μl of DEPC-treated water giving a 50pg/μl solution and so forth. The different concentrations of competitor were stored at –80°C before use.

Reverse transcriptions were carried out in 25μl reaction. The reagents involved in the reaction were at the following concentrations in the final volume: 1/5 5x reverse transcription buffer (Invitrogen), 1/10 5mM dNTPs (MBI Fermentas), 1/10 100μM random hexanucleotides (RH) (Anachem), 1/10 0.1M DTT (Invitrogen), and 1μl of Superscript Rnase H^- Reverse Transcriptase (Invitrogen). These were added to an amount of water determined by the volume and concentrations of competitors and sample added.

As an example a typical mastermix for a reverse transcription for NGF may be made in the following way: 100μl 5xBuffer, 50μl dNTPs, RH and 0.1M DTT, 3μl 50fg NGF competitor, 1μl 50pg GAPDH competitor, and 106μl of water. This can be divided into 20x18μl tubes, to which 6μl of sample can be added, followed by 1μl of reverse transcriptase giving a total volume of 25μl. A mastermix ensures uniformity of conditions for each sample.

The possibility of DNA contamination in the samples or reagents was assessed by using samples and reverse transcription mix without adding any reverse transcription enzyme to some tubes.

For the reverse transcription, samples (without RT enzyme) were transferred to a thermal cycler. This was used to initially heat the samples to 90°C for 2 minutes in order to remove any tertiary structures in the RNA that could interfere with the transcription, before adding the RT enzyme (on ice) to each sample and incubating at 37°C for 90 minutes. This was followed by 6 minutes at 95°C.

Before doing an RT-PCR reaction for all the samples in a reaction, a small amount of samples was usually used to do a titration, adding various concentrations of competitor to determine the one closest to the concentration in the samples. The concentration of competitor used needed to be close to the sample concentration because the analysis system was not sensitive enough to quantify differences of more than four fold.

All the samples were then run using the same volume of RNA added to the mixture with the closest competitor concentrations as determined in the titration. The volume of each sample was then altered to aim and make it as close as possible to the concentration of GAPDH competitor used. This calibrated all the samples to the same concentration of GAPDH, enabling relative estimation of the amount of gene expression of interest. The variations in added sample volume were compensated for by addition of more or less water to each sample, so that the total volume added to each mix remained the same.

2.3.4 PCR

Specific oligonucleotide primers for the cDNA of interest were ordered from MWG. Primers are listed in table 2.2. Primer stock was made by diluting the oligonucleotide

powder to 1μg/μl and mixing the forward and reverse primer 1/1. This stock was then diluted by 50 to obtain the working solution of primers.

PCR reactions were performed in 50μl volumes, made up of 45μl of reaction mix and 5μl of sample. The total mix per reaction was composed of 33.875μl of distilled water, 5μl of 10xBuffer (Helena Biosciences), 5μl primers (see above), 1μl dNTPs, and 0,125μl taq DNA polymerase (Helena Biosciences). As for the reverse transcription a mastermix was made to ensure uniformity of conditions for each sample. After mixing, 2 drops of mineral oil were added to the top of the reaction mix to prevent evaporation during the cycles of the PCR. The samples were then transferred to a thermal cycler. The cycles for the PCR reaction were done as follows: The cDNA was initially denatured at 95°C for 1 minute. This was followed by a 1 minute annealing step, which varied in temperature depending on the reaction (figure 2.7), and a 1 minute elongation step at 68°C. After the last cycle a last elongation step was performed for 10 minutes. The number of cycles required for each reaction to be able to detect the cDNA without entering the plateau phase of amplification depended on the initial concentration and nature of the RNA. An indication of the number of cycles used for each reaction is given in table 2.2.

Following the PCR reaction, 10μl of 6x blue loading dye was added to the products.

Table 2.2: Primers used for PCR reactions, annealing temperatures and cycle numbers

Reaction	Forward primer	Reverse primer	Annealing temp.	Cycle number
GAPDH	5'–TCC AGT ATG ACT CCA CTC AC–3'	5'–TCC TGG AAG ATG GTG ATG G–3'	51°C	18 – 27
trkA	5'–CGT CAT GGC TGC TTT TAT GG–3'	5'–ACT GGC GAG AAG GAG ACA G–3'	56°C	28-33
trkB+	5'–ACG CAG TGC TGA TGG CAG–3'	5'–TAC CTG CTG CGA TTT GCT G–3'	52°C	30-32
trkC+	5'–CCC ACC AAA GAC AAG ATG–3'	5'–TAT CCA GTC CAC ATC AGG–3'	52°C	30-32
p75	5'–CCG ATA CAG TGA CCA CTG TGA TG–3'	5'–AGC AGC CAA GAT GGA GCA ATA GAC–3'	60°C	27
NGF	5'–AGC ATT CCC TTG ACA CAG–3'	5'–GGT CTA CAG TGA TGT TGC–3'	50°C	24-30
BDNF	5'–ACT TGG CCT ACC CAG GTG TG–3'	5'–TGT CGT CGT CAG ACC TCT CG–3'	60°C	27-31
NT-3	5'–TAC TAC GGC AAC AGA GAC G–3'	5'–GTT GCC CAC ATA ATC CTC C–3'	55°C	29-32

2.3.5 Making and running of acrylamide gels

The products of the PCR-reaction were analysed by running them on a 8% polyacrylamide gel (National diagnostics) in V15.17 Horizontal gel tanks (Whatman). The gels were prepared by adding 90µl of 20% Ammonium Persulphate (APS) (Sigma) per 25ml gel followed by 30µl of TEMED (Sigma). The mixture was then mixed and poured between two glass-plates before inserting a comb and leaving it to set for at least half an hour. Once set, the gels were placed in their gel tank and the samples with loading buffer loaded into the preformed wells together with a DNA ladder. The gels where then run for 1 hour to 1½ hours at ~400V. The blue loading dye served as an indicator for the distance the samples had run, thus telling when the gels had to be stopped depending on the size of the amplified DNA fragment. Once run, one of the glass-plates was removed, the gel cut to size, and stained using Nucleic Acid Stain (Molecular Probes). SYBR®Gold labels the

amplified cDNA and shines at different brightnesses under a UV light source, depending on the amount of nucleic acid present. The gels were then washed under tap water and the DNA visualised using a UV-illuminator connected to a camera, which allowed pictures to be taken of the bands on the gel using the Phoretix Grabber computer program.

2.3.6 Analysis of relative mRNA levels

Analysis of the pictures with the endogenous and competitor cDNA bands on them was done using the Phoretix Quantifier computer program. This program enables the analysis of the intensity with which the bands were seen under the UV light, hence enabling quantification of the endogenous cDNA by direct comparison with the competitor cDNA. The intensity value returned by Phoretix Quantifier for the endogenous cDNA was divided by the intensity value for the competitor cDNA. This was then multiplied by the known concentration of the competitor, to give the amount of endogenous cDNA present. This was done for the mRNA expression of interest and for the GAPDH mRNA run with all samples. The relative expression of the gene of interest was then quantified as fg mRNA of interest / pg gapdh mRNA.

2.4 Origin, breeding and genotyping of knockout mice

This section aims at providing a background as to how the knockout mice used in the experiments were generated, what their phenotype is, and an explanation of the way these mice were bred in order to generate the desired knockouts.

2.4.1 Background

Knockout mice, lacking specific regions of the genome containing a gene of interest, have become extremely useful. They give a valuable insight into the specific function of a gene and its protein product *in vivo*.

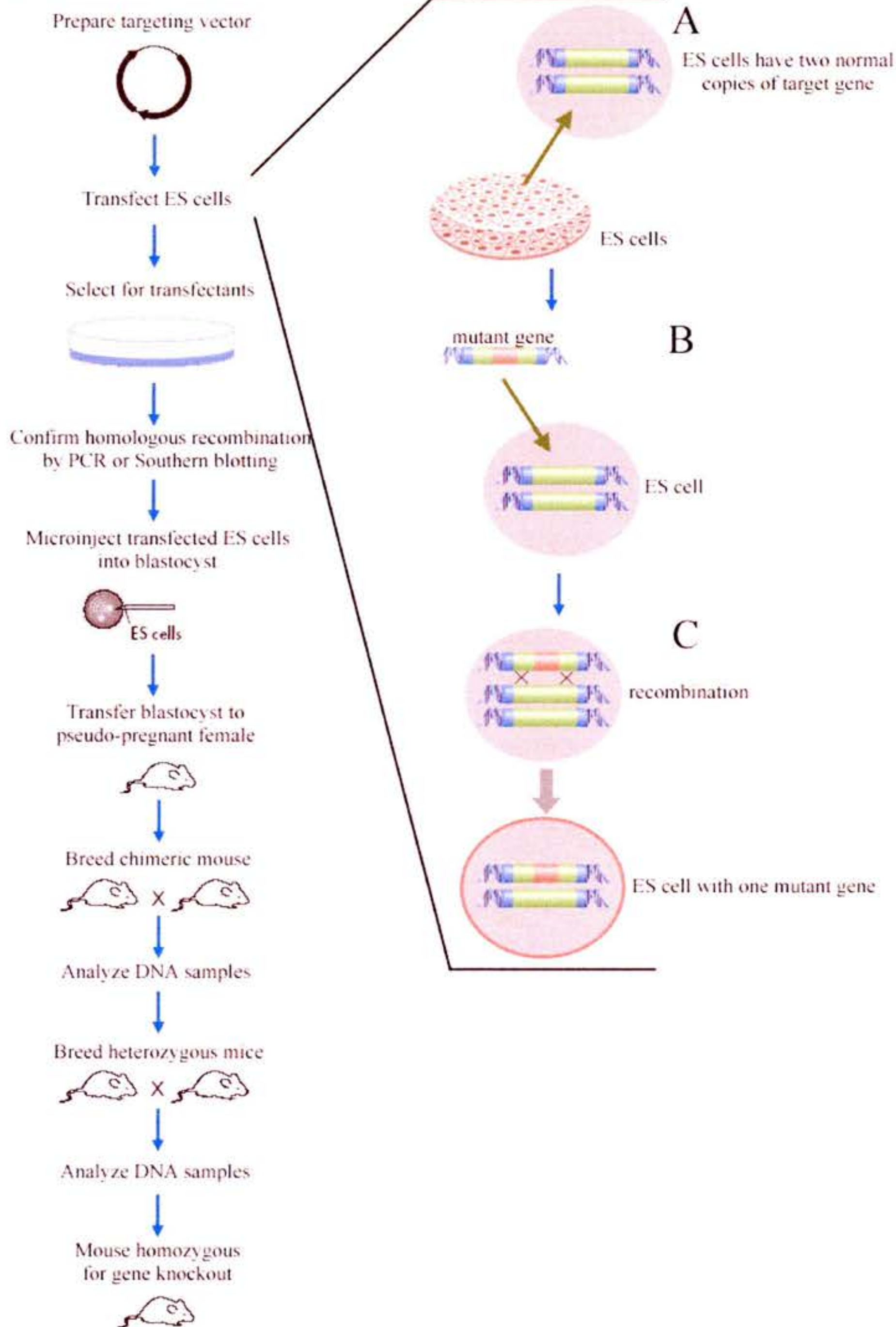
Knockout animals are usually generated by transfecting a targeting construct into pluripotent embryonic stem (ES) cells to replace the desired gene or a crucial part of it. The targeting construct contains two regions of homology that are located at either end of the 'vector' and are complementary to specific sites on the genome surrounding the gene of interest. The disrupted gene of interest and antibiotic selection markers are located between these regions of homology on the targeting construct. These homologous regions enable the targeting construct to bind to the genome and allow the exchange of the real gene with the disrupted gene and the antibiotic selection markers by homologous recombination. Selection for transfected cells is done by testing antibiotic resistance conferred by the antibiotic selection markers, and by polymerase chain reaction (PCR) and Southern blotting to certify homologous recombinants. The successfully transfected ES cells are then injected into blastocysts, which are inserted into the uterus of a surrogate mother. The resulting offspring will have some tissues derived from the blastocysts wildtype cells and other tissues derived from the manipulated ES cells. These mice are known as

chimera. If the progeny of the incorporated ES cells form germ-line cells, the mutations contained in them can be passed on to that mouse's offspring. The procedure involved in generating knockout mice is summarised in Figure 2.8.

2.4.2 trkA knockouts

The trkA knockout was generated by Smeyne et al. in 1994, by ablating the gene in D3 embryonic stem cells by homologous recombination. 51 amino acids from the trkA kinase domain were replaced with a phosphoglycerate kinase 1 *neo*-cassette in the opposite orientation. Lack of trkA leads to sensory and sympathetic neuropathies and most knockout mice die within a month of birth. They fail to respond to stimulation of their whisker pads and rear paws and develop myotic pupils and slight ptosis. Their fur becomes mottled and numerous scabs appear over the entire body; their paws are covered by ulcerations and digits are missing, thought to be due to self-mutilation. Also, some internal organs are abnormal. The phenotype includes decrease in the cholinergic basal forebrain projections to the hippocampus and cortex, and severe cell loss in the sympathetic (severely shrunken by P10), trigeminal (70-90% at P0) and dorsal root ganglia (70-90% at P0). Mice with this mutation were used in a CD1 background (Mice obtained from Rüdiger Klein, European Molecular Biology Laboratory, Heidelberg, Germany).

Figure 2.8 Procedure for generating knockout mice



Summary of the steps involved in the generation of knockout mice. The general procedure is shown on the left, while the right column shows events during transfection. Cultured ES cells have two normal copies of the target gene (A). When a disrupted version of the target gene is introduced (B), recombination between the related sequences of DNA swaps the disrupted version of the gene into the mouse genome (C). Successfully transfected ES cells are identified, and microinjected into a host blastocyst that is transferred to a pseudo-pregnant female. If the mutant gene contributes to the germ line in the resulting 'chimeric' mice, the next mouse generation will have one non-functional copy of the gene – these are heterozygous for the mutation. Interbreeding two heterozygous mice will result in some mice with two non-functional copies of the target gene; these are referred to as knockout mice (Figure combined and adapted from Qiagen News, Issue 1, 2002 and the Wellcome Trust website at http://www.wellcome.ac.uk/en/genome/technologies/hg17b005.html).

2.4.3 trkB knockouts

The trkB knockout was generated by Klein et al. in 1993, by ablating the gene in D3 embryonic stem cells by homologous recombination. 33bp of K2 (the second exon of the protein kinase domain of trkB) were replaced with a phosphoglycerate kinase 1 *neo*-cassette inserted in the same transcriptional orientation as the trkB gene. Most knockout animals die by P1, although some survive into the first postnatal week. TrkB animals appear to lack the sucking reflex observed in their wildtype littermates, and attempts to manually feed them were unsuccessful due to the animals inhaling instead of swallowing. These observations indicate sensory deficits as well as deficits in the swallowing motor system. Neuronal numbers in the trigeminal ganglion were reduced by ~60%, with larger differences in the anterior half of the ganglion. Further neuronal deficits were observed in facial motor neurons (~70% less), the DRG (~30% less), and spinal motor neurons (~25% less neurons).

Mice with this mutation were used in a CD1 background (Mice obtained from Rüdiger Klein, European Molecular Biology Laboratory, Heidelberg, Germany).

2.4.4 Partial p75 knockouts

The p75 knockout was generated by Lee et al. in 1992, by ablating exon III of the gene in J1 embryonic stem cells by homologous recombination. A phosphoglycerate kinase 1 *neo*-cassette was inserted in the third exon encoding cysteine rich repeats essential for ligand binding. Knockout mice are viable, although by 4 months they develop skin alterations on all extremities and tests reveal they are insensitive to heat. It was discovered that they have decreased sensory innervation of the skin

explaining some of these observations. In contrast to the observed sensory deficits there appear to be no effects on sympathetic ganglia and innervation.

Mice with this mutation were used in a C57/BL6 background and referred to as p75L (Mice obtained from Kuo-Fen Lee, Whitehead Institute for Biomedical Research, Nine Cambridge Center, USA).

2.4.5 Full p75 knockouts

As described above, Lee et al. generated a p75 knockout by manipulating the third exon of the gene. Schack et al. (2001) however, demonstrated that there was a protein isoform arising from alternative splicing of exon three, and that this protein is left intact in the partial knockouts described above. This s- (short) p75 receptor differs from the full-length receptor in that it only possesses one of the four cysteine-rich domains found in the extracellular domain of the full-length receptor.

To assess the role of this receptor, Schack et al. generated a knockout for both the full length and short p75 receptor by targeting exon four of the p75 receptor locus. In the first postnatal weeks the knockout mice generated were found to be substantially smaller than their littermates and had impaired motility due to hind limb ataxia. Analysis of the knockout mice revealed a greater decrease in cross-sectional area of the sciatic nerve (~53%) compared to the reduction seen in the partial knockouts (40%). The authors also noted a greater decrease of neuronal numbers in P3/P4 DRGs, with a 54% decrease compared to a 39% decrease with just the full length receptor lacking. Full knockouts also display partial perinatal lethality and defects in the vascular system, which the partial knockouts do not display.

Mice with this mutation were used in a C57/BL6 background and referred to p75D (Mice obtained from Yves-Alain Barde, Max Planck Institute of Psychiatry, Martinsried, Germany).

2.4.6 ErbB3 knockouts

The ErbB3 knockout was generated by Riethmacher et al. in 1997, by ablating the gene in E14-1 embryonic stem cells by homologous recombination. The *neo* cassette contained termination codons, which directly fused to coding sequences thereby preventing the production of ErbB3 protein.

Analysis of the phenotype of ErbB3 knockout mice showed that most homozygous embryos die between E11.5 and E13.5, while surviving embryos survived to term. Born knockout pups were slightly smaller than littermates, cyanotic, displayed no reaction to tactile stimuli, and did not breathe. DRG ganglia in the ErbB3 knockouts appeared normal in E12.5 embryos, but subsequently displayed a marked neuronal cell loss (E14.5: 70%, E18.5: 82% loss). Motor neurons of the ventral horn appeared normal up to E16.5, but were reduced by E18.5. Cranial ganglia and nerves were abnormal from E10, an effect attributed to the absence of neurons derived from hindbrain neural crest rather than death of neurons.

Mice with this mutation were used in a CD1 background (Mice obtained from Carmen Birchmeier, Max-Delbrueck-Center for Molecular Medicine, Berlin, Germany).

2.4.7 Bax knockouts

The Bax knockout was generated by Knudson *et al.* in 1995, by ablating the gene in embryonic stem cells by homologous recombination. A phosphoglycerate kinase 1 *neo*-cassette was inserted replacing a 3.7kb fragment containing exons 2 to 4 and part of exon 5 of *Bax*, preventing formation of a functional protein. Although externally indistinguishable from wildtype mice, knockout mice display hyperplasia of lymphoid tissues and enlarged spleens. Also, Bax knockout males are infertile and adult testes atrophic with no mature sperm production. Analysis revealed a marked increase in apoptosis and disorganized spermatogenesis in the testes of Bax knockouts, in contrast to the lymphoid tissues and spleen, which indicates that the same molecule can have positive or negative effects depending on the cell type. Knudson *et al.* (1995) speculate that the massive apoptosis and disorganized spermatogenesis may also indicate a role for *Bax* in regulating the meiotic cycle.

Mice with this mutation were used in a CD1 background (Mice obtained from Stanley Korsmeyer, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, USA).

2.4.8 Bad knockouts

The Bad knockout was generated in the laboratory of Stanley Korsmeyer (there are currently no publications on this mouse and no information on its generation). Mice with this mutation were used in a CD1 background (Mice obtained from Stanley Korsmeyer, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, USA).

2.4.9 Breeding

Mice in a C57/BL6 background were bred into a CD1 mouse background (trkA, trkB, ErbB3), by breeding heterozygous mice with CD1 wildtype mice, generating an F1 generation. The heterozygous mice from these litters were then crossed repeatedly with new CD 1 wildtypes until an F4 generation was reached and the mice displayed no characteristics of the original C57/BL6 mice (coloured fur, smaller size). The mice were backcrossed into a CD1 background because these mice have far greater litter sizes than C57/BL6 mice (14-22 compared to 4-10), which facilitates obtaining a sufficient number of knockouts for experimental studies, especially when studying double knockouts. Other mouse colonies used in these experiments had already been bred into a CD1 background (Bad, Bax) and some were studied in the original C57/BL6 background (p75Lee, p75 Dechant).

Single knockouts were generated by breeding two mice heterozygous for a mutation to generate litters containing heterozygotes, wildtypes and knockouts.

Double knockouts were generated by breeding mice heterozygous for one mutation with mice heterozygous for the other mutation. This would generate mice heterozygous for one mutation, wildtypes, and mice heterozygous for both mutations. The latter were paired to produce litters with a variation of genotypes including wildtypes, single knockouts and double knockouts.

Table 2.3 shows the total number of embryos collected for each of the mutations studied together with the obtained frequency of wildtype and knockout embryos.

Table 2.3: Total number of embryos collected in knockout experiments and distribution of genotypes

ErbB3

Age	Total number	Wildtype	ErbB3-Knockout	%-Wildtype	%-knockout
E11	87	29	27	33%	31%
E12	139	33	24	24%	17%
E13	109	26	20	24%	18%
E14	30	9	2	30%	6.7%

P75L

Age	Total number	Wildtype	P75L-Knockout	%-Wildtype	%-knockout
E12	21	7	5	33%	24%
E14	35	8	10	23%	26%
E16	20	7	5	35%	25%
E18	25	8	8	32%	32%

P75D

Age	Total number	Wildtype	P75D-Knockout	%-Wildtype	%-knockout
E12	29	5	9	17%	31%
E14	30	5	4	17%	13%
E16	19	5	5	26%	26%
E18	19	6	3	32%	16%

TT

Age	Total number	Wildtype	trkA -KO	trkB -KO	TrkA/trkB -KO	%-WT	%-trkA-KO	%-trkB-KO	%-trkA/trkB-KO
E12	68	8	3	6	3	12%	4%	9%	4%
E14	74	8	4	8	3	11%	5%	11%	4%
E16	74	12	7	6	3	16%	9%	8%	4%
E18	86	12	11	7	0	14%	13%	8%	0%

Bad/Bax

Age	Total number	Wildtype	Bax-KO	Bad-KO	Bad/Bax-KO	%-WT	%-Bax-KO	%-Bad-KO	%-Bad/Bax-KO
E12	161	11	12	10	8	7%	7%	6%	5%
E14	77	4	5	8	3	5%	6%	10%	4%
E16	183	11	10	11	4	6%	5%	6%	2%

Bad

Age	Total number	Wildtype	Bad-Knockout	%-Wildtype	%-knockout
E12	57	17	14	30%	25%
E14	46	13	15	28%	33%
E16	37	9	8	24%	22%

Bax

Age	Total number	Wildtype	Bax-Knockout	%-Wildtype	%-knockout
E12	26	9	7	35%	27%
E14	32	6	14	19%	44%

trkA

Age	Total number	Wildtype	trkA-Knockout	%-Wildtype	%-knockout
E12	60	19	18	32%	30%
E14	53	15	11	28%	21%
E16	57	16	9	28%	16%

trkB

Age	Total number	Wildtype	TrkB-Knockout	%-Wildtype	%-knockout
E12	46	7	17	15%	37%
E14	45	15	10	33%	22%
E16	56	12	9	21%	16%

2.4.10 Extraction of DNA

The genotype of mice and embryos used in knockout experiments was determined by tail-tipping the mice and keeping some embryonic tissue left after dissection. These tissues were digested overnight in 200µl of proteinase K / SDS solution at 56° C. Lysis could be accelerated by occasional vortexing of the samples. One additional tube, with the proteinase K mix but without any sample, was added at this stage to serve as a negative control. The remainder of the extraction was done using a Nucleospin® Tissue Kit (Macherey-Nagel), based on DNA binding to a silica

membrane in spin columns. Following digestion 200µl of distilled water (300µl for embryos) were added to the solution and the mix spun for 5min at 15000g. 200µl of the solution were then transferred to a new tube, ensuring that residual tissue in the bottom of the tube was taken up. After addition of a B3 buffer (containing chaotropic salts) the samples were vortexed and incubated at 70° C for 10 minutes, followed by addition of 210µl ethanol (99%) and further vortexing. The chaotropic salts and ethanol added in these steps fascilitate DNA binding to a silica membrane. The entire solution was then transferred to a Nucleospin® Tissue column in a 2ml collecting tube. The tubes were spun at 11000g for 1 min and the flow through discarded. Two buffers, one salt based, one ethanol based, were used to wash the silica membrane, by spinning the buffers through the spin tube as with the original solution. The silica was then dried by further spinning, before the spin tube was placed into a 1.5ml microcentrifuge tube, and 100µl (60-80µl for DNA from very young embryos) of elution buffer preheated to 70° C was added. The slightly alkaline elution buffer enabled dissociation of DNA from the silica membrane. Thus when the spin tubes placed in the microcentrifuge tubes were spun at 11000g for a further minute, the concentrated DNA was passed into the microcentrifuge tube ready for genotyping.

2.4.11 Genotyping

The extracted DNA was amplified using the polymerase chain reaction (PCR), allowing the amplification of specific regions of DNA using synthetic oligonucleotide primers, specifically designed from portions of the gene surrounding the gene of interest. The DNA is denatured and the primers bind to the DNA and are

extended in a 5' direction by DNA polymerase, thus producing a copy of the target sequence and effectively doubling the concentration of DNA in the tube. This is achieved by alterations in temperature, with denaturation (strand separation), primer binding and extension by DNA polymerase all requiring specific temperatures to be at their optimum and to avoid unspecific primer binding. These changes in temperature are repeated until the DNA is sufficiently amplified for visualisation. For genotyping three primers were employed, of which one was unique to the wild-type allele, one to the knockout allele and one was common to both alleles. The components of the PCR reaction were: Hotstart DNA polymerase (Biogene), primers (MWG Biotech.) deoxynucleoside triphosphates (dNTPs) (Promega), 10x Buffer, $MgCl_2$ (both Biogene) and in some reactions the PCR enhancer MR (Biogene).

PCR reactions were conducted in 20µl reactions with 1µl of the DNA concentrate added to a mix of the above components. The proportions of these ingredients per PCR reaction are represented in table 2.4, and the primers used are listed in table 2.5. It should be noted that for the Bad genotyping two separate reactions were used to amplify the wildtype (Bad1) and knockout (Bad2) DNA.

Table 2.4: Volume of reagents (in µl) used per 20µl genotyping reaction

Strain	p75D	P75L	trkA	TrkB	Bax	Bad 1	Bad 2	ErbB3
dH_2O	9.35	9.35	13.17	9.55	8.725	9.41	13.57	12.2
MR sol.	3.8	3.8	-	3.8	3.8	3.8	-	-
10x Buffer	2	2	2	2	2	2	2	2
$MgCl_2$	2	2	2	2	2	2	2	2
dNTPs	1	1	1	1	1	1	1	1
Primer 1	0.3	0.3	0.2	0.16	0.45	0.33	0.165	0.6
Primer 2	0.2	0.2	0.2	0.16	0.3	0.33	0.165	0.5
Primer 3	0.2	0.2	0.23	0.23	0.525	-	-	0.3
DNA polymerase	0.15	0.15	0.2	0.1	0.2	0.13	0.1	0.4

Table 2.5: Primers used for the genotyping reactions

Primer	Primer Name	Sequence
p75D primer 1	mp75ex4s3	5'- GAT GGA TCA CAA GGT CTA CGC – 3'
p75D primer 2	pGK2	5' – AAG GGG CCA CCA AAG AAC GG – 3'
p75D primer 3	2590	5' – TGT TGG AGG ATG AAT TTA GGG – 3'
p75L primer 1	koD2	5' – CCC CTT CCC AGC CTC TGA GC – 3'
p75L primer 2	U1	5' – AGC CGT GCA AGC CGT GCA CC – 3'
p75L primer 3	D1	5' – AGG GTA GGC ACG GGT CCA CG – 3'
trkA primer 1	P095-4	5' – CGG ACC TCA GTG TTG GAC AGC TGG – 3'
trkA primer 2	P096-0	5' – GAC CCT GCA CTG TCG AGT TTG C – 3'
trkA primer 3	P097-0	5' – GCT CCC GAT TCG CAG CGC ATC G – 3'
trkB primer 1	P080-0	5' – AGA CCA TGA TGA GTG GGT CGC C – 3'
trkB primer 2	P048-0	5' – TCG CGT AAA GAC GGA ACA TGA TCC – 3'
trkB primer 3	P081-0	5' – GAT GTG GAA TGT GTG CGA GGC C – 3'
Bax primer 1	In5R	5' – TTG ACC AGA GTG GCG TAG – 3'
Bax primer 2	Ex5F	5' – GCT GAT CAG AAC CAT CAT G– 3'
Bax primer 3	NeoR	5' – GCT TCC ATT GCT CAG CG – 3'
Bad1 primer 1	BAD 1	5' – ACG GGA TCC CCG AAG GAT GAG CGA TGA GTT TG – 3'
Bad1 primer 2	BAD 2	5' – AGC GAA TTC TCA GCC TGC GCT CTT TGG GCG AGG – 3'
Bad2 primer 1	BAD 3	5' – TCG TGC TTT ACG GTA TCG CC – 3'
Bad2 primer 2	BAD 4	5' – CAT TGC CCA AGT CAC CTT C – 3'
ErbB3 primer 1	Neo	5' – CGA ATT CGC CAA TGA CAA GAC GCT GG – 3'
ErbB3 primer 2	INA 1	5' – GGG TGT CTG AGT CTT TGA AGC TGG AG – 3'
ErbB3 primer 3	EXA2	5' – ACC TGT ATT CTC CCG ACT GTC CTG AA – 3'

Following addition of 1μl of DNA concentrate to 19μl of the mix of PCR reagents and mixing, 40μl of mineral oil was added to the tube in order to prevent evaporation of the reactants. The samples were then placed in a thermal cycler, which then took the samples through the temperature-dependent steps described above. Reactions for each sample were done in duplicate to ensure accuracy of the obtained genotype, and

run with the negative control (to account for the possibility of contamination) and a positive control (a heterozygous sample with both the wildtype and knockout allele).

The cycling temperatures for the different strains were as follows (Steps 1 + 2a: Denaturation of DNA, Step 2b: Annealing of primers, Step 3 + 2c: Synthesis of new DNA strands):

p75D reaction

Step 1: 95°C - 10 minutes

Step2: a: 95°C – 40 seconds

b: 59°C – 1 minute 10 seconds

c: 72°C – 1 minute 40 seconds

Number of cycles a-c: 41

Step 3: 72°C - 10minutes

4°C soak

p75L reaction

Step 1: 95°C - 10 minutes

Step2: a: 95°C – 40 seconds

b: 62°C – 1 minute 10 seconds

c: 72°C – 1 minute 40 seconds

Number of cycles a-c: 39

Step 3: 72°C - 10minutes

4°C soak

trkA reaction

Step 1: 95°C - 10 minutes

Step2: a: 95°C – 40 seconds

b: 65°C – 1 minute 10 seconds

c: 72°C – 1 minute 40 seconds

Number of cycles a-c: 39

Step 3: 72°C - 10minutes

4°C soak

trkB reaction

Step 1: 95°C - 10 minutes

Step2: a: 95°C – 40 seconds

b: 59°C – 1 minute 10 seconds

c: 72°C – 1 minute 40 seconds

Number of cycles a-c: 37

Step 3: 72°C - 10minutes

4°C soak

Bax reaction

Step 1: 95°C - 10 minutes

Step2: a: 95°C – 45 seconds

b: 50°C – 1 minute 20 seconds

c: 72°C – 2 minute 20 seconds

Number of cycles a-c: 43

Step 3: 72°C - 10minutes

4°C soak

Bad1 reaction

Step 1: 95°C - 10 minutes

Step2: a: 95°C – 40 seconds

b: 62°C – 1 minute 20 seconds

c: 72°C – 1 minute 50 seconds

Number of cycles a-c: 41

Step 3: 72°C - 10minutes

4°C soak

Bad2 reaction

Step 1: 95°C - 10 minutes

Step2: a: 95°C – 45 seconds

b: 64°C – 1 minute 20 seconds

c: 72°C – 1 minute 40 seconds

Number of cycles a-c: 40

Step 3: 72°C - 10minutes

4°C soak

ErbB3 reaction

Step 1: 95°C - 10 minutes

Step2: a: 95°C – 40 seconds

b: 68°C – 1 minute 50 seconds

Number of cycles a-b: 41

Step 3: 72°C - 10minutes

4°C soak

After the PCR reaction 4μl of 6x gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol in deionized water) were added to the product.

2.4.12 Electrophoresis and visualisation of DNA

The amplified DNA fragments were separated using electrophoresis through agarose gels. Due to its negative charge DNA will move towards the positive electrode when a current is applied. Agarose gels have pores, which when current is applied allow smaller DNA fragments to travel through the agarose at a quicker pace than heavier fragments, thus separating the DNA according to size. The size of the fragments was estimated through comparison with DNA bands of known size known as a DNA ladder. The DNA fragments on the agarose gel were visualised by incorporating ethidium bromide in the agarose gel, which binds to the DNA, and glows orange under ultra-violet (UV) light.

Agarose gels were prepared by dissolving molecular grade agarose (Helena BioSciences) in 1x tris-acetate (TAE) prepared from 50x stock. The 50xTAE stock was made with 242 grams of Tris base, 57.1 ml glacial acetic acid, and 100ml 0.5M EDTA (pH8.0) in 1 litre of deionised water. The concentrations of agarose gel used for the different strains of knockout mice can be seen in Table 2.6, together with the size of the bands from the wildtype and knockout DNA fragments.

The agarose in TAE was melted using a microwave oven at medium power. This was followed by the addition of ethidium bromide at 0.5mg/ml. This and the following steps were carried out in a fume hood due to the dangers of ethidium bromide. The

agarose solution was then poured into a plastic gel tray sealed at both ends with masking tape and containing plastic combs to form the wells that the PCR product can be loaded into. The gels were then left to set for a minimum of 30 minutes.

The gel was then placed in an electrophoresis tank filled with 1xTAE with 0.5mg/ml ethidium bromide. The PCR reaction product with the loading buffer were loaded in the wells made by the combs together with a DNA ladder for comparison. The tank was then put under current and the samples were run at 200V and 400mA for 40 minutes to 1 hour depending on the size of the fragments and the difference between the wildtype and knockout fragments The blue loading buffer gave an indication of how far down the gel the samples had run.

Finally the gel was visualised under a UV light, revealing the wildtype and knockout bands thus enabling identification of wildtype, heterozygous and knockout animals. Figure 2.9 shows examples of genotyping gels illustrating these three genotypes.

Table 2.6: Concentration of agarose gels used and size of bands

	Agarose concentration	Wildtype band	Knockout band
p75D	2%	345bp	475bp
p75L	2%	285bp	600bp
trkA	2%	400bp	800bp
trkB	1%	800bp	900bp
Bax	1.5%	307bp	507bp
Bad1	2%	550bp	
Bad2	2%		550bp
ErbB3	2%	489bp	712bp

Figure 2.9 **Examples of genotyping gels for the knockout mice studied, illustrating the appearance of wildtype and knockout bands**

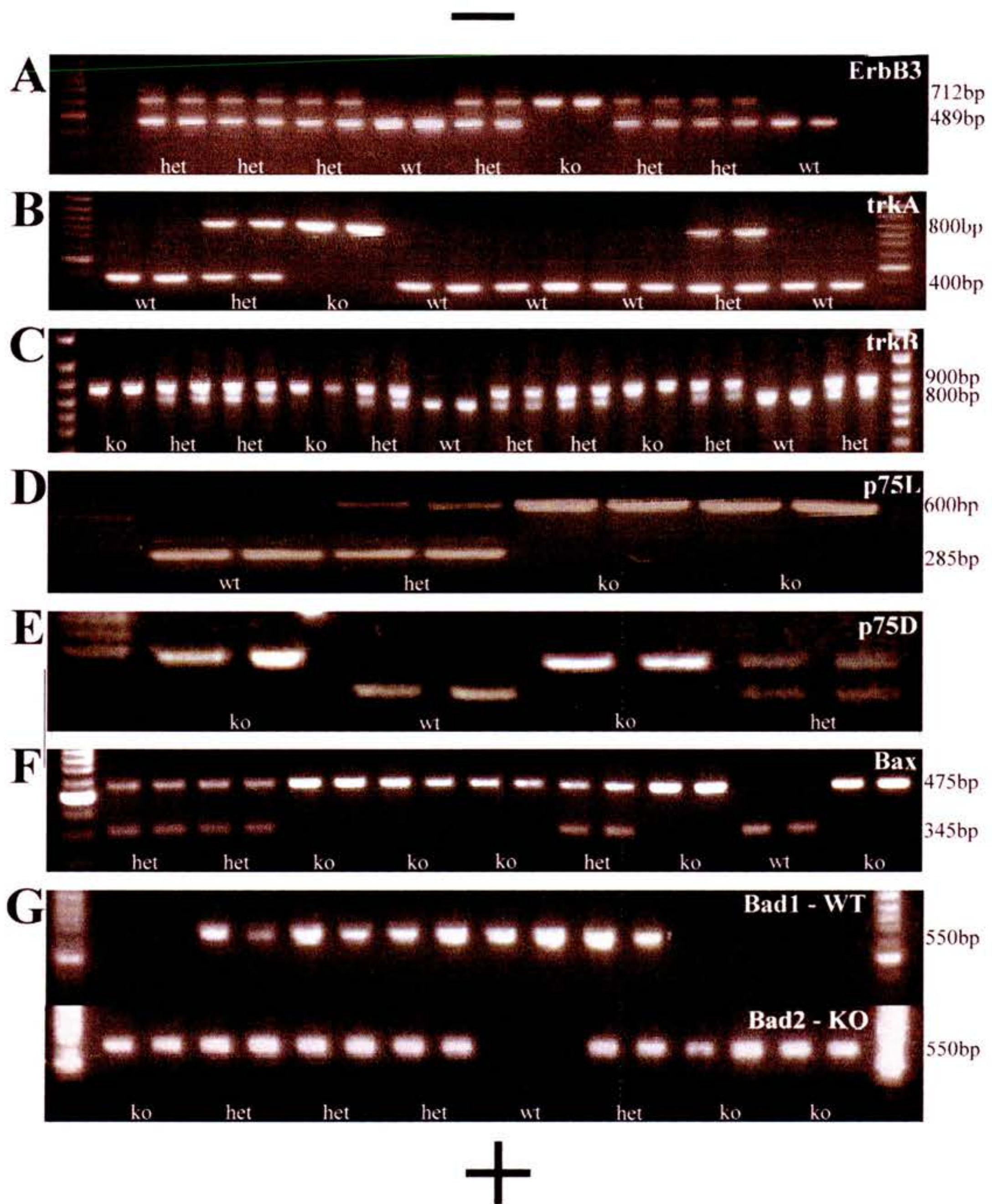


Figure showing examples of genotyping gels from litters obtained by breeding two heterozygous mice from the ErbB3 (A), trkA (B), trkB (C), p75L (D), p75D (E), Bax (F) and Bad (G) colonies together. DNA moves towards the positive electrode (+) when current is applied, with smaller fragments of DNA moving through the agarose gel faster than larger ones. In all the knockout mice studied, apart from the Bad colony, the knockout band is larger than the wildtype band and therefore moves towards the positive electrode at a slower rate, thus allowing the identification of wildtype (wt), heterozygous (het) and knockout (ko) mice. In the case of mice in the Bad colony, the wildtype (Bad1) and knockout (Bad2) bands are of the same size and are therefore run on separate agarose gels. One or two 100bp DNA ladder are shown with each gel.

2.5 Fixation, processing, cutting and staining of embryos for histological analysis

2.5.1 Background

The advent of knockout mice has resulted in extensive use of histological techniques for analysis of the morphological and quantitative changes in various cell types occurring in a variety of tissues when a particular protein is deleted. Furthermore, the development of specific, sensitive immunohistochemical techniques has facilitated this analysis by providing the opportunity to identify particular cells or proteins of interest.

The general histological stain used in these experiments was Cresyl Violet Acetate, also known as Cresyl Fast Violet (CFV). CFV is a Nissl stain (staining blue-purple). Nissl granules are basophilic granules, which represent rough endoplasmatic reticulum, the RNA of ribosomes, giving rise to the basophilia, which are distributed in the cell body and dendrites of neurones. The stain is a mixture of 2 or more basic dyes, which vary in their alkyl groups from the structure. It is based on coulombic attractions between the coloured cations of the dye and the anions present in the RNA. Staining is dark violet for nucleoli and Nissl substance and pink for cartilage.

Immunohistochemistry uses antibodies to recognise cellular or tissue constituents (antigens). This works by using a lock and key principle, with the antibody fitting the antigen of interest, and, ideally, not binding to any other constituents of the tissue. Antibodies possess variable and hypervariable domains which bind epitopes on the antigen to which the antibody was raised against. Antibodies are 'labelled' with an enzyme or chromogen to permit detection of bound antibody.

The method used in the experiments described in this thesis is known as indirect immunohistochemistry, which means that an unlabelled primary antibody was used to recognise the antigen of interest and the location of bound primary antibody was detected by a second labelled antibody raised in a different species against the constant domains of the primary antibody. This method provides a higher sensitivity than direct recognition of the primary antibody, since more than one secondary antibody is able to bind to each primary antibody hence increasing the detectability of antigens. The method also potentially enables the use of the same secondary antibody to detect a variety of primary antibodies raised in the same species. The detection system used here employed the avidin biotin system, the glycoprotein avidin having a high affinity for the vitamin biotin due to four biotin-binding hydrophobic pockets. A high number of biotin molecules can be linked to any antibody, thus increasing the amplitude of staining further without losing specificity, allowing more sparing use of the primary antibody. The visualisation of the above described complexes is done using biotinylated enzymes, in this case horseradish peroxidase in combination with a chromogen such as 3,3'-diaminobenzidine tetrahydrochloride (DAB), giving a brown staining of the protein of interest. The preformed macromolecular complexes between avidin and the biotinylated enzyme are referred to as ABC complexes (Hsu et al., 1981).

The primary antibody used in immunohistochemistry experiments is directed against a peptide corresponding to the C-terminus of β-III-Tubulin, which is synthesized exclusively by neurons of higher vertebrates (Lee et al., 1990). The β-III-Tubulin antibody (Promega) has been tested to perform in immunohistochemistry of frozen and paraffin-embedded sections and has been found to exhibit excellent signal-to-

noise ratios in both. It cross-reacts with most mammalian species and does not label non-neuronal cells (Promega, Neural Notes 11, 1998).

Non-specific binding of the secondary antibody was assessed by omitting the primary antibody from the staining procedure. This resulted in the absence of staining on neuronal sections.

2.5.2 Fixation of embryos

Before any staining can be done, freshly collected tissue had to be fixed and processed to enable it to be cut into thin sections using a microtome.

The aim of fixation is to suppress autolysis and bacterial attack, preserve the tissue and its constituents as close as possible to its natural size and shape (during fixation and after), and to enable ease of staining. Different fixatives may be better at some of these than others, so a fixative should be chosen carefully depending on the tissue studied and the staining to be performed.

After various tests performed by Dr. Lucia Piñón in this laboratory, it was found that neutrally buffered formalin (NBF) gave the best resolution for CFV staining, and that Carnoy's fixative was best suited to immunohistochemistry and for identification of neurons in early embryos using CFV staining. NBF belongs to the aldehydes and reacts with the basic amino-acid residues of proteins, causing cross-linking between the proteins. Carnoy's is a mixture of 60% Ethanol, 30% Chloroform, and 10% glacial acetic acid. The ethanol in Carnoy's coagulates proteins and precipitates nucleic acids, while the acetic acid minimises shrinkage induced by ethanol. Acetic acid also decalcifies the tissue, facilitating later processing. The chloroform bleaches

the tissue, thus improving contrast of subsequent immunohistochemistry. Neuronal numbers in embryos up to age E14 were counted using immunohistochemistry and hence fixed in Carnoy's, whereas neurons in older embryos (E16 and E18) were counted on CFV stained sections and thus fixed using NBF. This was done because CFV-stained neurons can be quite difficult to differentiate from some non-neuronal cells before E14, and hence immunohistochemistry was employed to minimise erroneous counting.

Different ages of embryo required different times in these fixatives for good fixation, while avoiding tissue shrinkage: E11 embryos were fixed in Carnoy's for 5 minutes at room temperature, E12 embryos for 15 minutes, and E13 and E14 embryos for 30 minutes. E16 and E18 embryos were fixed in NBF (BDH) for approximately one week.

2.5.3 Processing and embedding of embryos

Older NBF fixed embryo heads needed to be decalcified prior to further processing, i.e. the mineral content of bones and other tissues in these embryos needed to be reduced to soften the tissue enough to be able to section. Acids may be used for this purpose, but may interfere with the staining of the tissue by CFV, interfering with nucleic acid staining. Hence ethylene-diaminetetracetic acid (EDTA), a chelating agent was used, which depletes the outer mineral layer by binding to ionised calcium. The depleted calcium is replaced by ions from within, which then in turn are also depleted until there are no ions to replace the depleted ones and decalcification is complete. This is a slow process and required 5-7 days in 10% EDTA (Sigma) for

E16 embryos and 7-10 days for E18 embryos. During this period the samples were moved to fresh EDTA every 3 days.

After fixation and decalcification the embryo heads were dehydrated through a graded alcohol series. From absolute alcohol embryo heads were transferred to an alcohol/chloroform mixture, before being taken through two further lots of chloroform. This process, known as clearing, prepared the tissue for processing through paraffin wax for embedding, since alcohol is insoluble in paraffin wax. Clearing agents such as xylene or chloroform are miscible with both alcohol and paraffin wax, thus preparing the tissue for the uptake of the wax. Chloroform was used in these experiments since it bleaches the tissue enabling better resolution in both CFV and immunohistochemical staining. Clearing was followed by three changes in Paraffin wax at 56^0C. As for the fixation, the time the embryos spent in alcohol, chloroform and wax was dependent on the age of the tissue. The times at each step for each age as determined by Dr. Lucia Piñón are displayed in table 2.7. Finally, the heads of the embryos were embedded in paraffin upside down using embedding moulds, to allow serial sections to be cut. No wax was used at temperatures exceeding 62°C, since temperatures above this could have damaged the antigens required for immunohistochemistry. Also, paraffin wax, alcohols and chloroform used in the processing of embryos were frequently changed to minimise build up of chloroform in the wax and to ensure that alcohols and chloroform remained as close to the intended concentration as possible. Purity of the paraffin wax used was ensured by filtration of melted Gurr® wax pastilles (BDH) prior to use in processing or embedding.

Table 2.7: Fixatives and processing times for embryo heads depending on age

Fixative	Carnoy's			NBF	
Embryonic Age	E11	E12	E13+E14	E16	E18
dH_2O	-	-	-	2x1 hr	2x1 hr
75% Alc.	-	-	-	Overnight	Overnight
96% Alc.	1 min	1 min	2 mins	15 mins	30 mins
96% Alc.	1 min	1 min	2 mins	20 mins	30 mins
96% Alc.	-	1 min	2 mins	-	-
100% Alc.	1 min	2 mins	5 mins	15 mins	30 mins
100% Alc.	3 mins	7 mins	5 mins	20 mins	30 mins
1/1 Alc./Chlor.	5 mins	10 mins	15 mins	30 mins	30 mins
Chlor. I	10 mins	10 mins	15 mins	2 hrs	2 hrs
Chlor. II	1 hr	1 hr	1 hr	Overnight	Overnight
Paraffin I	10 mins	30 mins	30 mins	1 hr	1 hr 30 mins
Paraffin II	10 mins	30 mins	45 mins	1 hr	2 hrs
Paraffin III	10 mins	30 mins	45 mins	1 hr 30 mins	2 hrs

2.5.4 Cutting of embryos

After letting the wax harden overnight at 4^0 C, 8μm sections were cut using a rotary microtome. The sections were straightened out using a water bath at approximately 30-40^0 C, and put on Polysine slides (for Cresyl Fast Violet staining) or Gold Seal® slides (for Immunohistochemistry) (both BDH). Gold Seal® slides are better at keeping the sections on the slide and were hence used because the tissue is subjected to a lot of strain during the washes involved in the immunohistochemical staining procedure. The slides were then dried overnight at 37^0 C. To be economical with the antibody and detection kit used for immunohistochemistry, only slides with the ganglion on them were stained. This was done by cutting the tissue so that there were always four sections on one slide, used for CFV, and six on another slide, used for immunohistochemistry (see Figure 2.10). The CFV stained slides were used to determine the position of the ganglion of interest, hence indicating which of the

immunohistochemistry slides could be stained.

Figure 2.10: Cutting of sections for immunohistochemistry

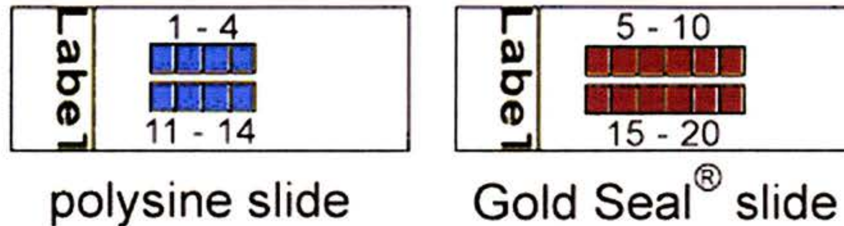


Figure illustrating the way sections were cut for immunohistochemistry. Four sections were cut on a Polysine slide, used for CFV staining, and six on another slide, used for immunohistochemistry. 1-4 are the first four sections to be cut and kept on a polysine slide. The following six sections are then kept on a Gold Seal® slide (5-10). The next 4 are then again kept on a polysine slide (11-14) and so forth all the way through the tissue.

2.5.5 Staining of sections using cresyl fast violet (CFV)

A 0.01% CFV solution with a few drops of acetic acid was prepared. This was done using Cresyl Violet Acetate powder (Sigma), which was dissolved to the required concentration before being mixed at 45° C for 2 hours. After addition of 3 drops/250ml of acetic acid the solution was filtered. Sections were cleared in Xylene (approx. 2x5 minutes) and rehydrated through a graded alcohol series (2x100%, 1x96%, 1x70%) followed by distilled water (approximately 2 minutes in each solution) before being transferred into the staining solution. The time the slides had to remain in the staining solution varied with embryonic age (older = longer), fixative (Carnoy's longer than NBF), and has even been reported to vary with genotype of the animal. Thus staining was checked at regular intervals to avoid over- or understaining. Following the staining and rinsing with distilled water (with 3

drops of acetic acid), the slides were differentiated using 96% alcohol with a few drops of glacial acetic acid, followed by absolute alcohol and chloroform. Slides were considered to be differentiated when the nucleoplasm of the neurons appeared free of stain, and the cytoplasm and dark nucleoli were clearly stained. The chloroform was washed out through 2 100% alcohols, before being mounted in DPX (BDH) after going through xylene. The typical appearance of CFV staining and the criteria for identifying neurons can be seen in Figure 2.13.

2.5.6 Immunohistochemical staining of sections

Immunohistochemical staining was done with anti-β-III-Tubulin mouse monoclonal antibody (Promega) as the primary antibody to identify neurons, using the Vectastain® ABC Kit (Vector Laboratories) as the avidin-biotin detection system. DAB (Vector Laboratories) was used as the chromogen. For E13 and E14 embryos a Vector® M.O.M. Immunodetection Kit was used, to minimise background-staining occurring at these older ages. The primary antibody was used at a concentration of 1/2000 in PBS, while the secondary antibody, complexing solution and chromogen were used at the manufacturers recommended concentrations.

PBS was used for washing of slides between the steps in the staining procedure. A quenching solution, made up freshly before use from 10% methanol and 0.3% hydrogen peroxide in PBS (De Dellis et al., 1979) was used to block any endogenous peroxidase activity in the tissue prior to staining, since this would interfere with the peroxidase reaction with the chromogen. A blocking solution was used to block non-specific binding of the antibody. Non-specific binding is caused by the affinity of

certain parts of the tissue for the immunoglobulins used due to hydrophobic and electrostatic forces. Non-specific binding can be inhibited by incubating with an immunoglobulin-rich serum, which will not react or interfere with the primary antibody. The serum used for blocking is usually from the species that the secondary antibody was raised in, in this case horse. Low concentration detergents, such as Triton-X, are usually added to the block in order to improve penetration of the blocking solution, as well as the penetration of the antibody. Hence the block used in the staining procedures was made from 10% horse serum, 0.4% Triton-X (both Sigma) in PBS. Staining solutions such as the block and the antibody dilutions were kept on the slides using either probe clips or hydrophobic barrier pens in humidity chambers.

The staining procedure started in the same way as with CFV staining, clearing the wax using xylene and then rehydrating through a graded alcohol series. This was followed by a wash with PBS before incubation in the quenching solution for approx. 20 minutes. Following 2 x 5 minute washes in fresh PBS the sections were incubated with blocking serum at 37°C for a minimum of 30 minutes. The blocking solution was then tipped off before adding the primary antibody at 1/2000 in PBS. The slides were then incubated with the primary antibody at either 37°C for 1 hr or 4°C overnight. The antibody solution was then washed off before washing the slides for two times five minutes in fresh PBS. During these washes the secondary antibody and the complexing solution were prepared: One drop (~50μl) of the secondary antibody stock supplied was added to 10 ml of PBS (1/200). The complexing solution was prepared by adding 2 drops of stock solution A (Avidin DH) to 10 mls of PBS before mixing well. 2 drops of stock solution B (biotinylated horseradish

peroxidase H) was then added before mixing. This solution was required to stand for at least 30 minutes before use, for the ABC complexes to from. After the washes the slides were incubated with the secondary antibody solution for 45 minutes at room temperature, before being washed as previously. They were then incubated with ABC solution for 30 minutes at room temperature before another wash and application of the chromogen. The DAB was prepared following the manufacturers recommendations, adding two drops of buffer to 5 ml of distilled water, followed by 4 drops of DAB stock and 2 drops of hydrogen peroxide. Sections were incubated with DAB for the time required for optimal staining, varying between 2 and 10 minutes, before stopping the chromogen reaction using tap water (5 minutes). The stained sections were then dehydrated as before through alcohols and xylene and mounted in DPX. The sections were then left to dry for at least two days before examination under a microscope.

The staining procedure using the M.O.M. Immunodetection Kit (Vector Laboratories) is similar to the one described above, but blocking occurred in a supplied mouse IgG blocking reagent, and antibodies were diluted in a special M.O.M. diluent. A different ABC complex solution was used too. Another difference was shortened incubation times for the primary antibody (30 minutes at 37°C), secondary antibody (10 minutes at room temperature) and ABC solution (5 minutes).

Figure 2.11 illustrates the appearance of anti-βIII-Tubulin-stained cells, using DAB as the chromogen, showing βIII-Tubulin antigens stained in the cytoplasm of the neurons. It also demonstrates the differences in neuronal size between different ganglia in the peripheral nervous system.

Figure 2.11: Typical appearance of βIII-Tubulin-stained ganglia

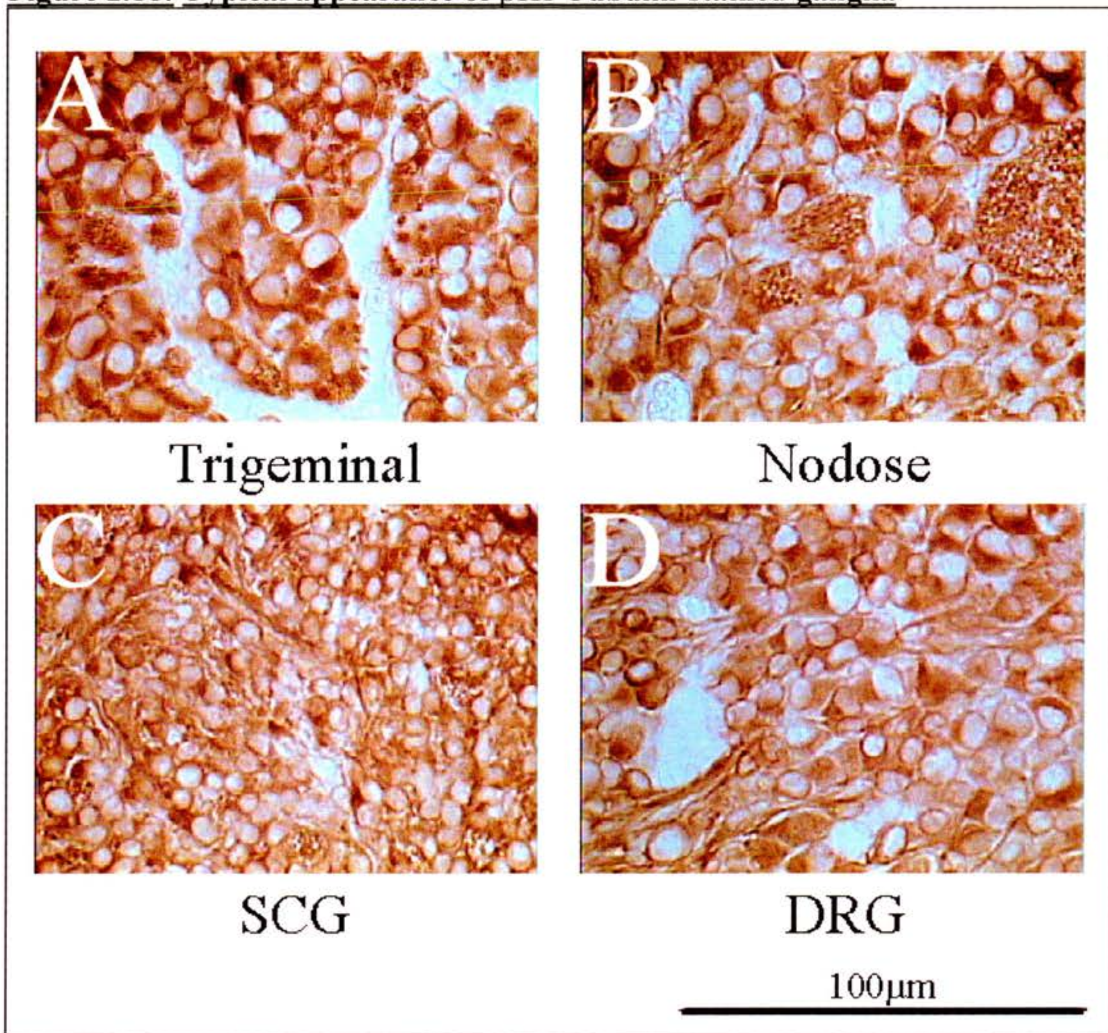


Figure illustrating βIII-Tubulin-staining in E14 trigeminal (A), nodose (B), superior cervical (SCG) (C) and dorsal root (DRG) (D) ganglia at 1000x magnification. Neurons are easily identifiable by the βIII-Tubulin-positive cytoplasm and fibres, and the clear nucleus. While the sensory ganglia (trigeminal, nodose and DRG) have comparably similarly sized neurons, the neurons in the sympathetic ganglion SCG are visibly smaller, and also appear more densely packed.

2.6 Counting of immuno- and CFV-stained neurons and pyknotic neurons

2.6.1 Background

Quantitative estimation of the neuronal number in the ganglia of interest was done using a stereology system. Stereology was developed to address two problems traditionally associated with quantitative microscopy: first, the magnification needed for counting only enables viewing of a restricted amount of the object of interest. This makes it difficult to derive a total count from the often tiny fraction of the total that has been counted. Second, the sectioning necessary to visualise the item of interest also decreases the dimensionality of the object, making it impossible to directly quantify the object from cross-sections. Stereology is a branch of mathematics and probability theory, interested in obtaining three-dimensional information from two-dimensional sections. It is an accepted unbiased (i.e. it makes no previous assumptions) method for collecting information about the number, length, surface area, and volume of objects. In these experiments a computer program was employed, 'Digital Stereology' supplied by Kinetic Imaging, for stereological assessment of the neuronal numbers in the ganglia of interest.

2.6.2 Counting of neurons on CFV and histochemically stained sections

The first step in the estimation of the neuronal number for the ganglion of interest is the estimation of the volume of the ganglion. This is done using the Digital

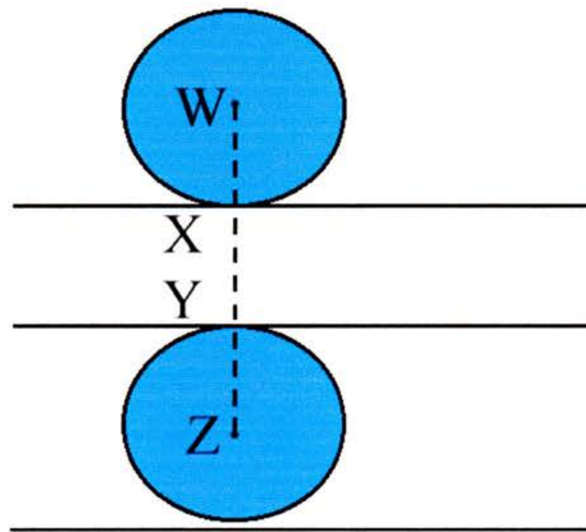
Stereology program. Two dimensional areas of the sections of the ganglia of interest were obtained by the computer program placing a point grid over the object in both dimensions with the same intervals between the points. The number of points in contact with the area of the ganglion were used to quantify the area in an unbiased way by multiplying the number of points in contact with the object of interest with the area associated with each gridpoint (known value). This has been found to be more accurate than the more traditional method of edge following (Gundersen et al., 1981). Every tenth section was counted in this way, starting with a random (unbiased) section. The distance between these sections was known (i.e.: every tenth 8µm section x10 = 80µm), it was thus sufficient to multiply the measured areas of the examined sections by this distance. The data were expressed in μm^3. For the trigeminal ganglion of E14 and E13 embryos every tenth section was assessed for volume determination. Volume of trigeminal ganglia of younger embryos, nodose, dorsal root and superior cervical ganglia were assessed using every fifth section.

The second step in estimating neuronal numbers using Digital Stereology was to determine the density of neurons per volume by introducing a motorised focus, thus allowing the position of sampling in a plane of focus to be determined, adding a depth dimension to the 2 dimensional picture seen and returning a value of neurons/unit volume. Random samples were taken in every fifth section, using different starting points, aiming to sample approximately a quarter of the area of interest in the section. The density data returned by the Digital Stereology Program was expressed as neurons/μm^3. The values from the first and second step were then multiplied to obtain a value for the total number of neurons/ganglion.

When counting neuronal nuclei in sections, not all of those visible represent whole nuclei. There will be a large number of nuclei that lie partly within the examined section, and it is not possible to distinguish whole nuclei and fragments. "One cannot therefore extrapolate from the mean apparent number of nuclei within the volume of a microtome section to the mean number within a given mass of tissue without exaggeration" (Abercrombie, 1946). The Abercrombie correction is aimed at rectifying the bias, with Abercrombie stating that the true density of a population can be regarded as the number of nuclear points within unit volume. The nuclear point is designated as any geometrical point in the same relative position in all nuclei. "A nuclear-point cannot overlap two adjacent sections, and the number of nuclear points can therefore be extrapolated to the number in any volume of tissue without error" (Abercrombie, 1946). The average number of nuclear points, representing the corrected number, is calculated by the formula: $N^C = N^U x(T/(P+T))$. N^C being the corrected number, N^U the uncorrected number, T the thickness of the section (8μm in this case), and P the mean nuclear diameter of the neurons, which varied depending on the ganglion, age and genotype of the embryo and was determined for each individual count (Range: 6-12μm). Nuclei whose nuclear-points are outside the section but which are nevertheless represented inside the section, have their nuclear-points extending through a volume of tissue of (on average) the thickness of the mean nuclear diameter, as shown in Figure 2.12 (Abercrombie, 1946). Below this Figure is an explanation of the derivation of the formula for Abercrombie correction. The mean diameter of the neurons (P) was determined by drawing a minimum of 100 nuclei per ganglion using a camera lucida at x40 magnification, together with a scale bar of known size. These drawings were then scanned and analysed on a Macintosh

computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). After setting the scale of the drawing using the known scale bar as a reference, individual nuclei were selected using the 'magic wand' tool in the program and measured. This returned the mean nuclear diameter of the nuclei, enabling correction.

Figure 2.12: Derivation of Abercrombie correction



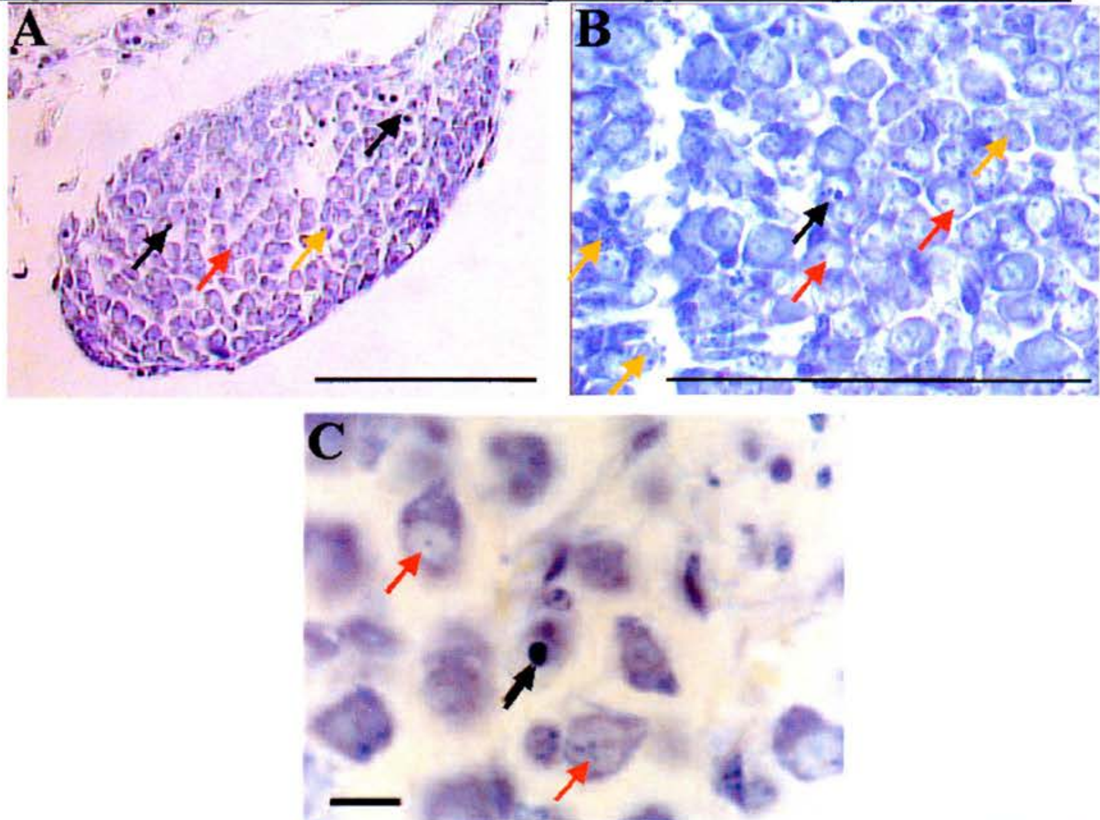
Diagrammatic side view of a microtome section that lies between the two horizontal lines. Two spherical nuclei of equal radius are so placed that they touch the surfaces of the sections X and Y. Any nucleus of this radius will be represented in this section if its center lies anywhere between W and Z (area designated by dotted line). Since a random distribution of nuclei can be assumed between the two indicated points, the proportion of all nuclei represented in the section whose centers lie within the section is given by XY/WZ, i.e. the section thickness/(section thickness + nuclear diameter) (from Abercrombie, 1946).

2.6.3 Counting of pyknotic cells

Pyknotic neurons were counted using CFV-stained slides. The total number of pyknotic neurons was counted, as identified by the large purple blebs of clumped

chromatin in a large pale nucleus surrounded by stained cytoplasm (figure 2.7). All the pyknotic neurons were counted on every fifth section of the ganglion at x40 magnification. The sum of these was then multiplied by five to obtain an approximation of the total number of pyknotic neurons in the ganglion. Pyknotic neurons were not counted in the SCG ganglia, since the density and small size of the neurons (see Figure 2.13) would make accurate identification of pyknotic neurons impossible. Due to their relatively small size, the counts of blebs of clumped chromatin are less likely to be found in more than one section, resulting in little bias and making Abercrombie's correction unnecessary for pyknotic neuron counts (Dr. Lucia Piñon, personal communication).

Figure 2.13: Illustration of CFV staining and appearance of pyknotic neurons



(A) Appearance of an E12 CFV-stained dorsal root ganglion at x400 magnification. Scale: 200μm (B) Appearance of E16 CFV-stained neurons at x1000 magnification. Scale: 100μm (C) Appearance of E19 CFV-stained neurons at x1000 magnification. Scale: 20μm (from Piñon, et al., 1996). Black arrows indicate the large purple blebs of clumped chromatin in a pale nucleus surrounded by stained cytoplasm, which identify pyknotic neurons. These can be distinguished from other neurons, with their less intensively stained nucleolus (red arrows) (Konigsmark, 1970). Also indicated are the smaller non-neuronal cells (orange arrows) with their smaller and darker nuclei.

2.7 Statistical Analysis

2.7.1 Statistical variables

Variables are the different classes of information of a data set. Variables that are manipulated by the experimenter are known as 'independent variables', and variables that are measured in an experiment are called 'dependent variables', as they dependent on the independent ones. In choosing statistical test to evaluate whether data sets come from different populations or if they do not (null hypothesis), it is important to identify the type of variable the data represents. Variables can be either quantitative or qualitative. Qualitative variables are concerned with classes or categories, such as age or gender. As the name suggests, quantitative variables measure quantities, i.e. amounts or counts. The data in this thesis are quantitative. Quantitative data can be further divided into discrete and continuous variables. Discrete variables consist of isolated points whose values are separated from each other. Counting variables are an example, as they will always be an integer such as 1, 2, 4, etc. and can never be 2.12 for instance. Continuous variables can take on any possible value within the limits of the variable range, and includes data such as length, speed or weight (Cann, 2003).

The first dependent variable measured is the expression of mRNA relative to the expression of a housekeeping gene. This measurement is quantitative, since it measures the absolute amount of something (by comparison to known amounts of competitor). Further, the variable is continuous, since it can take on any possible value within the limits of the variable range.

The second dependable variables measured are the counts of neuronal and pyknotic nuclei. Counts are strictly speaking discrete variables, since one cannot possibly get half a neuron. However, the counts of neuronal nuclei obtained in these experiments are estimates of numbers rather than counts, with values such as 4495.817 neurons in an E13 DRG as an example (following Abercrombie correction). Since the combination of the estimated numbers with the Abercrombie correction could theoretically give rise to any value within the limits of the variable range, estimates of neuronal nuclei can be considered a continuous variable. Pyknotic neuron estimates by contrast are not corrected and are therefore only able to take on discrete values.

Before making comparison between groups of data the significance level needs to be set, where a difference between the groups is considered to be significant. This was set at $p \leq 0.05$ (5%).

2.7.2 Parametric versus non-parametric

There are two types of statistical tests, depending on the nature of the data: parametric and non-parametric tests. Parametric tests depend on estimates of the parameters of populations or probability distributions. These tests include the student's t-test, ANOVA (analysis of variance) and correlation analysis. These tests are only meaningful for continuous data that can be meaningfully added and is sampled from a population where an underlying normal distribution is assumed, or whose distribution can be rendered normal by mathematical transformation (Lewis and Traill, 1999; Cann, 2003). Non-parametric tests use ranks and require fewer

assumptions about a population or probability distribution and are applicable in a wider range of situations (Cann, 2003). These tests include the Mann-Whitney test and the Kruskal-Wallis test. When both parametric and non-parametric tests are applicable, statisticians usually recommend using parametric methods because they tend to provide better precision. Parametric tests make use of more of the available data and are more 'powerful', i.e. they are less likely to reject the null hypothesis when it is false (Lewis and Traill, 1999; Cann, 2003).

As stated, parametric methods require continuous data, hence the pyknotic neuron counts, which are discrete data, cannot be analysed using parametric methods and require the use of non-parametric tests.

Data points need to be suitable for meaningful addition and other calculations (Lewis and Traill, 1999), which can be problematic with relative measurements, such as the ones in the competitive RT-PCR experiments. However, such operations can be performed with relative measurements as long as the denominator (bottom line) remains the same (Lewis and Traill, 1999), which is the case here, as all measurements were relative to 1pg of GAPDH.

In the chapters examining neuronal counts some data are expressed as percentages and ratios. Ratio and percentage measurements are special cases, in that percentages from 0 to 100% or proportions from 0 to 1 form a binominal distribution, rather than a normal one. The deviation from normality tends to be greater for small or large percentages (Zar, 1999). Non-parametric statistics were used on these types of data, as they do not have a normal distribution.

2.7.3 Parametric tests

The Student's t-test is a parametric test that compares the means of two groups of samples by calculating the probability of there being no difference between them (null hypothesis). The smaller this probability, the higher the likelihood that there is a difference. This test, devised by William Gosset ('Student') in 1908 (cited in Cann, 2003), has two variations: one for paired samples and one for unpaired or independent samples. The version used here is the one for unpaired samples.

The t-test can only be used to compare two groups. If more than two groups have to be compared, other tests must be used, such as the analysis of variance between groups (ANOVA). A different test is necessary for more than two groups, because the multiple pair-wise comparisons (such as t-tests) that would have to be used increase the possibility of committing a type one error (rejection of the null hypothesis erroneously). ANOVA can compare the means of three or more samples, thus overcoming this problem (Cann, 2003). ANOVA is similar to the t-test, calculating the probability of there being no difference between group means. There are different versions of ANOVA, depending whether more than one type of variable is being assessed. When more than one type of response to a treatment is assessed, a two-way ANOVA is used, whereas a one-way ANOVA is performed when only one type of variable is being considered. In the experiments presented in this thesis only one type of variable was measured, allowing the use of a one-way ANOVA.

An ANOVA only indicates whether there is a difference between at least one of the means of groups, not giving information between which of the groups the differences lie. Post-hoc tests (also known as multiple comparison tests) that make pair-wise comparisons are required to establish this. There are a variety of post-hoc tests that

can be used for ANOVA. The post-hoc employed here was the Fisher's LSD (least significant difference) procedure, which performs all possible comparisons of group means. Fisher's LSD was preferred to the Tukey HSD (honestly significant difference) test, as it does not require equal sample sizes (Kirk, 1982).

A parametric statistic used to compare the correlation between variables, such as nuclear and neuronal size, is the Pearson's correlation coefficient. This test is a measure of the strength of the association between two variables. It returns a measure of the linear relationship between two variables, which ranges from $r = +1$ (perfect positive linear relationship) to $r = -1$ (perfect negative linear relationship).

2.7.4 Non-parametric tests

Non-parametric tests are useful in situations where the assumptions required by parametric tests appear questionable. The non-parametric equivalent to the t-test is the Mann-Whitney U test. It uses the median as a measure of central tendency, rather than the mean used by parametric methods, and is based on the ranking of measurements, with both groups being considered jointly (pooled) for ranking (Wardlaw, 2000). The non-parametric equivalent to the one-way ANOVA is the Kruskal-Wallis test, which is a generalisation of the Mann-Whitney U test. If significant differences are detected using the Kruskal-Wallis test, these need to be investigated using post-hoc tests. The test used here was the Dunn's Multiple Comparisons Test.

The graphs and statistical analysis presented in this thesis were done using the computer software Minitab® 14.12.0 (Minitab Inc.), StatView® 4.57 (Abacus Concepts Inc.) and Microsoft® Excel 2000.

CHAPTER III

Intrinsic and extrinsic mechanisms in the control of trkA expression

3.1 Introduction

Precise temporal and spatial regulation of gene expression are key features of neuronal development. The timing of neurotrophin responsiveness, for example, is tightly controlled in developing sensory neurons. Neurotrophin independence and the timing of neurotrophin survival upon target field innervation appear to be controlled by an intrinsic timing mechanism in placode-derived sensory neurons (Davies and Vogel, 1991; Vogel and Davies, 1991). Similarly, other populations of sensory neurons switch their dependence from one set of neurotrophins to another during an early stage in development (e.g. Buchman and Davies, 1993; Buj-Bello et al., 1994; Piñon et al., 1996), the switch being controlled in part by cell-cell interactions within the ganglion (Enokido et al, 1999).

The trigeminal ganglion comprises populations of neurons and supporting cells that have been thoroughly studied in relation to the periods of neurotrophin independence, and dependence on particular neurotrophic factors in relation to the timing of target field innervation (Davies, 1997). In vitro studies have demonstrated that these neurons are initially neurotrophin independent, before becoming transiently dependent on BDNF and NT-3 at the onset of target field innervation. Subsequent to target field innervation they become dependent on NGF, whose limited supply is believed to govern developmental cell death in this population of neurons (Buchman and Davies; Paul and Davies, 1995). The expression of trk receptors mirrors these changes in neurotrophin dependence. TrkB and trkC (BDNF and NT-3 high affinity receptors respectively) are highly expressed in the trigeminal ganglion at early developmental stages, whereas trkA (NGF high affinity receptor) is

expressed at later stages (Ernfors et al., 1992; Arumae et al., 1993; Wyatt and Davies, 1993). Consistent with this pattern of expression and the sequential action of neurotrophins, more neurons undergo cell death in *trkB*-deficient embryos early in development, whereas more neurons are lost in *trkA*-deficient mice at later stages in development (Piñon et al., 1996; chapter 5).

The trkA receptor is of particular interest since NGF signalling through trkA is believed to regulate the survival of the majority of neurons during the period of developmental cell death. The expression of trkA begins shortly after the commencement of target field innervation (~E11/E12 in the mouse) and increases in the early stages of target field innervation (Wyatt and Davies, 1993). Some of the mechanisms controlling trkA have been found to be intrinsic, or regulated by cell-cell interactions within the ganglion (Wyatt and Davies, 1993; Enokido et al., 1999). The study presented in this chapter was concerned with establishing whether mechanisms intrinsic to the ganglion control the entire developmental programme of trkA expression. The developmental time-course of trkA expression has been examined previously (Wyatt and Davies, 1993). There has, however, been no detailed study over the time-period of trkA increase in vitro, assessing the importance of intrinsic mechanisms. The study presented here addressed this issue by culturing intact trigeminal ganglia at different ages of development to ascertain whether the direction and magnitude of changes in trkA expression in culture mirrored in vivo changes. Using this approach, it appeared that changes in trkA expression are strongly regulated by intrinsic mechanisms from within the ganglion. To investigate whether the central (hindbrain) and peripheral (maxillary process)

target fields of the trigeminal ganglion influence trkA expression, early ganglia were cultured with or without these target fields. Surprisingly, it was found that the presence of both the peripheral and/or central target fields reduced trkA expression in the ganglion at E11 due to the release of a diffusible substance. Such an effect was also detected at subsequent ages, although to a lesser extent. The developmental significance of this effect is unclear.

3.2 Results

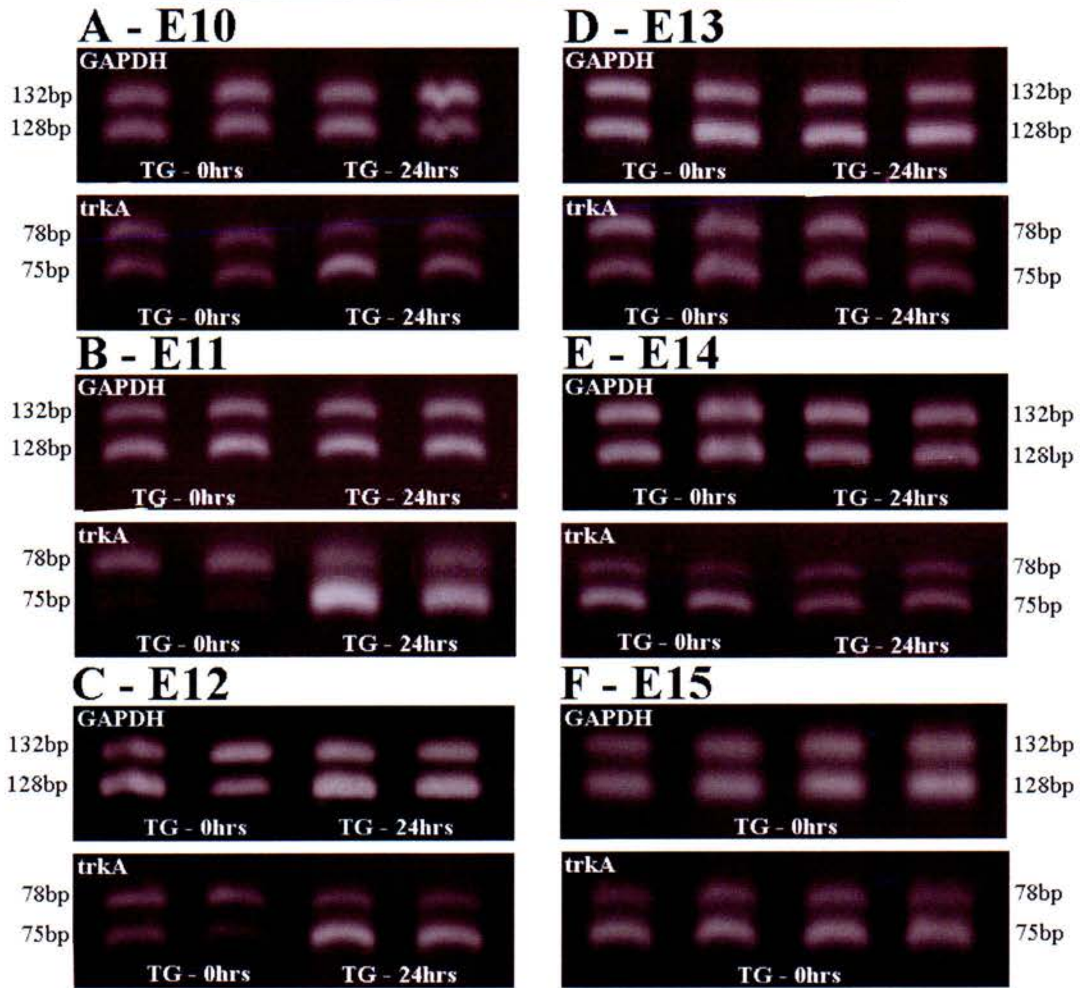
3.2.1 trkA expression in cultured trigeminal ganglia

To assess whether there is an intrinsic mechanism within the trigeminal ganglion that regulates expression of trkA during development, trigeminal ganglia were grown by themselves for 24hrs and the expression of trkA mRNA was compared to the expression seen in vivo at the corresponding age.

The ganglia were grown with the neurotrophins (NGF, BDNF, and NT-3), to maximize their survival. TrkA mRNA levels were expressed relative to GAPDH mRNA to correct for any differences in the size/mass of dissected ganglia or differences in the yield of mRNA from extractions. Examples of gels assessing relative trkA mRNA levels after 24hrs in culture are shown in Figure 3.1.

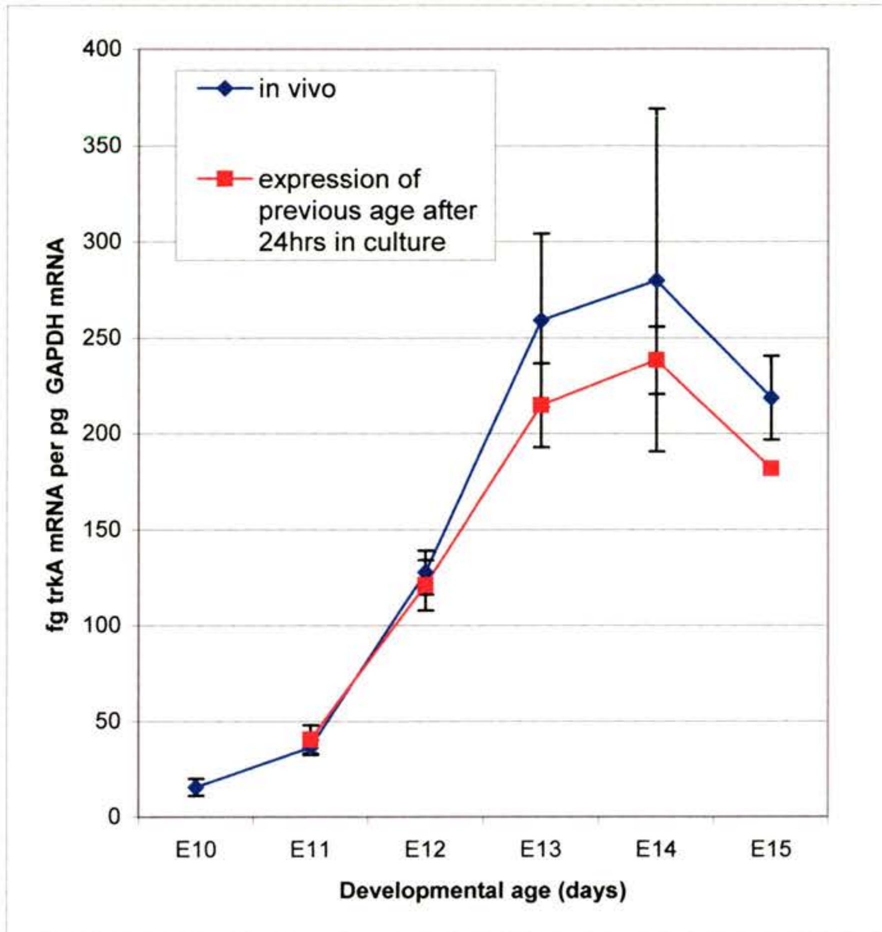
Figure 3.2 shows that very similar changes in trkA mRNA expression occurred in the trigeminal ganglia cultured for 24 hours, as took place over the same 24 hours in vivo (there were no significant differences in the levels of trkA at the equivalent in vitro and in vivo stages at all the time points studied (t-test; $p>0.05$ at E11, E12, E13, E14 and E15)). This suggests that trkA expression in the trigeminal ganglion is regulated at least in part by factors, cell-cell interactions or genetic programmes that are intrinsic to the ganglion, and are not controlled by extrinsic factors released from their target fields or tissues. Thus both the increase in trkA expression during the period of target innervation from E11 to E13, and the decrease between E14 and E15 associated with developmental cell death appear to be predetermined within the ganglion.

Figure 3.1: Examples of gels showing relative trkA expression in the trigeminal ganglion in vivo and after 24 hrs in vitro



SYBR®Gold (Molecular Probes) stained gels showing the products of RT-PCR reactions amplified with either GAPDH-specific or trkA-specific primers. (A) Reactions containing 62,5fg of the GAPDH competitor RNA (132bp) and 2.1fg of the trkA competitor (78bp) plus total RNA from E10 trigeminal ganglia (TG-0hrs) or E10 trigeminal ganglia cultured for 24 hrs (TG – 24hrs) (GAPDH: 128bp; trkA: 75bp). (B) Reactions containing 300fg of the GAPDH competitor RNA (132bp) and 30fg of the trkA competitor (78bp) plus total RNA from E11 trigeminal ganglia (TG-0hrs) or E11 trigeminal ganglia cultured for 24 hrs (TG–24hrs) (GAPDH: 128bp; trkA: 75bp). (C) Reactions containing 520fg of the GAPDH competitor RNA (132bp) and 104fg of the trkA competitor (78bp) plus total RNA from E12 trigeminal ganglia (TG-0hrs) or E12 trigeminal ganglia cultured for 24 hrs (TG–24hrs) (GAPDH: 128bp; trkA: 75bp). (D) Reactions containing 625fg of the GAPDH competitor RNA (132bp) and 167fg of the trkA competitor (78bp) plus total RNA from E13 trigeminal ganglia (TG-0hrs) or E13 trigeminal ganglia cultured for 24 hrs (TG–24hrs) (GAPDH: 128bp; trkA: 75bp). (E) Reactions containing 833fg of the GAPDH competitor RNA (132bp) and 208fg of the trkA competitor (78bp) plus total RNA from E14 trigeminal ganglia (TG-0hrs) or E14 trigeminal ganglia cultured for 24 hrs (TG–24hrs) (GAPDH: 128bp; trkA: 75bp). (F) Reactions containing 937fg of the GAPDH competitor RNA (132bp) and 167fg of the trkA competitor (78bp) plus total RNA from E15 trigeminal ganglia (TG-0hrs) (GAPDH: 128bp; trkA: 75bp).

Figure 3.2: trkA expression in the trigeminal ganglion in vivo compared to the expression in the ganglion grown for 24 hrs in vitro



Expression of trkA in trigeminal ganglia after 24hrs in culture with growth factors (purple line), or in vivo at the age corresponding to 24hrs after the ganglia where put into culture (blue line). (i.e. the first in vitro data point at E11 is the level of trkA expressed in E10 ganglia cultured for 24 hrs and so on). Bars represent the mean ± standard error (n=4-16 per data point).

3.2.2 Effect of the peripheral and central target fields of the trigeminal ganglion on trkA expression

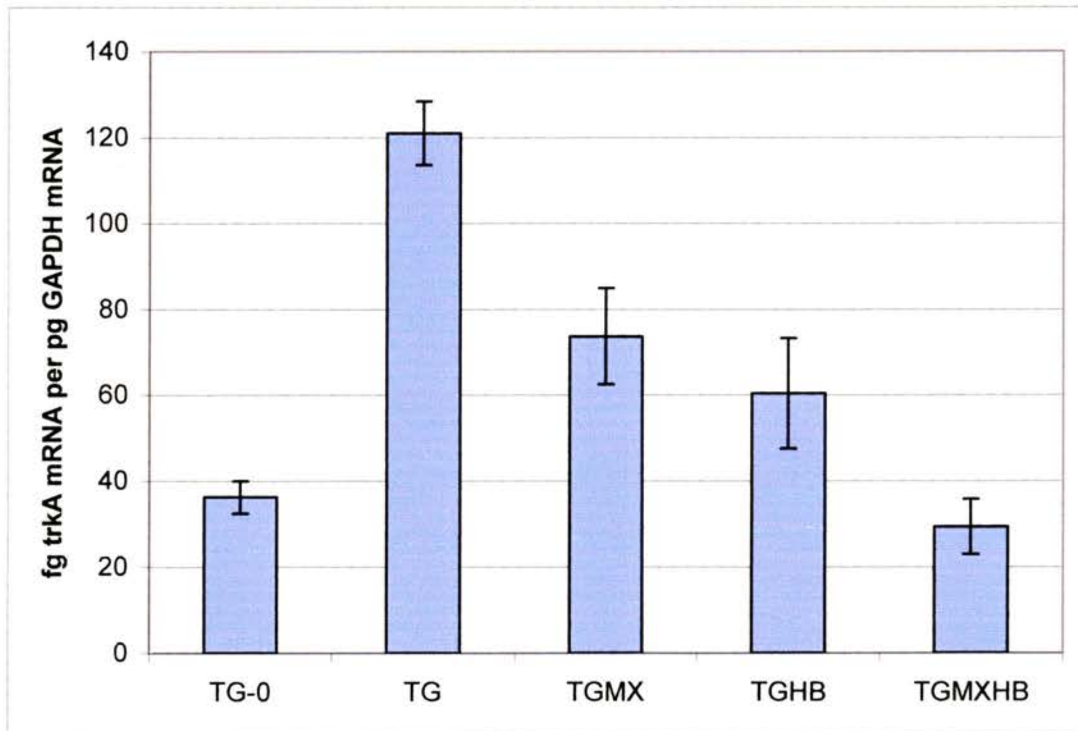
To ascertain whether the target fields of the trigeminal ganglion affect trkA expression, E11 ganglia were grown with or without their target fields in culture for 24 hours. As above, NGF, BDNF and NT-3 were added to the medium to ensure maximal survival of neurons in both experimental conditions. Subsequent experiments demonstrated that these factors have no influence on the results presented below (preliminary data shown in appendix – Figure A.1). This may be related to the large proportion of neurons that survive independently of neurotrophins at this stage (Piñon et al., 1996; chapter 5). The age of E11 was chosen for the following reasons: 1. E11 is the stage at which a marked increase in trkA receptor expression is first observed (Figure 3.3). 2. It coincides with the onset of target innervation in vivo (Davies and Lumsden, 1984). 3. It lies immediately prior to the main period of 'switching' in neurotrophin dependence of the neurons in the ganglion (Buchman and Davies, 1993).

The target fields used were the hindbrain, the central target field of the trigeminal ganglion, and the maxillary process, its most densely innervated peripheral target field. Ganglia and target fields were dissected with their neural connections intact as described in chapter 2 (Figure 2.4A, Figure 3.4).

An ANOVA revealed significant differences between the trigeminal ganglion grown alone and with its target fields attached ($p<0.0001$). Paradoxically, it was found that the presence of either of these target fields resulted in a reduction in the expression levels of trkA (Figure 3.3). Whereas expression in the ganglion reached in vivo levels when grown alone (TG), the attached maxillary process (TGMX) and

hindbrain (TGHB) reduced trkA mRNA in the ganglion. The above targets reduced trkA mRNA levels in the trigeminal ganglion by almost 40% (Fisher's LSD; $p<0.001$) and 50% (t-test; $p<0.0001$), respectively, and with both target fields attached (TGMXHB) there was a 75% reduction (Fisher's LSD; $p<0.0001$), maintaining trkA mRNA to levels observed in ganglia collected prior to cultures (TG-0).

Figure 3.3: trkA expression in the E11 trigeminal ganglion after 24 hrs in culture without or with its peripheral and central targets attached

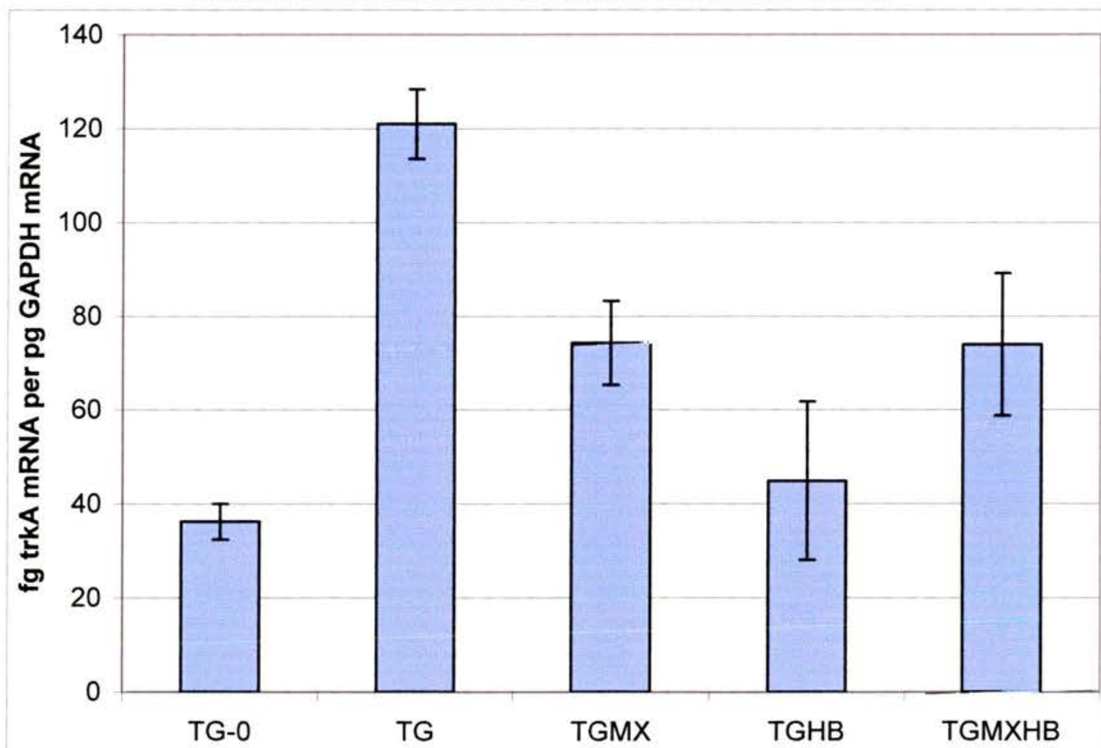


Expression of trkA in E11 trigeminal ganglia after 24hrs in culture with NGF, BDNF and NT-3. TG-0: Expression in ganglia before culture. TG: Expression in the ganglion grown on its own. TGMX: Expression in the ganglion grown with the maxillary process. TGHB: Expression in the ganglion grown with the hindbrain. TGMXHB: Expression in the ganglion grown with both target fields. Bars represent the mean ± standard error (n=6-16 per data point).

3.2.3 The target fields effect reductions in trkA expression by acting through a diffusible substance

To determine whether the unexpected in vitro effect of the target fields on trkA expression was due to physical connections between the trigeminal ganglion and its targets or due to a diffusible substance being released from the targets, E11 ganglia and their central and peripheral targets were dissected separately and placed back into contact (see Figure 2.4B, Figure 3.5) or left at a slight distance of approximately 500μm (figure 2.4C, figure 3.6). This was done by stabilizing the structures in Matrigel® for the 24 hour culture period.

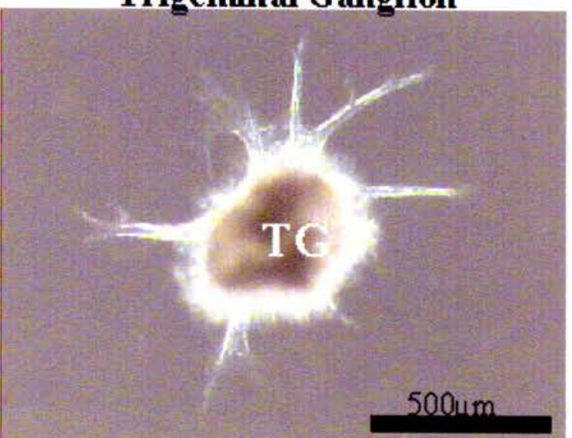
Figure 3.7: trkA expression in E11 trigeminal ganglia after 24 hrs in culture without their target fields or with their peripheral and central target fields separated and repositioned in contact



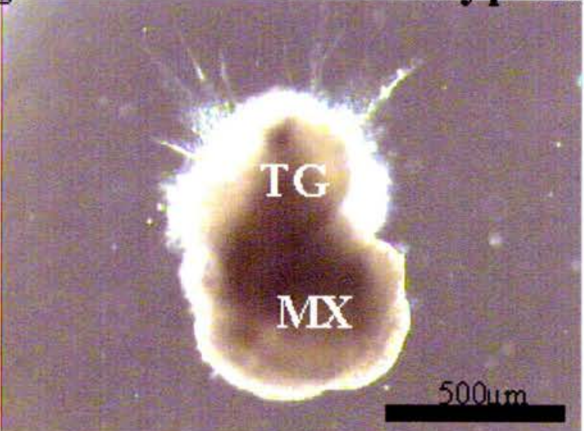
Expression of trkA in E11 trigeminal ganglia after 24hrs in culture with NGF, BDNF and NT-3. TG-0: Expression in ganglia before culture. TG: Expression in the ganglion grown on its own. TGMX: Expression in the ganglion grown with the maxillary process. TGHB: Expression in the ganglion grown with the hindbrain. TGMXHB: Expression in the ganglion grown with both target fields. Bars represent the mean ± standard error (n=4-16 per data point).

Figure 3.4: **The trigeminal ganglion cultured by itself or attached to its target fields as in figure 2.8A**

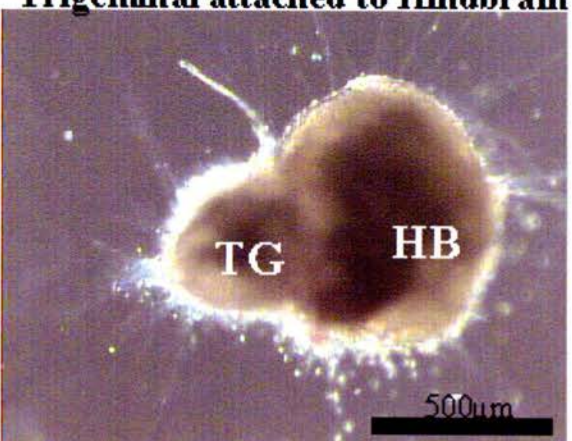
Trigeminal Ganglion



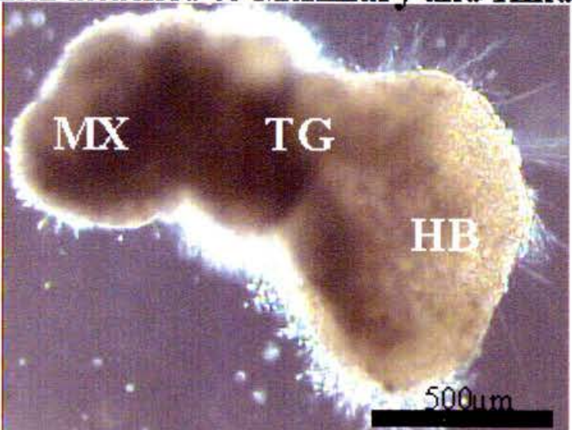
Trigeminal attached to Maxillary process



Trigeminal attached to Hindbrain



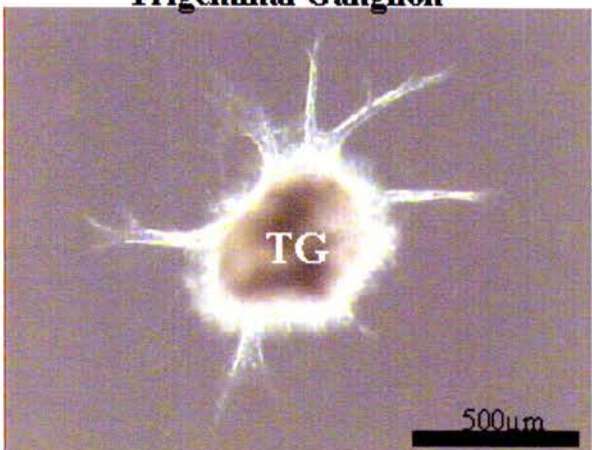
Trigeminal attached to Maxillary and Hindbrain



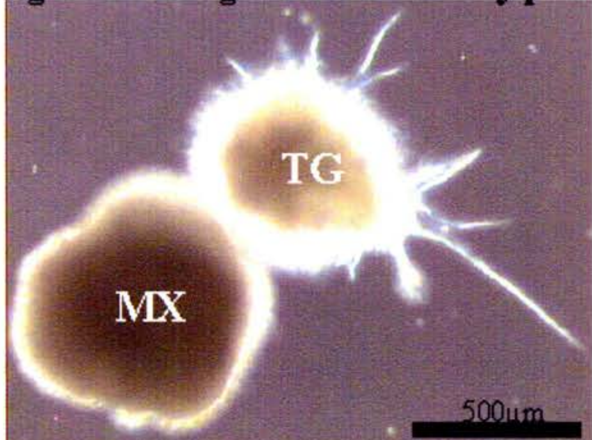
TG: Trigeminal, MX: Maxillary process, Hb: Hindbrain.

Figure 3.5: **The trigeminal ganglion cultured by itself or with its separated target fields in proximity**

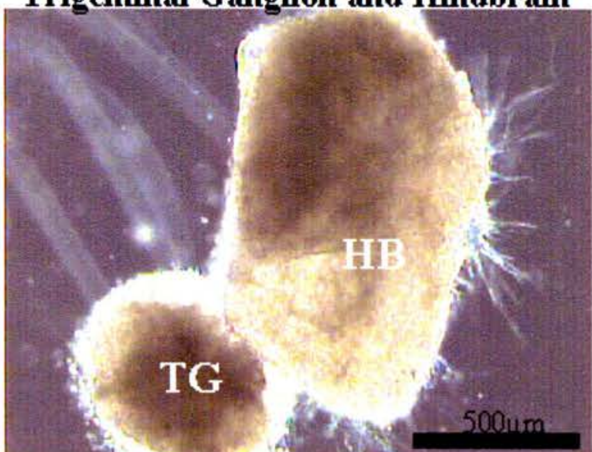
Trigeminal Ganglion



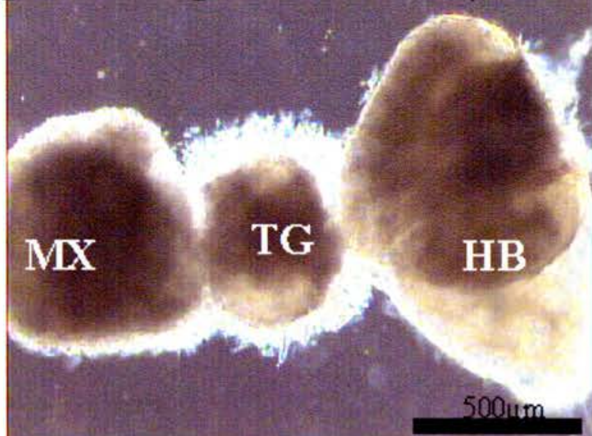
Trigeminal Ganglion and Maxillary process



Trigeminal Ganglion and Hindbrain



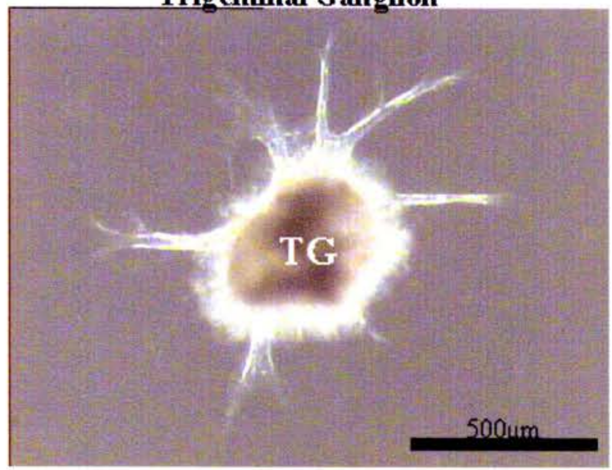
Trigeminal Ganglion and Maxillary and Hindbrain



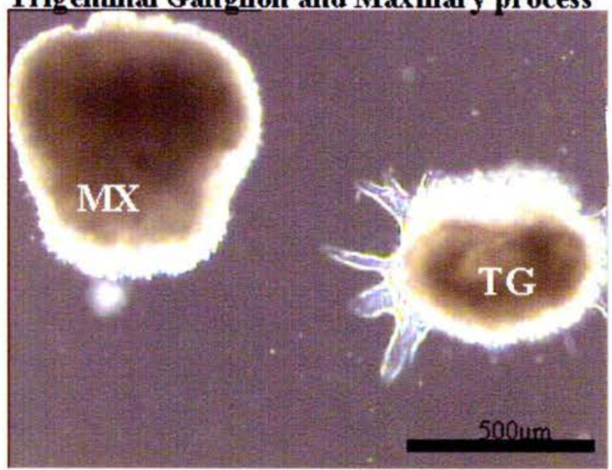
TG: Trigeminal, MX: Maxillary process, Hb: Hindbrain.

Figure 3.6: The trigeminal ganglion cultured by itself or with its separated target fields at a slight distance

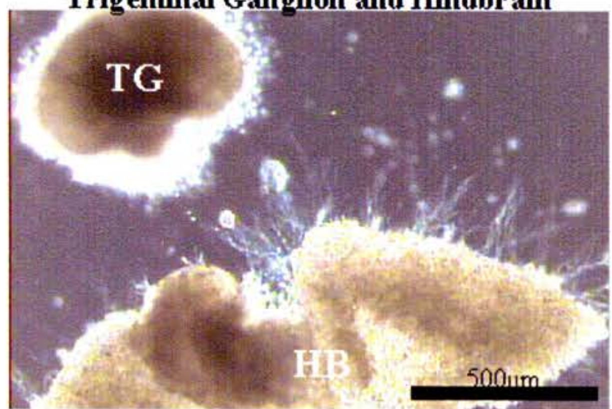
Trigeminal Ganglion



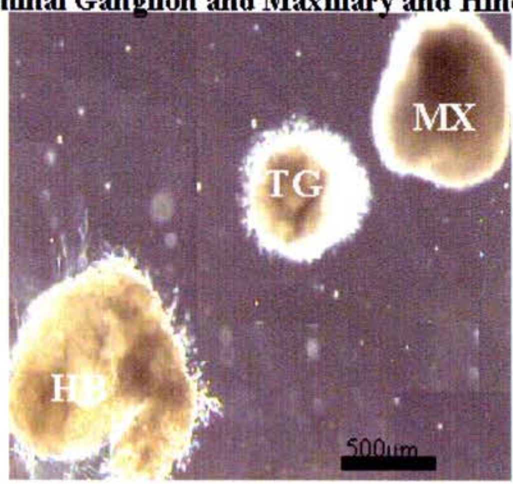
Trigeminal Ganglion and Maxillary process



Trigeminal Ganglion and Hindbrain



Trigeminal Ganglion and Maxillary and Hindbrain



TG: Trigeminal, MX: Maxillary process, Hb: Hindbrain.

When the target fields were separated and placed back in contact with the trigeminal ganglion (Figure 3.7), there were still significant effects (ANOVA; $p<0.0001$). The maxillary process still mediated a 40% reduction in trkA mRNA levels (Fisher's LSD; $p<0.05$), whereas the hindbrain mediated a 60% reduction (Fisher's LSD; $p<0.001$) and both together a 40% reduction (Fisher's LSD; $p<0.01$). Thus neural connections to the target field were not needed for the effect to occur. Indeed, effects were almost as strong as with intact connections. While some of the effect may be explained by re-growth of axons into the target field, the strength of the remaining effect would suggests that at least some of it was mediated by a diffusible substance.

When the target fields were separated and left at a slight distance from the trigeminal ganglion (Figure 3.8), the effects were greatly reduced, although some effect remained (ANOVA; $p<0.0001$). When the ganglion was grown with the hindbrain (20% reduction; Fisher's LSD; $p<0.001$) or both target fields (25% reduction; Fisher's LSD; $p<0.05$), the reduction in trkA mRNA levels was still within significance limits. The maxillary process had no significant effect (Fisher's LSD; $p>0.05$)

The above results indicate that the substance mediating the effect of the target fields on trkA expression does not require neuronal connections to the ganglion and is diffusible. Preliminary studies using conditioned medium support these observations, with hindbrain homogenate reducing trkA expression in trigeminal ganglia (see appendix – Figure A.2).

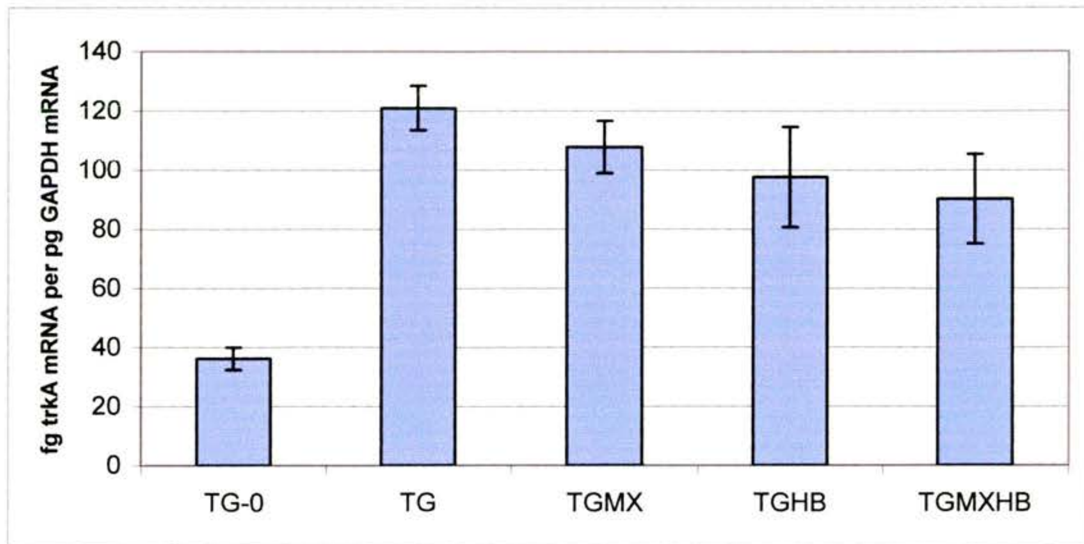
To ensure that the substance reducing trkA expression was expressed in the target fields in vivo, and not induced in vitro due to some effect of the dissection or the

culture medium, homogenates of E11 maxillary processes and hindbrains were prepared as described in chapter 2 (2.2.2). The supernatant was diluted to different extents in fresh medium. The results of this experiment can be seen in Figure 3.9.

The homogenate supernatant also reduced trkA expression in the trigeminal ganglion (ANOVA; $p<0.0005$), indicating that the substance presumably released in vitro appears to be synthesized in vivo as well. All dilutions of homogenate studied reduced trkA expression by 40-60%, although these reductions were not significant for diluted (50% and 10%) maxillary process homogenates (Fisher's LSD; $p>0.05$). Both 100% maxillary process and all hindbrain homogenates displayed significant reductions in trkA expression (ANOVA followed by Fisher's LSD; $p<0.05$), Different homogenate concentrations were not significantly different from each other (Fisher's LSD; $p>0.05$). The effects of the homogenates suggests that the diffusible substance mediating reductions in trkA mRNA levels is produced in vivo. Homogenate studies could provide a starting point for the isolation of such a substance.

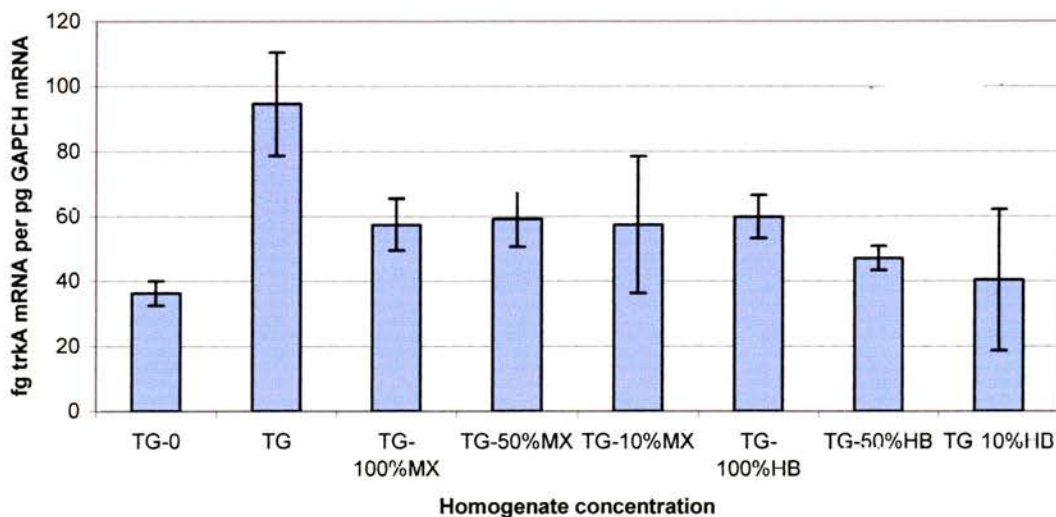
Another interesting observation made in cultures grown with target field homogenate was the almost complete absence of neurite outgrowth from the ganglion when grown with homogenate of either target field (Figure 3.10). This was observed in all the homogenate cultures at every homogenate concentration, and may indicate the presence of substances in the maxillary processes and hindbrains that would normally prevent neurons from growing further once they reach their target field.

Figure 3.8: trkA expression in E11 trigeminal ganglia after 24 hrs in culture without their target fields or with its peripheral and central target fields separated and repositioned at a distance



Expression of trkA in E11 trigeminal ganglia after 24hrs in culture with NGF, BDNF and NT-3. TG-0: Expression in ganglia before culture. TG: Expression in the ganglion grown on its own. TGMX: Expression in the ganglion grown with but separated from the maxillary process. TGHB: Expression in the ganglion grown with but separated from the hindbrain. TGMXHB: Expression in the ganglion grown with but separated from both target fields. Bars represent the mean ± standard error (n=6-16 per data point).

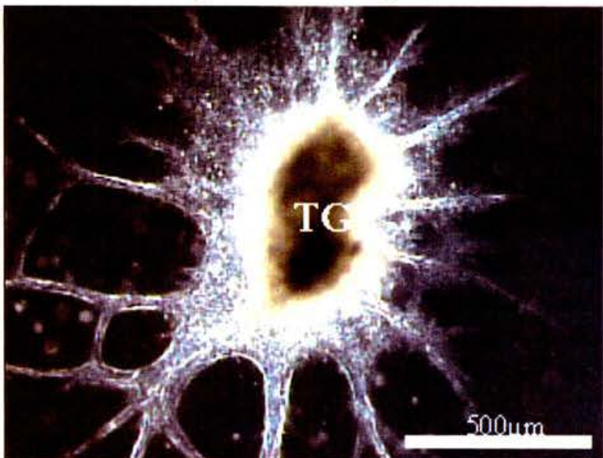
Figure 3.9: trkA expression in E11 trigeminal ganglia after 24 hrs in culture with different concentrations of maxillary process and hindbrain homogenate



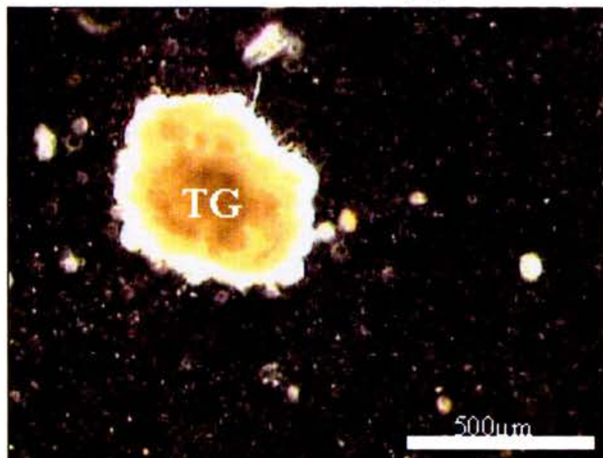
Expression of trkA in E11 trigeminal ganglia after 24hrs in culture with NGF, BDNF and NT-3 in maxillary and hindbrain homogenate. TG-0: Expression in ganglia before culture. TG: Expression in the ganglion grown on its own in normal medium. TG-x%MX: Expression in the ganglion grown with maxillary process homogenate. TG-x%HB: Expression in the ganglion grown with different concentrations of hindbrain homogenate. Bars represent the mean ± standard error (n=2-11 per data point).

Figure 3.10: The trigeminal ganglion cultured by itself in normal medium, or in maxillary process or hindbrain homogenate

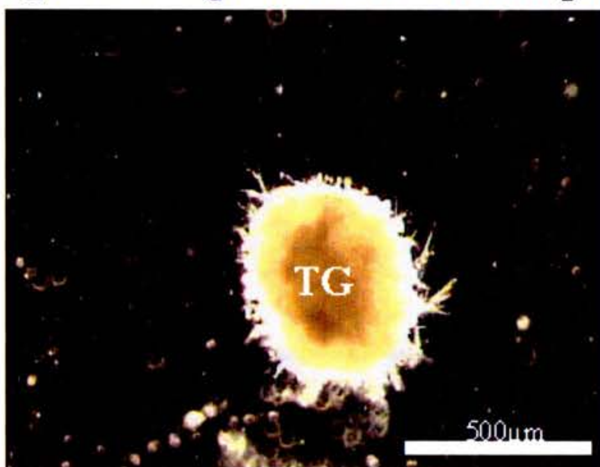
Trigeminal Ganglion



Trigeminal Ganglion in Maxillary process homogenate



Trigeminal Ganglion in Hindbrain homogenate



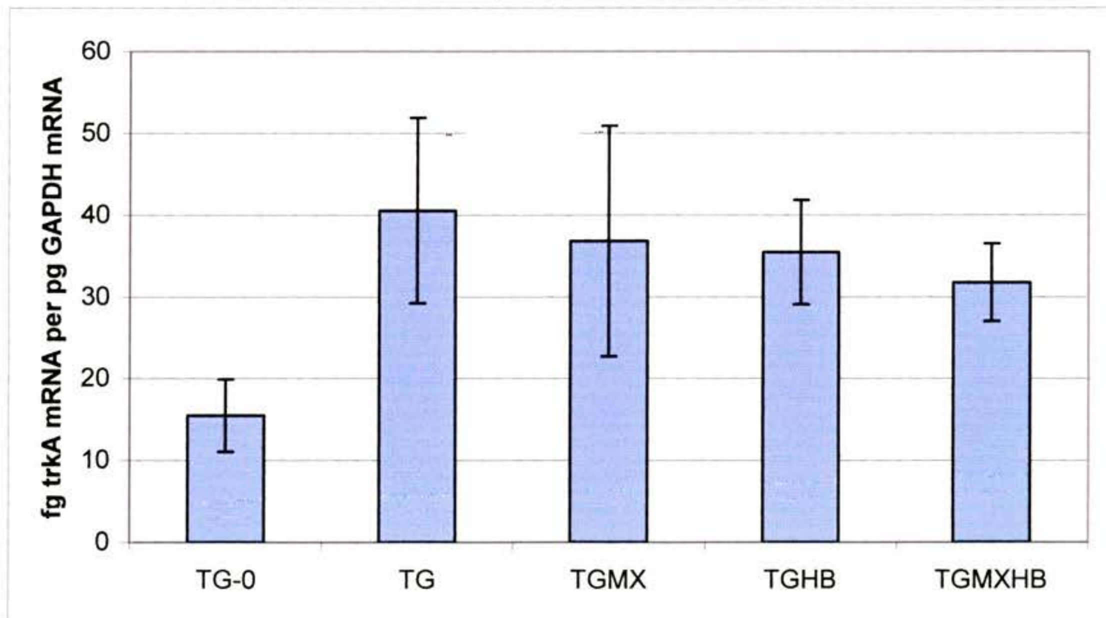
3.2.4 Age-related regulation of trkA expression

To investigate whether the reduction of trkA mRNA by the central and peripheral target fields was confined to the age of E11 or if it applied to other ages, trigeminal ganglia were grown with their target fields at E10, and at E12 to E15. These cultures were performed with intact neural connections between target field and ganglion, as seen in Figure 2.4A (chapter 2). NGF, BDNF and NT-3 were added to the medium to ensure maximal survival of neurons in both experimental conditions at all ages.

Figure 3.11 shows trkA mRNA levels in E10 cultures after 24hr incubation. E10 cultures proved difficult to study due to the tendency of the ganglion to dissociate in culture. Some ganglia could be grown, but parts of the ganglia may have been lost in some instances, explaining the unusually large variations obtained (large error bars). The E10 data should thus be considered with caution. It was observed that there was no significant regulatory effect of the target field on trkA expression at E10 (ANOVA; $p>0.05$), although as stated, this may be an artefact due to difficult culture conditions. As already established in section 3.2.2 (Figure 3.3), at E11 there were ~42% and ~50% reductions in trkA expression in the maxillary process and hindbrain, respectively, with both together resulting in a ~75% reduction compared to the trigeminal ganglion alone. Figure 3.12 shows trkA expression in E12 trigeminal ganglia prior to culture and after 24 hours with or without their central and peripheral targets. An ANOVA indicated there were significant differences between the sample groups (ANOVA; $p<0.0005$). No significant effect of the maxillary process on trkA mRNA levels could be observed at this age (Fisher's LSD; $p>0.05$), whereas a ~25% reduction was maintained with the hindbrain (Fisher's LSD; $p<0.05$) and with both target fields (~50%; Fisher's LSD; $p<0.0001$). Figure 3.13

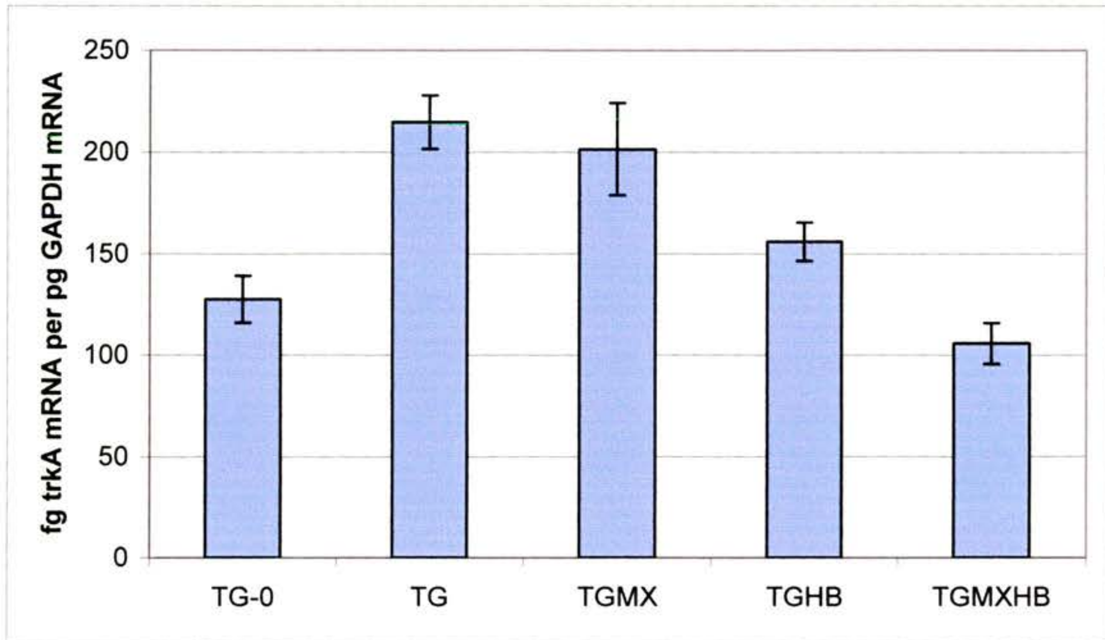
displays trkA expression in cultures from trigeminal ganglia cultured in the same manner at the age of E13 (ANOVA; $p<0.01$). There was no significant effect of the maxillary process (Fisher's LSD; $p>0.05$), with a remaining effect with the hindbrain (Fisher's LSD; $p<0.05$)) or both target fields attached (Fisher's LSD; $p<0.01$). At E14 (Figure 3.14) and E15 (Figure 3.15) no significant effects of the peripheral and central target field on trkA mRNA levels in the trigeminal ganglion after 24 hrs in culture could be observed (E14: ANOVA; $p>0.05$). There was a significant decrease in trkA expression in the trigeminal ganglion compared to levels before culture however, an effect that was found both with the trigeminal ganglion alone and with the trigeminal ganglion grown with its target fields (ANOVA followed by Fisher's LSD; $p<0.05$)

Figure 3.11: trkA expression in E10 trigeminal ganglia after 24 hrs in culture without or with their peripheral and central target fields attached



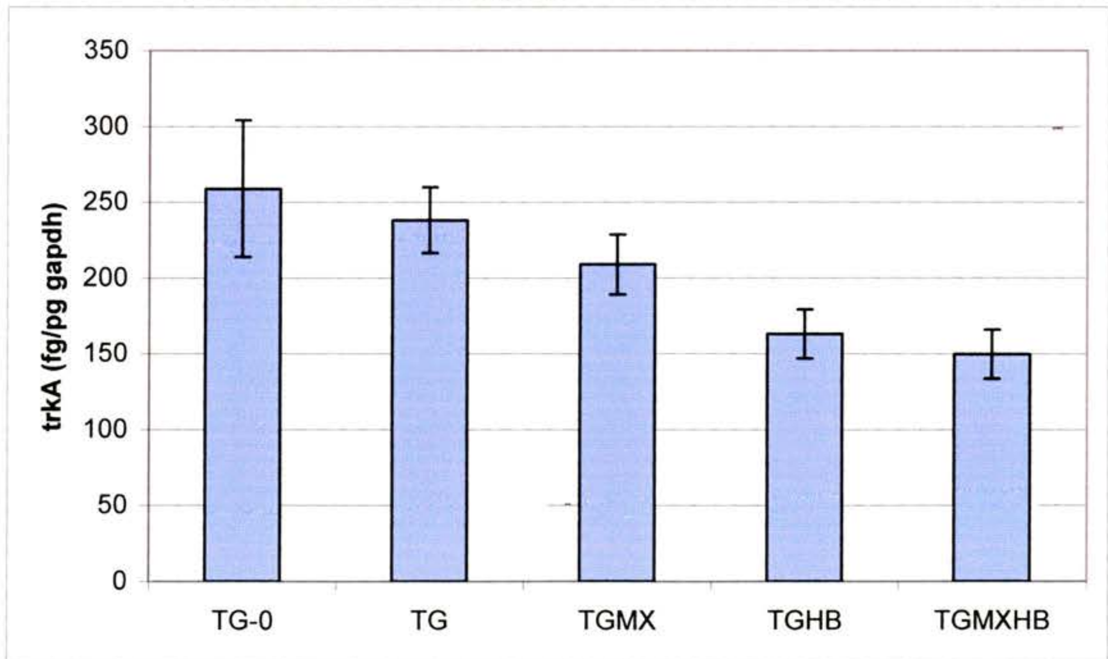
Expression of trkA in E10 trigeminal ganglia after 24hrs in culture with NGF, BDNF and NT-3. TG-0: Expression in ganglia before culture. TG: Expression in the ganglion grown on its own. TGMX: Expression in the ganglion grown with the maxillary process. TGHB: Expression in the ganglion grown with the hindbrain. TGMXHB: Expression in the ganglion grown with both target fields. Bars represent the mean ± standard error (n=3-4 per data point).

Figure 3.12: trkA expression in E12 trigeminal ganglia after 24 hrs in culture without or with their peripheral and central target fields attached



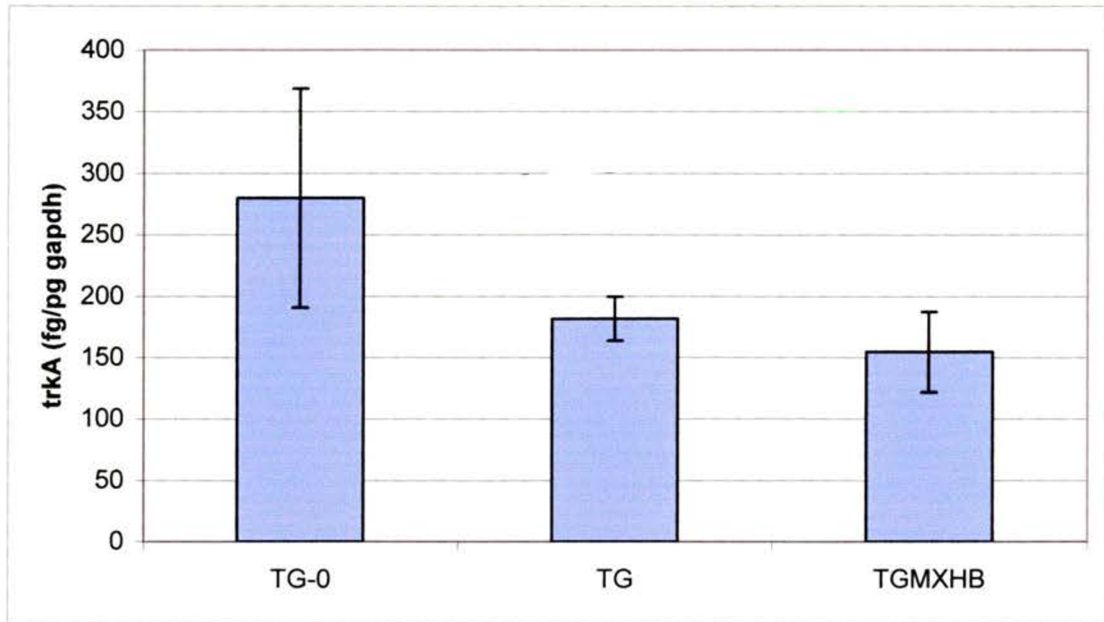
Expression of trkA in E12 trigeminal ganglia after 24hrs in culture with NGF, BDNF and NT-3. Symbols as above. Bars represent the mean ± standard error (n=4 per data point).

Figure 3.13: trkA expression in E13 trigeminal ganglia after 24 hrs in culture without or with their peripheral and central target fields attached



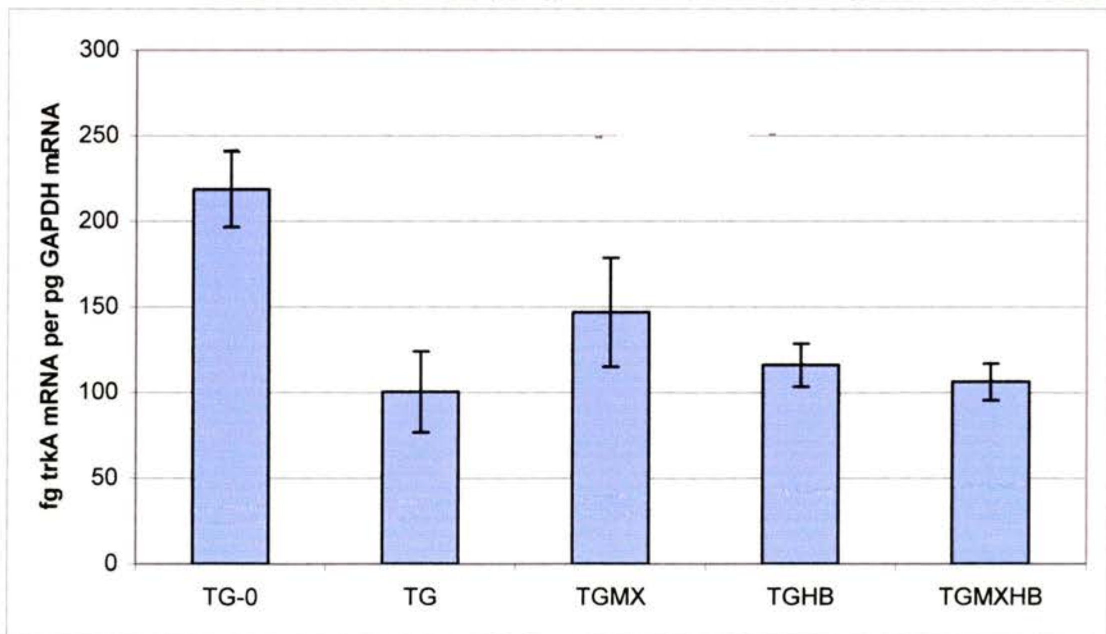
Expression of trkA in E13 trigeminal ganglia after 24hrs in culture with NGF, BDNF and NT-3. Symbols as before. Bars represent the mean ± standard error (n=7-8 per data point).

Figure 3.14: trkA expression in E14 trigeminal ganglia after 24 hrs in culture without or with their peripheral and central target fields attached



Expression of trkA in E14 trigeminal ganglia after 24hrs in culture with NGF, BDNF and NT-3. TG-0: Expression in ganglia before culture. TG: Expression in the ganglion grown on its own. TGMXHB: Expression in the ganglion grown with both target fields. Bars represent the mean ± standard error (n=4 per data point).

Figure 3.15: trkA expression in E15 trigeminal ganglia after 24 hrs in culture without or with their peripheral and central target fields attached



Expression of trkA in E15 trigeminal ganglia after 24hrs in culture with NGF, BDNF and NT-3. TGMX: Expression in the ganglion grown with the maxillary process. TGHB: Expression in the ganglion grown with the hindbrain. Other symbols as above. Bars represent the mean ± standard error (n=4 per data point).

The main peripheral and central target field of the trigeminal ganglion thus appear to down-regulate trkA expression in vitro after 24 hrs. This effect was most pronounced at E11, with some less effect at E12 and E13. Earlier (E10) and later (E14 and E15) in the development of the trigeminal ganglion there appeared to be no effect by the target fields on trkA mRNA levels. It is possible that this effect was due to the release of neurotoxic substances from the target field in culture. However, there appeared to be no effect at later ages, which together with preliminary findings that the target fields did not significantly affect trkB and trkC expression in the ganglion (see appendix – Figures A.3 and A.4), would argue against such a neurotoxic substance (although there was a downregulation of p75 mRNA with the target fields, Appendix – Figure A.5). Also, homogenates of the maxillary process and the hindbrain succeeded at inhibiting the increase in trkA. These tissues were homogenized immediately after collection, making any build up in neurotoxic substance(s) an unlikely possibility.

3.3 Discussion

The effects observed in this study are difficult to explain because one part of the study seems to indicate that trkA in the trigeminal ganglion is controlled by mechanisms intrinsic to the ganglion, whereas the studies involving the target field appear to suggest that target fields are capable of down-regulating trkA expression during early development. I shall thus discuss each finding in turn before attempting to find explanations for the contrasting effects observed.

3.3.1 Intrinsic regulation of trkA expression in the mouse trigeminal ganglion during development

The time-course of trkA expression in vivo in the studies reported here closely resembles the time-course previously established for the trigeminal ganglion (Wyatt and Davies, 1993), confirming the accuracy of the methods used. During the initial development of the trigeminal ganglion between E10 and E11, very little increase in trkA expression was observed, consistent with most neurons at this stage in development being either independent of neurotrophins or depending on BDNF and NT-3 for survival (Buchman and Davies, 1993; Piñon et al., 1996). TrkA expression subsequently increased sharply between E11 and E13, which is consistent with neurons switching their survival dependence from BDNF and NT-3 to NGF. Interestingly, in vitro, the levels of trkA mRNA expressed by the ganglion alone precisely mimicked this developmental expression in vivo. This confirms earlier observations of a switch in survival dependence between E11 and E12 that requires cell-cell interactions within the trigeminal ganglion (Enokido et al., 1999). Preliminary results mentioned in the results section and shown in the appendix

(Figure A.5) suggest that p75 undergoes a similar sharp increase in expression between E11 and E12 in vitro, whereas no significant increases are observed at this stage in the expression patterns of trkB and trkC in vitro (Figures A.3 and A.4). Thus, events within the cultured trigeminal ganglion appear to trigger the onset of NGF dependence and the proportional loss in trkB responsiveness during this stage in development. Whereas the previous study (Enokido et al., 1999) demonstrated this by assessing the responsiveness of neurons to the different neurotrophins, the data presented here shows that this intrinsic change in responsiveness is accompanied by an intrinsic change in receptor expression in the ganglion. It is unlikely that the increases in the expression levels of trkA and p75 are related to the expression of these receptors by non-neuronal cells, since it has been demonstrated that non-neuronal cells in the trigeminal ganglion at this age do not express either of these two receptors (Wyatt and Davies, 1993). The finding that trkA increases sharply between E12 and E13 also confirms previous findings of increasing trkA expression in dissociated neuronal cultures at these ages, at least during the first 18 hours (Wyatt and Davies, 1993)

The decreasing levels of trkA expression between E14 and E15 in vivo could be due to a reduction in the number of trkA-dependent neurons as a proportion of other cells in the ganglion. This could result from death of trkA expressing neurons or a relative increase in the number of satellite and Schwann cells. The mean level of trkA mRNA per neuron could also decrease with age. Further ages will need to be assessed to determine whether this intrinsic decrease in trkA expression continues later into the period of developmental cell death.

The observation that cultured ganglia of different ages follow almost exactly the same changes in trkA expression as seen in vivo, suggests an extremely tightly controlled intrinsic mechanism in the control of trkA expression. This would confirm the importance of mechanisms intrinsic to the neurons or the ganglion during neuronal development. Similar findings have been made in placode-derived sensory neurons in the chick, where the onset of neurotrophin dependence appears to be intrinsically pre-set, at least in part (Vogel and Davies, 1991; Davies and Vogel, 1991). The finding that trkA expression in the trigeminal ganglion is intrinsically regulated also correlates with previous findings suggesting that the maturation of sensory neurons from the trigeminal ganglion is target independent (Stainier and Gilbert, 1991); further evidence for the independence of this neuronal population from their target fields.

3.3.2 Downregulation of trkA by a diffusible substance released from the target fields

The finding that the target fields of the trigeminal ganglion release a diffusible substance that has a profound effect on trkA mRNA levels in the ganglion in vitro seems at odds with the idea that trkA expression is predominantly regulated by intrinsic mechanisms within the ganglion. The relation between these two sets of results will be discussed later in this chapter, with this section focusing on the potential significance of the target field effects in vitro.

The developmental period during which the target field has an effect on reducing trkA expression in the trigeminal ganglion in vitro appears to be restricted to E11 to

E13, with the strongest effects observed at E11. Thus the diffusible substance appears to act between the onset of innervation and the period immediately prior to the onset of developmental cell death. This makes it unlikely that this putative substance is actively involved in promoting neuronal death. Rather, one could speculate that the time window of its action in vitro suggests a possible role for this substance in maintaining neurons alive before the period of developmental cell death by delaying the onset of trkA expression and NGF dependence. It may ensure low trkA levels in neurons whose axons have reached their target field prior to developmental cell death, keeping these neurons independent of NGF and enabling neurons to compete with later arriving ones for limited supplies of NGF over the same period of development. This would maximise the 'choice' of the target field, enabling the selection of neurons on the basis of the appropriateness of their axonal terminations in the target field.

Of the known substances present in the central and peripheral target fields of the trigeminal ganglion at this stage in development, only BDNF has been shown to decrease trkA expression in trigeminal neurons (Wyatt and Davies, 1993). BDNF is highly unlikely to cause the observed effect however, since it was present in the culture medium in which trigeminal ganglia grown alone quite readily increased their trkA mRNA levels. Other substances known to affect trkA expression include retinoic acid, which has been found to up-regulate trkA in some tissues whereas in others it down-regulates trkA (e.g. Wyatt et al., 1999; Holst et al., 1995; 1997). It is unlikely to be involved in the effects observed however, since it has been shown to have no effect on trkA expression in the trigeminal ganglion at the ages of E11 and E12, i.e. the stages at which the most significant effect was observed (Wyatt et al.,

1999). NGF has also been proposed to be involved in alterations in trkA expression (e.g. Holtzman et al., 1992). However, the levels of trkA mRNA in the trigeminal ganglion of NGF null mutants appears normal, indicating that NGF has no effect on trkA expression (Davies et al., 1995). Thus very little is known about factors influencing trkA expression in vivo or in vitro, making the identification of the substance involved in the downregulation of trkA by the target field in vitro difficult. Analysis of the culture medium from homogenised target fields obtained in the experiments may assist in the identification of such substances.

3.3.3 Conclusion

This conclusion section will attempt to find an explanation for the apparently contradictory effects observed in the trigeminal ganglion in the studies conducted.

One possible explanation for the observed reductions in trkA expression in the co-culture experiments is that there are substances in vivo that up-regulate trkA expression to higher levels than would develop intrinsically. A substance from the target fields down-regulating trkA would then be necessary to maintain trkA at appropriate levels. If this were the case, why would there be such a tightly controlled pre-determined intrinsic mechanism of trkA expression in isolated ganglia in vitro? However, positive and negative modulators of physiological and developmental processes are widespread.

It is possible that the release of the putative repressor of trkA expression from the target fields is normally very tightly regulated in vivo and that the regulatory mechanisms controlling its release may be absent in vitro, hence causing excessive release and the pronounced effects observed in vitro.

Perhaps one of the functions of the putative repressor of trkA expression in vivo may be to delay the onset of NGF dependence of the early innervating neurons and thereby sustain their survival until the majority of neurons innervates the target field so as to maximize competition for NGF and select the most appropriately connected neurons. This potential function is illustrated in Figure 3.16. The alternative explanation to the above is that the target field-derived repressor of trkA expression is an in vitro artefact that has no physiological parallel in vivo. It is curious, however, that the production and/or response to the repressor appear to be highly developmentally restricted, arguing in favour of its importance.

In future studies it will be important to carry out heterochronic co-cultures to clarify to what extent the synthesis/release of the putative repressor and the response of the neurons to it are developmentally restricted. It will also be interesting to assess whether the expression of trkA in vitro continues to follow that observed in vivo at later stages in development. This will be particularly interesting since it could potentially demonstrate that trkA is intrinsically downregulated as part of developmental cell death. Secondly, the expression of other neurotrophin receptors will also need to be assessed, to see whether these are tightly controlled by intrinsic mechanisms as well. Thirdly, it will be useful to identify the inhibitory substance released from the target fields in vitro, using the homogenate as a starting point. Once the substance is identified, it should be possible to assess its significance in vivo. Hopefully further study of both these observations will lead to an explanation for the different effects observed in this study.

Figure 3.16: Potential function of trkA downregulation by the target field prior to trophic factor competition

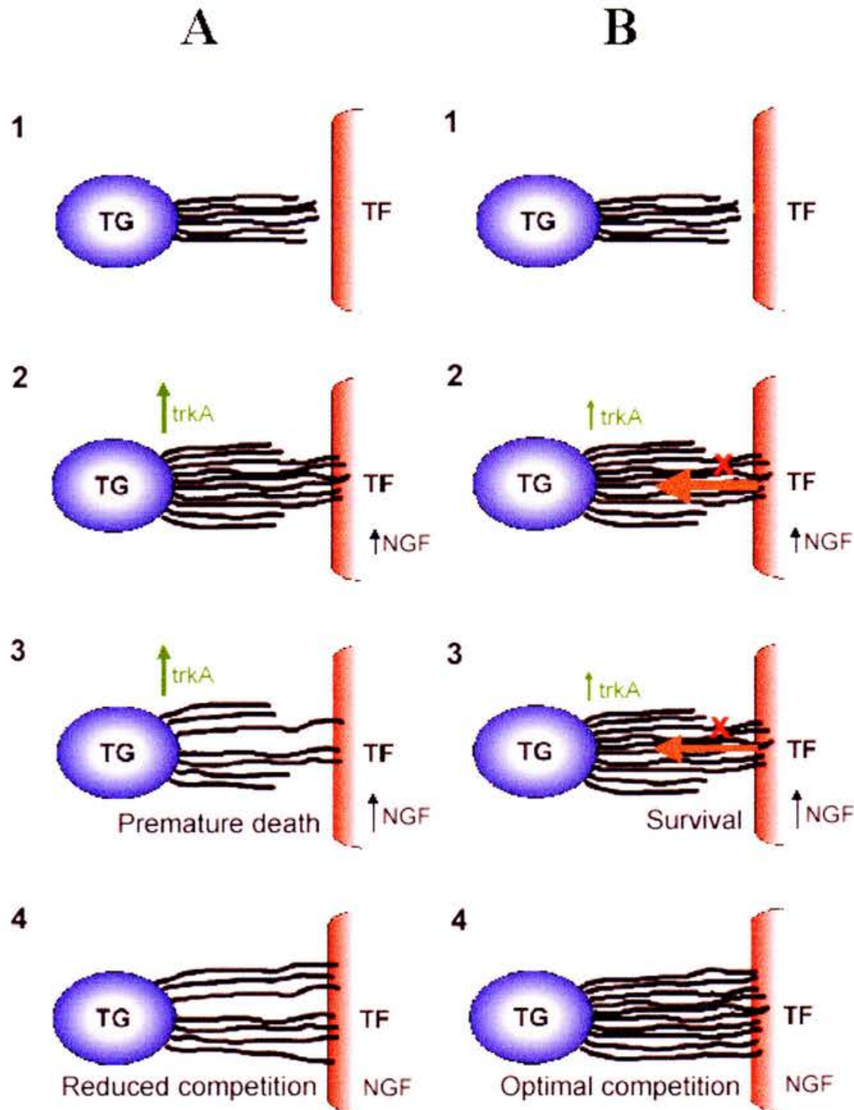


Figure illustrating the potential benefit of trkA downregulation during the phase of target innervation, before competition for limited amounts of NGF, assuming that trkA expression confers dependence on NGF for survival. (A) shows the possible situation in the absence of a repressor of trkA expression. (1) Axons approach their target field. (2) As axons reach the target field, neurons increase trkA expression, while the target field begins expressing NGF. (3) As NGF expression is restricted because of its role as a survival factor in limited supply during developmental cell death (PCD), increasing trkA expression in neurons prior to PCD may result in excessive numbers of neurons being trkA-dependent. If too many neurons are dependent on trkA-signalling for their survival prior to PCD (while axons are still extending to the target fields), neurons that do not receive sufficient NGF will die at this stage. (4) This results in reduced competition of neurons for survival factors from the target field during PCD, with some inappropriately connected neurons surviving due to them reaching the target field at a later stage and not having to compete with more appropriately connected neurons. (B) shows the benefits of a substance (x, red arrow) that reduces any excessive upregulation of trkA. (1) Axons approach their target field. (2) As axons reach the target field, increases in trkA expression are inhibited by a substance that effects a reduced magnitude of trkA increase. (3) Restricted NGF expression does not kill neurons prior to PCD, as less neurons express trkA (less neurons are dependent on NGF for their survival at this stage). (4) This results in maximizing competition for NGF between all neurons and enabling the selection of the most appropriately connected neurons.

CHAPTER IV

Influence of early sensory innervation on neurotrophin expression in the target field

4.1 Introduction

Neuronal numbers in the developing peripheral nervous system are regulated by extrinsic growth and differentiation factors. One such factor is nerve growth factor (NGF). During embryonic development, most cutaneous sensory neurons depend for their survival on a supply of NGF synthesised in their peripheral targets. NGF promotes survival by binding to the trkA receptor tyrosine kinase, whose signalling is modulated by the common neurotrophin receptor p75. In the trigeminal system trkA is expressed by sensory neurons shortly after axons reach their targets (Wyatt et al, 1993) and NGF expression begins in the target area with the arrival of the earliest axons (Davies et al., 1987).

The synthesis of NGF in target fields has been found to coincide with the commencement of innervation by NGF-dependent neurons (Davies et al., 1987), as determined in the maxillary target field, a target of sensory neurons from the trigeminal ganglion. The final innervation density of the target fields of NGF-dependent neurons is proportional to the amount of NGF synthesised in them. Cutaneous targets requiring high innervation density have higher levels of NGF mRNA than targets with lower innervation density (Harper and Davies, 1990).

Classic studies clearly demonstrated a role for the target in governing the number of neurons supplying it. Complete or partial removal of target fields correlates with a reduction in the survival of innervating neurons during the period of naturally occurring cell death in development, most likely due to a reduced production of a trophic factor, such as NGF, normally synthesized by the target field (Oppenheim,

1981; 1985; Hamburger and Oppenheim, 1982). Increases in target field availability during development result in an increase in the number of neurons surviving the period of naturally occurring cell death, most likely due to increased availability of trophic factors (Oppenheim, 1981; 1985; Betz, 1987; Lamb, 1984).

However, the relationship between the amount of target field removed / added, and the number of neurons innervating it undergoing increased / decreased cell death has often been found to be non-linear. Increasing the target availability by doubling its size for example might be expected to result in virtually no cell death in neuronal populations that normally lose half their numbers during the period of naturally occurring cell death (Oppenheim, 1991). This situation, however, has never been found, only partial rescue of neuronal numbers with doubled target availability has been found (Oppenheim, 1981; 1985; Lamb, 1984; Sperry and Grobstein, 1985; Tanaka and Landmesser, 1986; Sohal et al., 1986). Likewise the reduction in neuron number following partial target field ablation is not linearly related to the target field size (Lamb, 1980; Tanaka and Landmesser, 1986; Farel, 1989).

One striking example of such an occurrence comes from experiments carried out in the hind-limb bud of the frog. Bilateral innervation of a single limb bud, by amputating the other limb and disrupting the barriers between the two sides, resulted in motor projections from both sides into the single limb bud. The number of motor neurons surviving after metamorphosis were normal on both sides, as long as the operation had been carried out prior to innervation (Lamb, 1980; 1981). This implied that a single limb bud was able to support twice the normal number of neurons after

the period of naturally occurring cell death. If there was competition for limited amounts of factor between the innervating populations of motoneurons, one would have expected less neurons to survive on each side in bilaterally innervated animals compared to unilaterally innervated animals. In contrast, sensory neurons innervating the single limb bud were found to behave in a fashion consistent with the neurotrophic hypothesis. That is, the number of neurons innervating the hind-limb bud after innervation and programmed cell death on both sides added up to the neuronal numbers usually observed on one side (Lamb et al., 1989). This suggests that neurons had died proportionally to a signal of predetermined strength from the target field. These findings illustrate how different population of neurons, even within the same species of animal, may behave differently.

The results of studies demonstrating non-linear relationships between target and cell death led Purves (1980) to speculate that increased quantity of initial innervation (in the case of a bilaterally innervated limb bud as an example) may alter the target field, allowing it to support an extra complement of neurons The extent to which neurons may be able to do so could vary between different neuronal populations, as appears to be the case for motoneurons and sensory neurons observed in the experimental model mentioned above.

In respect of the observations mentioned above, it is interesting that a study analysing NGF mRNA synthesis in the whisker pad (maxillary target field) found NGF mRNA synthesis increased rapidly in maxillary explants cultured from E11.5 onwards, whereas in E10/E10.5 explants very little increase in NGF mRNA synthesis

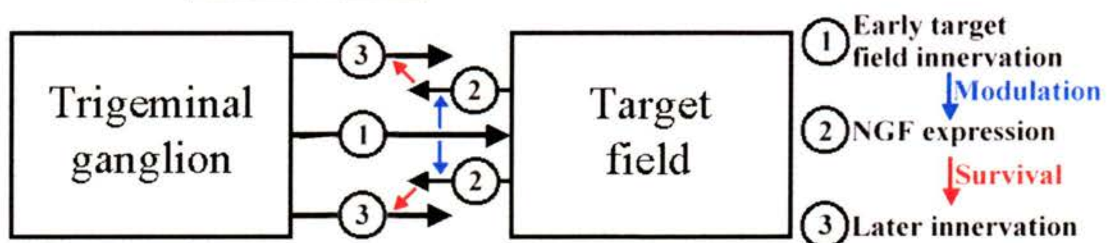
was observed (Rohrer et al., 1993). NGF expression in the maxillary target field begins with the arrival of the first axons at ~E10.5/E11 (Davies et al., 1987), i.e. at the point between these two very distinct observations. Thus, it is possible that initial innervation may play a role in either initiating or up-regulating expression of NGF.

The aim of the study presented in this section was to assess the possibility that the initial innervation density may influence the number of neurons the target field can support by regulating the level of trophic factor synthesis. The maxillary target field of the trigeminal ganglion leads itself particularly well to this type of study, due to its high density of innervation and high levels of neurotrophic factor (Davies et al., 1987; Harper and Davies, 1990). The time course of innervation is well established, and developmental changes in the levels of neurotrophic factor mRNA and protein have been studied intensively (Buchman and Davies, 1993). Prior to innervation, trigeminal ganglion sensory neurons require BDNF and NT-3, whereas shortly after innervation they become dependent on NGF for survival (Buchman and Davies, 1993). BDNF and NT-3 mRNA are expressed in the maxillary target field prior to the arrival of the first axons, with their levels peaking at E12 and E13, respectively (relative to actin mRNA). NGF mRNA is first detected at E10.5, reaching peak expression at E13 and NGF protein is also first detected at E11 and peaks at E13 (Davies et al., 1987).

To investigate if the trigeminal ganglion influences the expression of NGF, BDNF and NT-3 in the maxillary target field, the target field was cultured with and without trigeminal ganglia at different stages of development. NGF mRNA levels in very

young target field explants increased to higher levels when grown with the ganglion compared with target field explants grown alone, raising the possibility that early target innervation influences NGF expression. No significant differences were observed in BDNF and NT-3 expression. The effects of neuronal numbers in the trigeminal ganglion were further investigated by measuring the level of NGF mRNA in ErbB3-/- mice, which display a large, early decrease in the number of trigeminal neurons. Consistent with this possibility, the level of NGF mRNA was significantly lower in the early target fields of these embryos compared with wild type. However, the levels of NGF mRNA were not significantly higher in the target fields of embryos in which the later period of naturally occurring neuronal death is reduced (Bax-/- and Bad-/- embryos) compared with wild type embryos, or in which a loss of neurons occurred during later stages of innervation (trkA-/-, trkB-/-, p75-/-). This suggests that very early changes in target field innervation may be able to modulate the expression of NGF in the maxillary target field, possibly to adjust intrinsic NGF levels to the timing of innervation or the initial number of neurons reaching the target fields. Later innervation has no such effect. This possible interaction between the initial neuronal innervation and the target field is represented in Figure 4.1.

Figure 4.1: Modulation of NGF expression in the maxillary target field by early innervation



Block diagram illustrating the potential modulation of NGF expression in the maxillary target field of trigeminal neurons by early innervation. Early innervating axons (1) modulate intrinsic NGF levels (2) to adjust the supply to the timing/number of later innervating neurons (3).

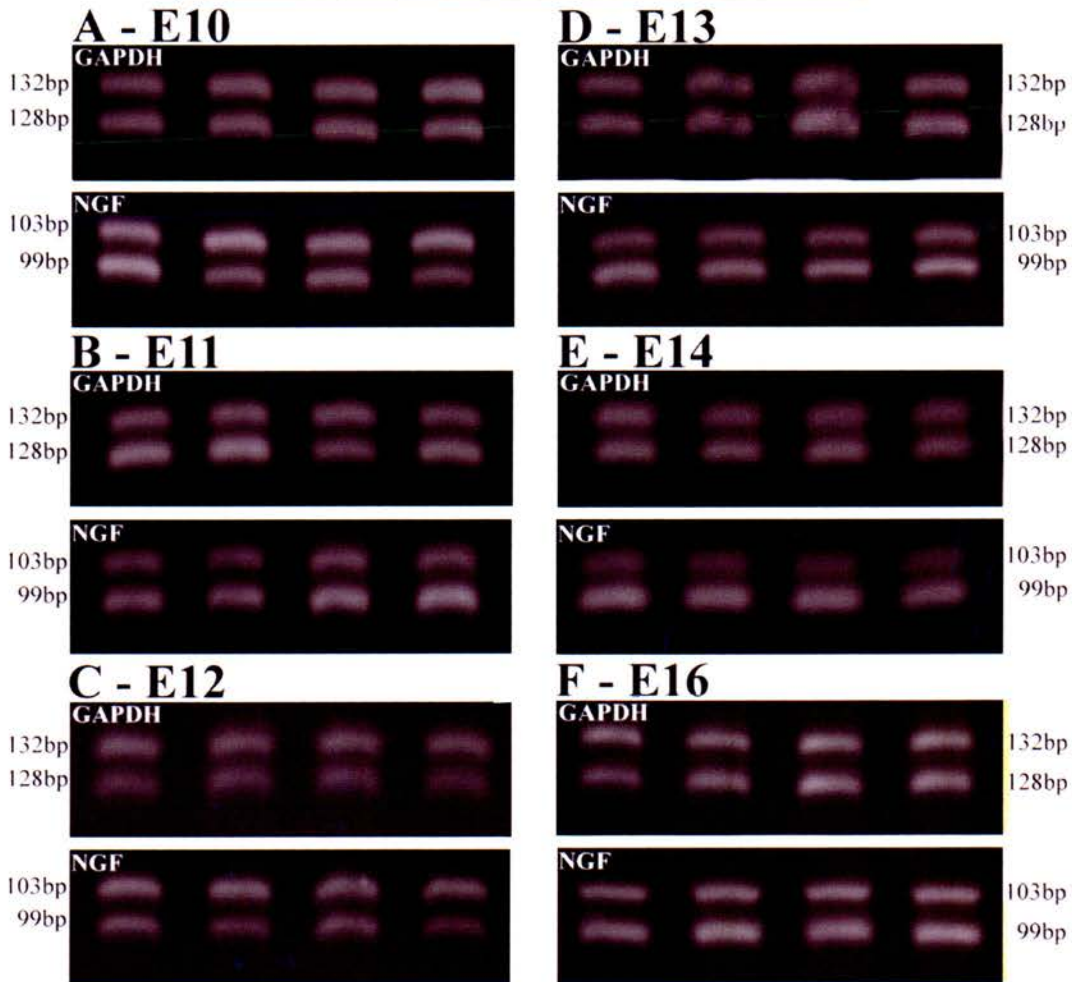
4.2 Results

The methods for dissection and culture of trigeminal ganglia and the maxillary target field, and determination of neurotrophin expression by competitive RT-PCR are described in chapter 2.

4.2.1 Expression of NGF mRNA in the maxillary target field in vivo

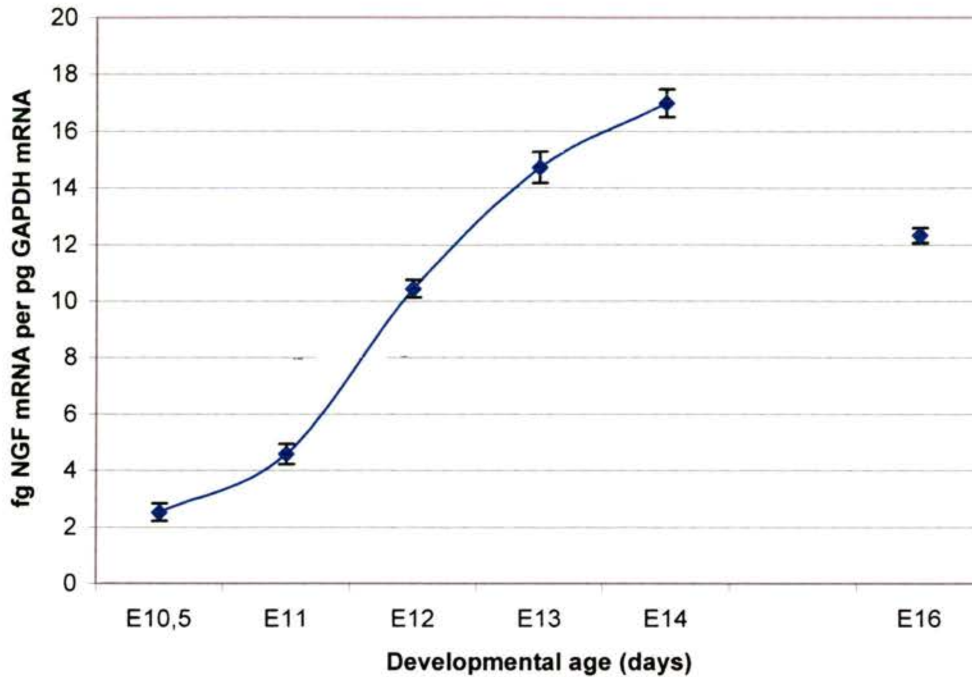
To ascertain the developmental increase in NGF mRNA in the maxillary target field of mice in relation to innervation in vivo, maxillary target fields and the whisker processes to which they give rise were collected and NGF mRNA was assessed using RT-PCR at stages from E10.5 to E15. Examples of these gels are shown in Figure 4.2. The expression of NGF mRNA relative to GAPDH mRNA is shown in Figure 4.3. NGF mRNA was detected at E10.5 at low levels and increased significantly (relative to GAPDH mRNA) by E11 (t-test; $p<0.001$). This was followed by further significant increases in NGF mRNA between E11 and E12 (t-test; $p<1E-22$), E12 and E13 (t-test; $p<5E-08$), and E13 and E14 (t-test; $p<0.05$). There was a significant decrease in NGF mRNA levels between E14 and E16 (t-test; $p<5E-13$). The developmental change in NGF mRNA expression observed in this study confirms previous observations (Davies et al., 1987; Buchman and Davies, 1994), with a marked increase in NGF mRNA between E10.5 and E12, coinciding with the arrival of the first axons (Davies and Lumsden, 1984; Buchman and Davies, 1993). This demonstrates that the competitive RT-PCR method used here was able to replicate results previously obtained using northern blotting, enabling the use of this more sensitive technique for the remaining experiments in this section.

Figure 4.2: Examples of gels showing the relative NGF expression in the trigeminal ganglion in vivo between E10 and E16



SYBR®Gold (Molecular Probes) stained gels showing the products of RT-PCR reactions amplified with either GAPDH-specific or NGF-specific primers. (A) Reactions containing 625fg of the GAPDH competitor RNA (132bp) and 1.6fg of the NGF competitor (103bp) plus total RNA from E10 maxillary processes (GAPDH: 128bp; NGF: 99bp). (B) Reactions containing 1.5pg of the GAPDH competitor RNA (132bp) and 6.5fg of the NGF competitor (103bp) plus total RNA from E11 maxillary processes (GAPDH: 128bp; NGF: 99bp). (C) Reactions containing 1.5pg of the GAPDH competitor RNA (132bp) and 15fg of the NGF competitor (103bp) plus total RNA from E12 maxillary processes (GAPDH: 128bp; NGF: 99bp). (D) Reactions containing 20pg of the GAPDH competitor RNA (132bp) and 250fg of the NGF competitor (103bp) plus total RNA from E13 maxillary processes (GAPDH: 128bp; NGF: 99bp). (E) Reactions containing 30pg of the GAPDH competitor RNA (132bp) and 400fg of the NGF competitor (103bp) plus total RNA from E14 maxillary processes (GAPDH: 128bp; NGF: 99bp). (F) Reactions containing 25pg of the GAPDH competitor RNA (132bp) and 250fg of the NGF competitor (103bp) plus total RNA from E16 maxillary processes (GAPDH: 128bp; NGF: 99bp).

Figure 4.3: Relative in vivo levels of NGF mRNA in the maxillary target field in early development of the mouse embryo



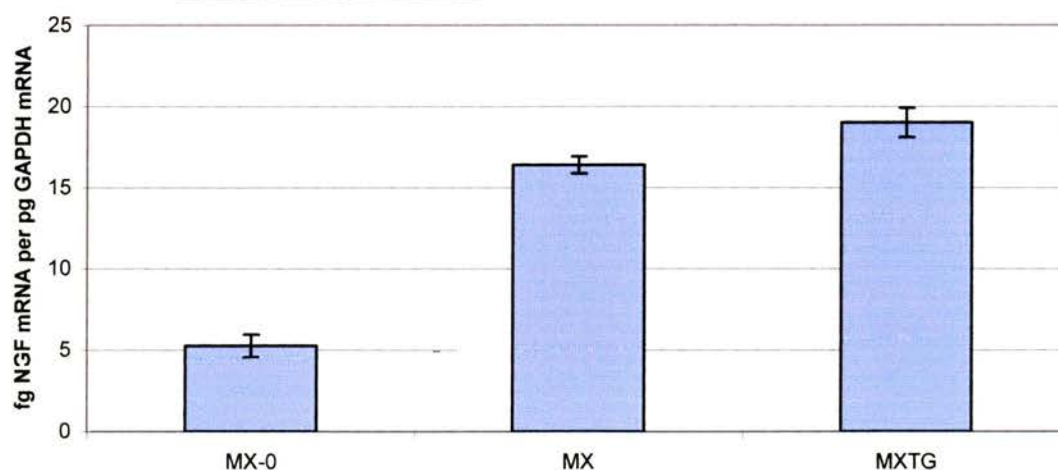
Mean levels of NGF mRNA relative to GAPDH mRNA in the maxillary target field of CD1mouse embryos. A rapid increase in NGF expression between E10.5 and E13 is clearly visible, as is the decrease in NGF expression between E14 and E16. Bars represent the mean ± standard error (n=16-69 for each data point).

4.2.2 Expression of NGF mRNA in the maxillary target field in vitro – effects of age and early innervation

Two ages at early stages of development were chosen to assess the effect of innervation on NGF expression in vitro: E10.5 and E11. Very few sensory axons are present in the maxillary target field at E10.5 and none have yet contacted the maxillary epithelium where the highest levels of NGF mRNA are expressed later in development. By E11, the earliest axons make contact with the maxillary epithelium (Davies and Lumsden, 1984). Maxillary target fields of these two ages were grown either alone, or with the trigeminal ganglion and its neuronal connections to the

maxillary target field intact. These explants were grown for 48 hours, (equivalent to E12.5 in vivo for E10.5 maxillary target field, and E13 for E11 maxillary target field), thus covering the main period of the increase in the level of NGF mRNA in vivo and the marked increase in innervation density. Figure 4.4 illustrates NGF mRNA expression relative to GAPDH mRNA in the E11 maxillary target field of mouse embryos prior to culture (MX-0) and after 48 hours in culture with and without the trigeminal ganglion (MX and MXTG respectively), with an ANOVA indicating significant differences ($p<0.0001$). The level of NGF mRNA in the E11 maxillary target field grown alone (MX) increased more than two-fold over the 48 hr culture period (Fisher's LSD; $p<0.0001$). The level of NGF mRNA in the maxillary target fields grown with the trigeminal ganglion attached was significantly higher than in processes grown alone (14% higher, Fisher's LSD; $p<0.05$). This suggests that innervation of the target field may be able to modulate the synthesis of NGF to some extent.

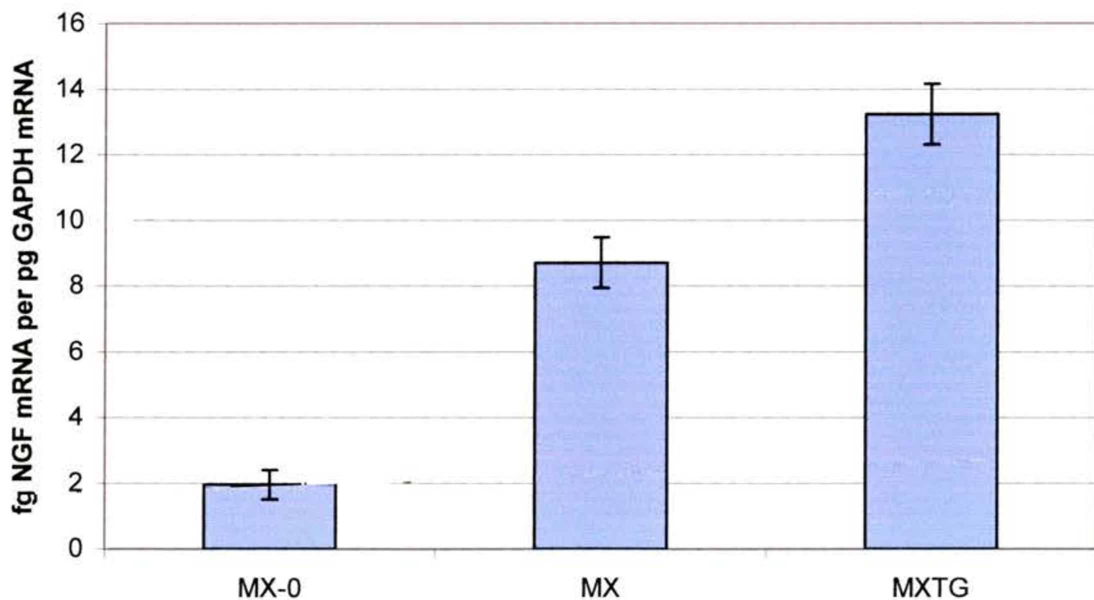
Figure 4.4: Relative levels of NGF mRNA in the E11 maxillary target field after 48 hrs in culture



Mean levels of NGF mRNA relative to GAPDH mRNA in maxillary target fields at E11 (MX-0), after 48 hrs in culture without the trigeminal ganglion attached (MX), or after 48hrs in culture with the trigeminal ganglion attached (MXTG). Bars represent the mean ± standard error (n=12-16 for each data point).

Figure 4.5 illustrates the level of NGF mRNA relative to GAPDH mRNA in the E10.5 maxillary target field of mouse embryos prior to culture (MX-0) and after 48 hours in culture with (MXTG) or without (MX) attached trigeminal ganglia, with an ANOVA indicating significant differences ($p<0.0001$). The level of NGF mRNA in maxillary target fields grown alone increased by over three-fold compared to its level in freshly collected tissue (Fisher's LSD; $p<0.0001$). The level of NGF expression in maxillary target fields grown with the trigeminal ganglion attached was greatly increased compared to the level in explants grown without the ganglion (52% increase, Fisher's LSD; $p<0.0005$). This not only suggests that innervation of the target field is able to modulate the synthesis of NGF, but together with the data in Figure 4.4 it suggests that early target innervation has more influence on NGF expression than later innervation, at least in vitro.

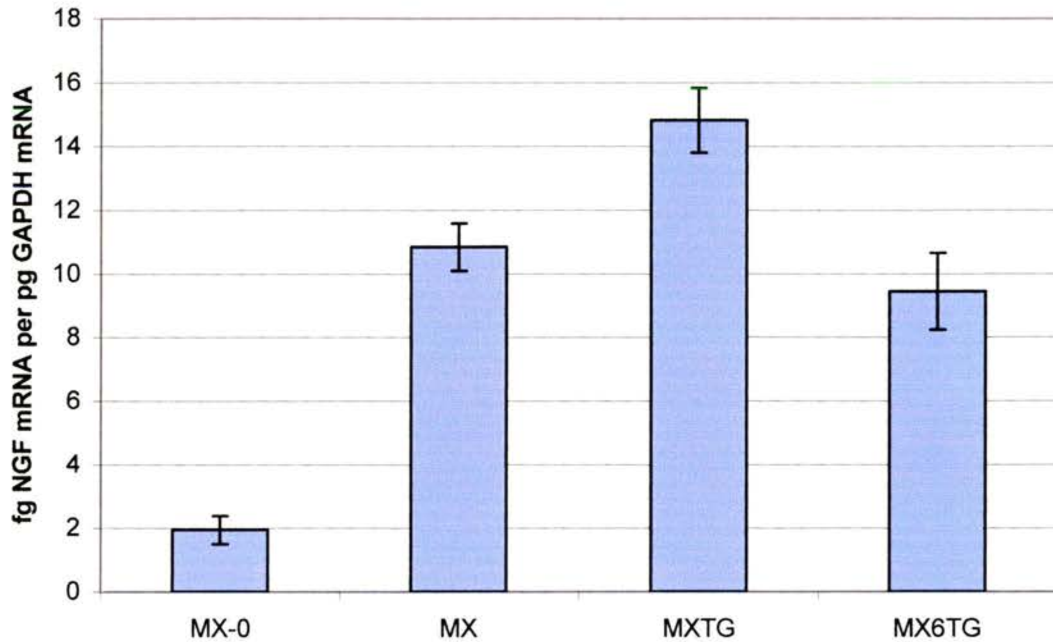
Figure 4.5: Relative levels of NGF mRNA in the E10.5 maxillary target field after 48 hrs in culture



Mean levels of NGF mRNA relative to GAPDH mRNA in maxillary target fields at E10.5 (MX-0), after 48 hrs in culture without the trigeminal ganglion attached (MX), or after 48hrs in culture with the trigeminal ganglion attached (MXTG). Bars represent the mean ± standard error (n=8-12 per data point).

To compare NGF mRNA levels in cultured E10.5 and E11 maxillary target fields at the equivalent in vivo stage, E10.5 maxillary target fields were grown for 60 hours (equivalent to E13 in vivo and E11 explants grown for 48 hrs). The results obtained from these cultures are shown in Figure 4.6, with an ANOVA indicating significant differences ($p<0.0001$). Although the increase in NGF mRNA expression in E10.5 explants after 60 hours in culture was slightly less than the increase observed in E10.5 cultures after 48 hours, the difference in NGF mRNA expression in E10.5 explants grown with and without attached trigeminal ganglia was still highly significant and relatively greater than in E11 cultures after 48 hrs (37% increase, Fisher's LSD; $p<0.005$). To ascertain whether the increase in NGF mRNA in maxillary target field co-cultured with trigeminal ganglia was due to innervation, E10.5 maxillary target fields were grown with 6 unattached trigeminal ganglia surrounding each process (MX6TG). As shown in Figure 4.6, no significant difference in NGF mRNA level was observed in these co-cultures compared with maxillary target fields grown alone (Fisher's LSD; $p>0.05$). This would strengthen the assumption that the initial innervation influences the levels of NGF mRNA rather than some diffusible substance from the ganglion.

Figure 4.6 Relative levels of NGF mRNA in the E10.5 maxillary target field after 60 hrs in culture



Mean levels of NGF mRNA relative to GAPDH mRNA in maxillary target fields at E10.5 (MX-0), after 48 hrs in culture without the trigeminal ganglion attached (MX), or after 48hrs in culture with the trigeminal ganglion attached (MXTG). Bars represent the mean ± standard error (n=8-12 per data point).

Since target field innervation appeared to have an effect on NGF expression, the relative levels of BDNF and NT-3 mRNA were also assessed in the same samples used for NGF mRNA analysis.

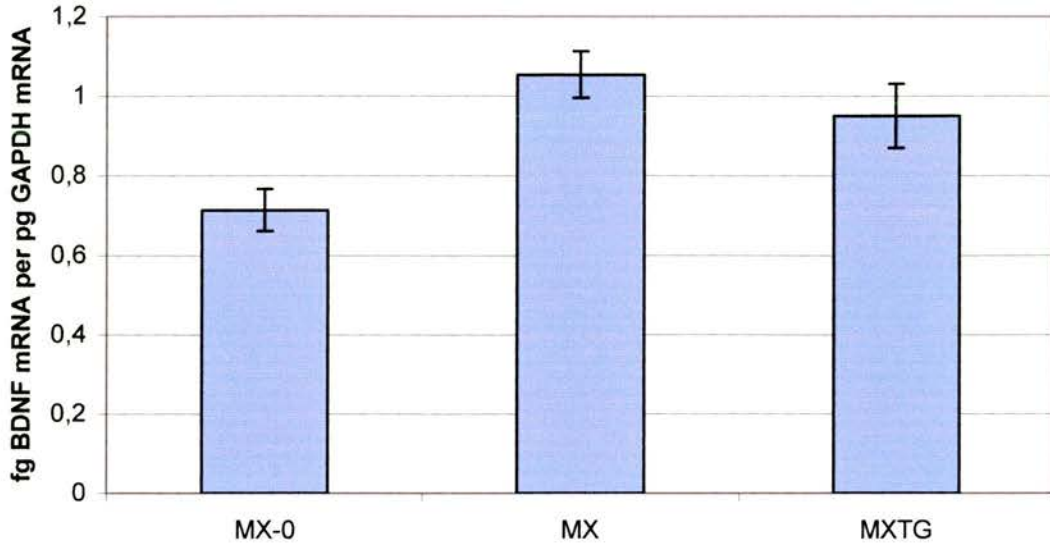
Figure 4.7 shows the level of BDNF mRNA relative to GAPDH mRNA in the E11 maxillary target fields of mouse embryos immediately after dissection and after 48 hours in culture with and without attached trigeminal ganglia), with an ANOVA indicating significant differences ($p \leq 0.001$). Although the level of BDNF mRNA in the E11 maxillary target field grown by itself (MX) increased over the 48 hour culture period compared to the level at the time the tissue was harvested (MX-0, Fisher's LSD; $p < 0.0005$), this increase was not as substantial as that observed for

NGF mRNA (<50% compared to >200% for NGF mRNA). Furthermore, the level of BDNF mRNA in the maxillary target field grown with the trigeminal ganglion attached (MXTG) was not significantly different to the level in explants grown without the ganglion (Fisher's LSD; $p>0.05$).

Figure 4.8 shows the level of BDNF mRNA relative to GAPDH mRNA in the E10.5 maxillary target field of mouse embryos before and after 48 hours in culture with and without attached trigeminal ganglia (ANOVA; $p<0.0001$). Although the level of BDNF mRNA in the E10.5 maxillary target field grown by itself (MX) increased over the 48 hour culture period compared to the level at the time the tissue was harvested (Fisher's LSD; $p<0.0001$), just as with E11 target fields, the presence of the trigeminal ganglion did not significantly alter the level of BDNF mRNA (Fisher's LSD; $p>0.05$). This suggests that early innervation of the maxillary target field has no effect on BDNF expression in vitro, suggesting that BDNF expression is either regulated by other factors, or is intrinsically regulated in the target field.

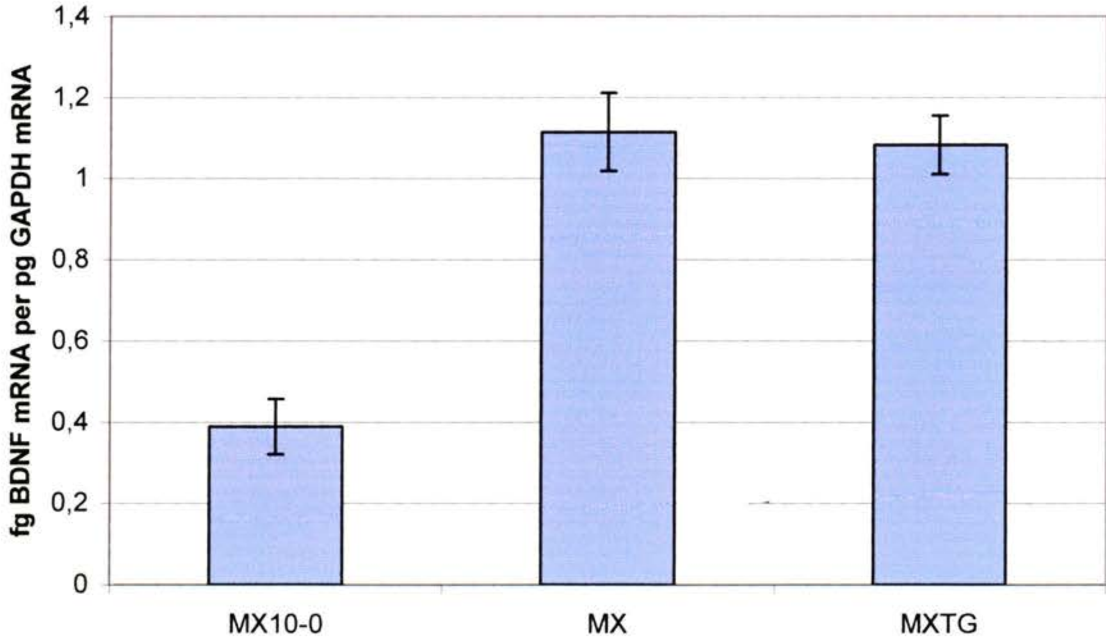
Figure 4.9 shows the level of NT-3 mRNA relative to GAPDH mRNA in the E11 maxillary target field of mouse embryos before and after 48 hours in culture with and without attached trigeminal ganglia (ANOVA; $p<0.0001$). Like NGF mRNA and BDNF mRNA, the level of NT-3 mRNA increased in the maxillary target field over this culture period (Fisher's LSD; $p<0.0001$). The relative levels of NT-3 mRNA in maxillary target fields grown with and without attached trigeminal ganglia were not significantly different (Fisher's LSD; $p>0.05$).

Figure 4.7: Relative levels of BDNF mRNA in the E11 maxillary target field after 48 hrs in culture



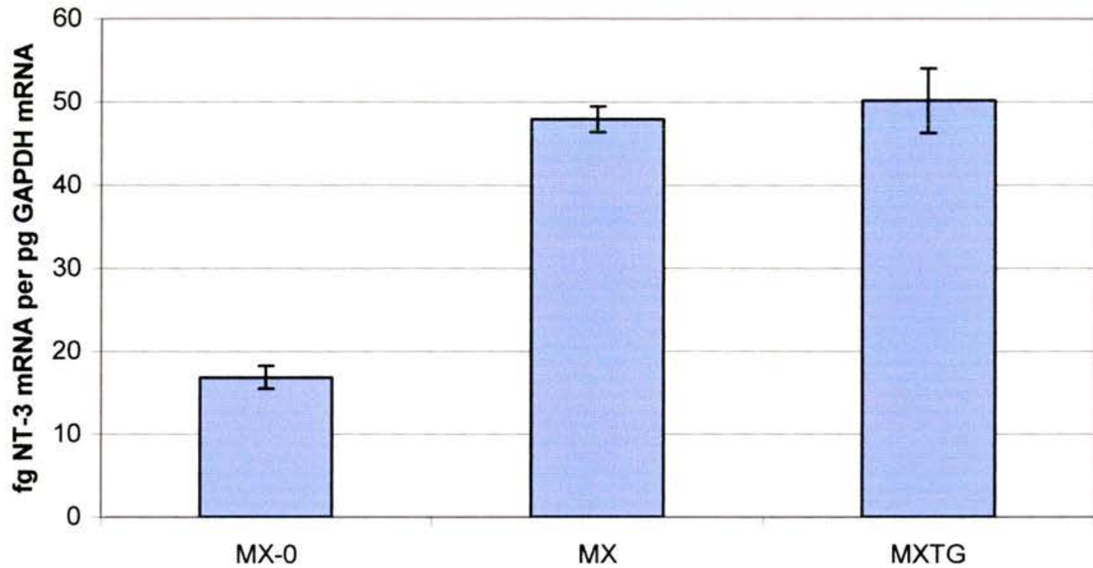
Mean levels of BDNF mRNA relative to GAPDH mRNA in maxillary target fields at E11 (MX-0), after 48 hrs in culture without the trigeminal ganglion attached (MX), or after 48hrs in culture with the trigeminal ganglion attached (MXTG). Bars represent the mean ± standard error (n=11-16 per data point).

Figure 4.8: Relative levels of BDNF mRNA in the E10.5 maxillary target field after 48 hrs in culture



Mean levels of NGF mRNA relative to GAPDH mRNA in maxillary target fields at E10.5 (MX-0), after 48 hrs in culture without the trigeminal ganglion attached (MX), or after 48hrs in culture with the trigeminal ganglion attached (MXTG). Bars represent the mean ± standard error (n=8-12 per data point).

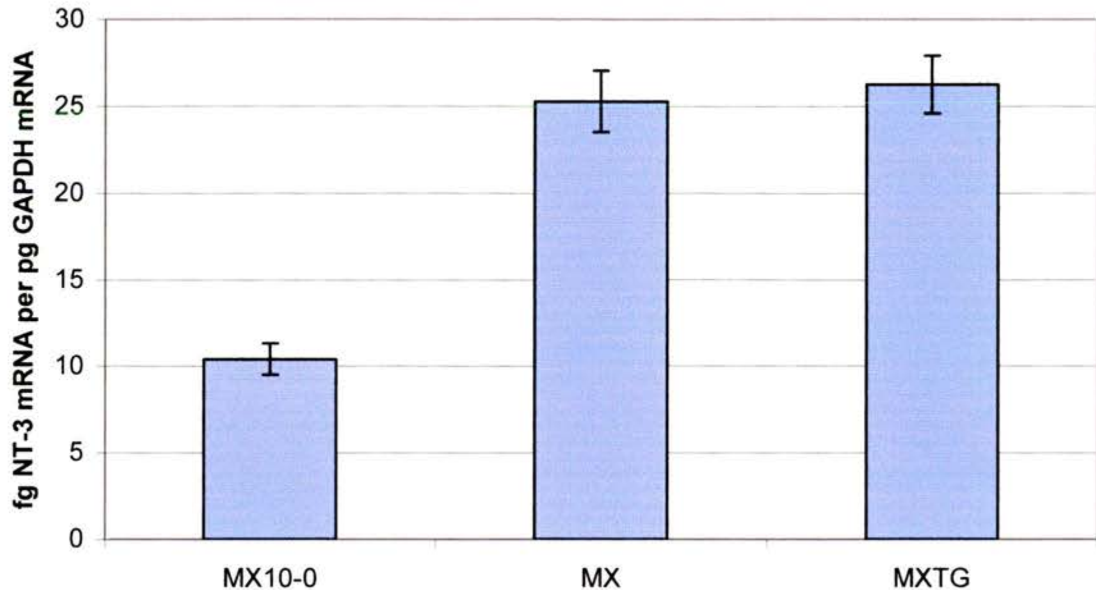
Figure 4.9: Relative levels of NT-3 mRNA in the E11 maxillary target field after 48 hrs in culture



Mean levels of NT-3 mRNA relative to GAPDH mRNA in maxillary target fields at E11 (MX-0), after 48 hrs in culture without the trigeminal ganglion attached (MX), or after 48hrs in culture with the trigeminal ganglion attached (MXTG). Bars represent the mean ± standard error (n=12-16 per data point).

Figure 4.10 shows the level of NT-3 mRNA expression relative to GAPDH mRNA in the E10.5 maxillary target field of mouse embryos after 48 hours in culture with or without the attached trigeminal ganglia (ANOVA; $p<0.0001$). Like NGF mRNA and BDNF mRNA, the level of NT-3 mRNA increased in the maxillary target field over this culture period (Fisher's LSD; $p<0.0001$), and just as with E11 maxillary target fields, the presence of the trigeminal ganglion did not significantly alter the relative expression of NT-3 (Fisher's LSD; $p>0.05$). This suggests that, like for BDNF, early innervation of the maxillary target field has no effect on NT-3 expression in vitro.

Figure 4.10: Relative levels of NT-3 mRNA in the E10.5 maxillary target field after 48 hrs in culture



Mean levels of NT-3 mRNA relative to GAPDH mRNA in maxillary target fields at E10.5 (MX-0), after 48 hrs in culture without the trigeminal ganglion attached (MX), or after 48hrs in culture with the trigeminal ganglion attached (MXTG). Bars represent the mean ± standard error (n=8-12 per data point).

The above results suggest that early innervation of the maxillary target field in vitro selectively modulates the expression of NGF but not other neurotrophins. Furthermore, the lack of effect of the trigeminal ganglion on the expression of BDNF and NT-3 mRNAs argues against the effect on NGF mRNA expression being caused by some kind of survival effect of the trigeminal ganglion on neurotrophin secreting cells in the maxillary target field, since this would be expected to affect the level of all neurotrophins.

In vitro studies provide only limited insight into the events occurring in vivo. The elevated level of NGF mRNA in E10.5 and E11 maxillary target fields co-cultured with trigeminal ganglia may not reflect an effect of innervation on NGF expression

in vivo. Indeed, in the co-culture models used in this study, the level of NGF expression does not increase to exactly the same levels seen at an equivalent stage in vivo. In E11 (figure 4.4), the levels of NGF mRNA in maxillary target fields co-cultured for 48 hours with the attached trigeminal ganglia rose above in vivo E13 levels (figure 4.3), whereas in E10.5 co-cultures grown for 60 hours (figure 4.5) it rose to levels almost exactly matching E13 in vivo levels (figure 4.3). This could be due to a variety of factors, such as slight variation in the ages the maxillary target fields were collected, or differences in the way explants of the two ages studied develop in culture. It is impossible to collect embryos at 'exact' stages, due to the difficulty in determining the exact time of mating. Only approximations based on the plug date and the visual developmental stage of the embryos can be made. Another potential complication of co-cultures is the elimination of adjoining tissues from the experimental system, which, in vivo, may have multiple effects on the development of the trigeminal ganglion and its target field.

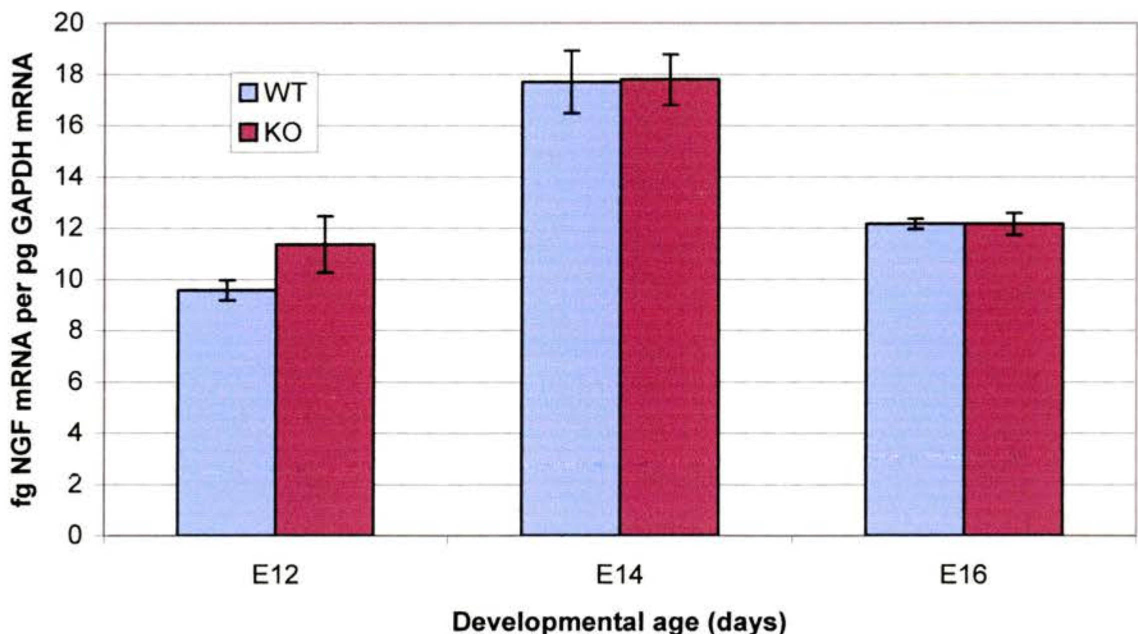
To assess whether there is an effect of innervation on NGF mRNA expression in vivo, knockout mice with either elevated or reduced neuronal numbers during development were used. This study was restricted to NGF mRNA, since it was the expression of this neurotrophin that was apparently affected by early innervation in vitro.

4.2.3 Expression of NGF mRNA in the maxillary target field of knockout mice with increased neuronal numbers in the trigeminal ganglion during development

NGF mRNA was assessed using competitive RT-PCR in two mutants with increased neuronal numbers during embryonic development, *bax*-/- and *bad*-/- mice, to ascertain whether this would result in increased levels of NGF mRNA in the target field.

Ablation of the *bax* gene of the mouse (Knudson et al., 1995) results in reduced death of neurons in the trigeminal ganglion (Middleton and Davies, 2001). NGF expression in the maxillary target field of wildtype and *bax* null mutant mice was assessed at E12, E14 and E16. The results are shown in Figure 4.11.

Figure 4.11: Relative levels of NGF mRNA in the maxillary target field of wildtype and *bax* null mutants

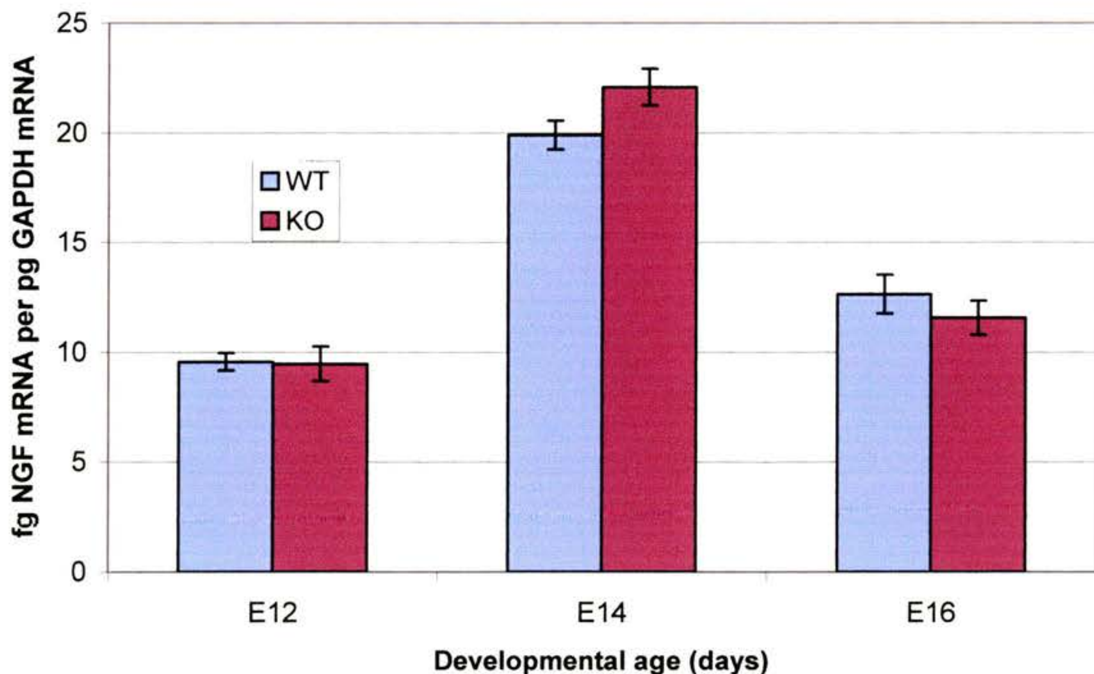


Mean relative levels of NGF mRNA relative to GAPDH mRNA in the maxillary target field of wildtype mice (WT-blue) and *bax*-/- mice (KO-red) at E12, E14 and E16. Bars represent the mean ± standard error (n=7-29 per data point).

A slight increase in NGF expression in the maxillary target field of the Bax null mutants compared to their wildtype littermates was observed at E12, although this was statistically insignificant (t-test; $p>0.05$). No differences in NGF mRNA were observed at the other ages examined.

Ablation of the *bad* gene of the mouse (provided by Stanley Korsmeyer, unpublished) has similarly been found to result in increased neuronal numbers in the trigeminal ganglion (Victoria Barker and Gayle Middleton, personal communication – not verified). NGF expression in the maxillary target field of wildtype and *bad* null mutant mice was assessed at the ages of E12, E14 and E16. The results can be observed in Figure 4.12.

Figure 4.12: Relative levels of NGF mRNA in the maxillary target field of wildtype and *bad* null mutants

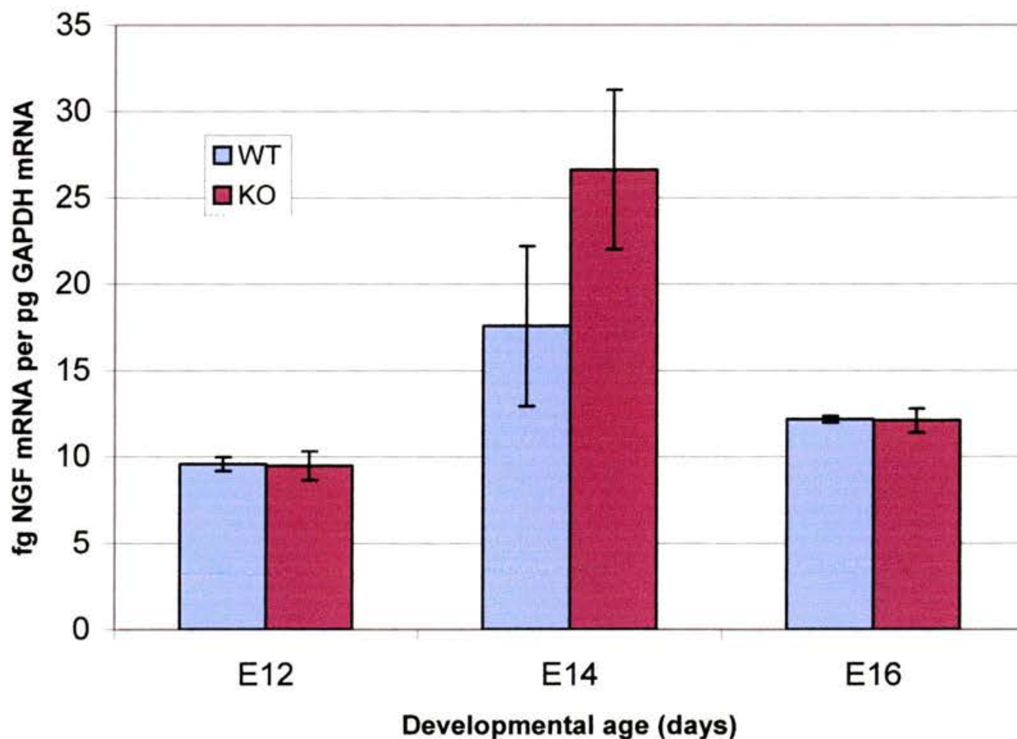


Mean relative levels of NGF mRNA relative to GAPDH mRNA in the maxillary target field of wildtype mice (WT-blue) and *bad-/-* mice (KO-red) at E12, E14 and E16. Bars represent the mean ± standard error (n=12-30 per data point).

A slight increase in NGF expression in the maxillary target field of the *bad* null mutants compared to their wildtype littermates was observed at E14, although this was statistically insignificant (t-test; $p>0.05$). No differences in NGF mRNA were observed at the other ages examined.

Finally, an attempt was made to collect *bad/bax* double mutant mice. This was hampered by the very low occurrence of double mutant mice, and especially the very low occurrence of wildtype and double mutants in the same litters, making a precise comparison difficult. The data for these double mutants can be seen in Figure 4.13, showing no significant differences in the levels of NGF mRNA.

Figure 4.13: Relative levels of NGF mRNA in the maxillary target field of wildtype and *bax/bad* double null mutants



Mean relative levels of NGF mRNA relative to GAPDH mRNA in the maxillary target field of wildtype mice (WT-blue) and *bad-/-; bax-/-* mice (KO-red) at E12, E14 and E16. Bars represent the mean ± standard error (n=2-30 per data point).

The results using null mutant mice with higher neuronal complements in the trigeminal ganglion did not appear to have any significant effect on NGF expression in the maxillary target field, in vivo. This may suggest that the observations in vitro do not replicate events in vivo. However, it appears that neuronal numbers in both the *bax* and the *bad* null mutants are not actually higher than in wildtype embryos until E14 at the earliest (Victoria Barker and Gayle Middleton, personal communication). The differences in NGF mRNA expression detected in vitro were most significant in much earlier co-cultures. Thus, if neuronal numbers in the trigeminal ganglion of *bax* and *bad* null mutants are only increased in the later stages of naturally occurring cell death, one would not expect these null mutants to display significantly different levels of NGF mRNA.

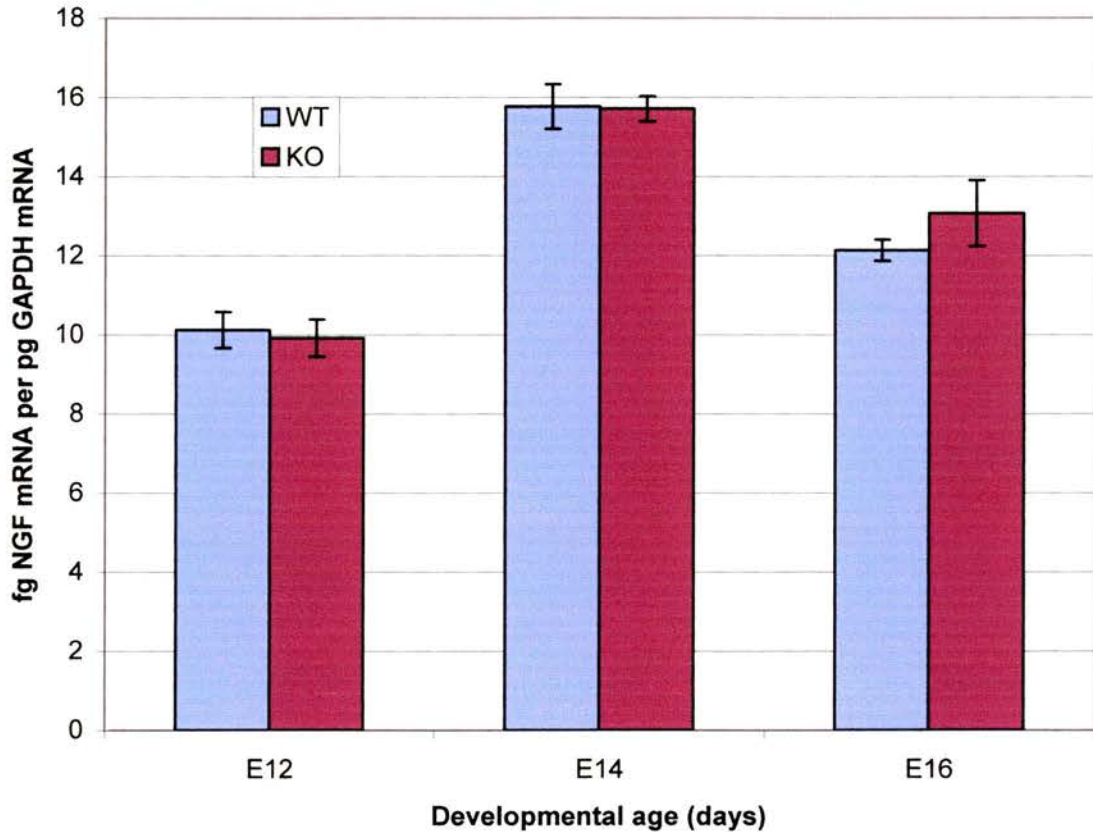
4.2.4 Expression of NGF mRNA in the maxillary target field of knockout mice with reduced neuronal numbers in the trigeminal ganglion during development

NGF mRNA was assessed using competitive RT-PCR in four mutants with reduced neuronal numbers at different stages of embryonic development, to ascertain whether this would result in decreased levels of NGF mRNA in the target field.

Ablation of the *trkA* gene in the mouse (Smeyne et al., 1994) causes increased neuronal death and decreased neuronal numbers in the developing trigeminal ganglion after the age of E13 (Piñon et al., 1996; chapter 5). NGF expression in the maxillary target field of wildtype and *trkA* null mutant mice was assessed at the ages of E12, E14 and E16. The results are shown in Figure 4.14. These results showed

that the reduction in neuronal numbers in the trigeminal ganglia of *trk*A null mutants, occurring during the later stages of target field innervation, had no effect on NGF mRNA levels in the maxillary target field. No significant differences were observed at any of the ages studied.

Figure 4.14: Relative levels of NGF mRNA in the maxillary target field of wildtype and *trkA* null mutants

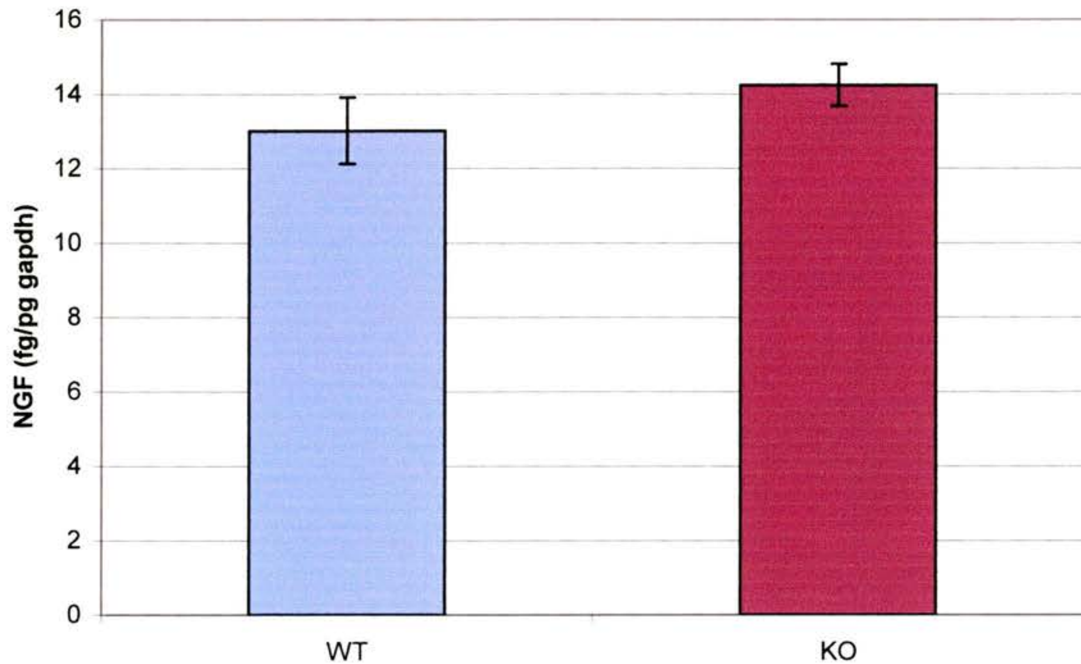


Mean relative levels of NGF mRNA relative to GAPDH mRNA in the maxillary target field of wildtype mice (WT-blue) and *trkA*-/- mice (KO-red) at E12, E14 and E16. Bars represent the mean ± standard error (n=9-16 per data point).

Like the study on *trkA* null mutants, embryos with a mutation in *p75* (Lee et al., 1992), which results in lower neuronal numbers in the trigeminal ganglion during the

latter stages of embryonic development (chapter 5), where found to have normal levels of NGF mRNA expression at E14 (t-test; $p>0.05$) (Figure 4.15).

Figure 4.15: Relative levels of NGF mRNA in the E14 maxillary target field of wildtype and *p75* null mutants

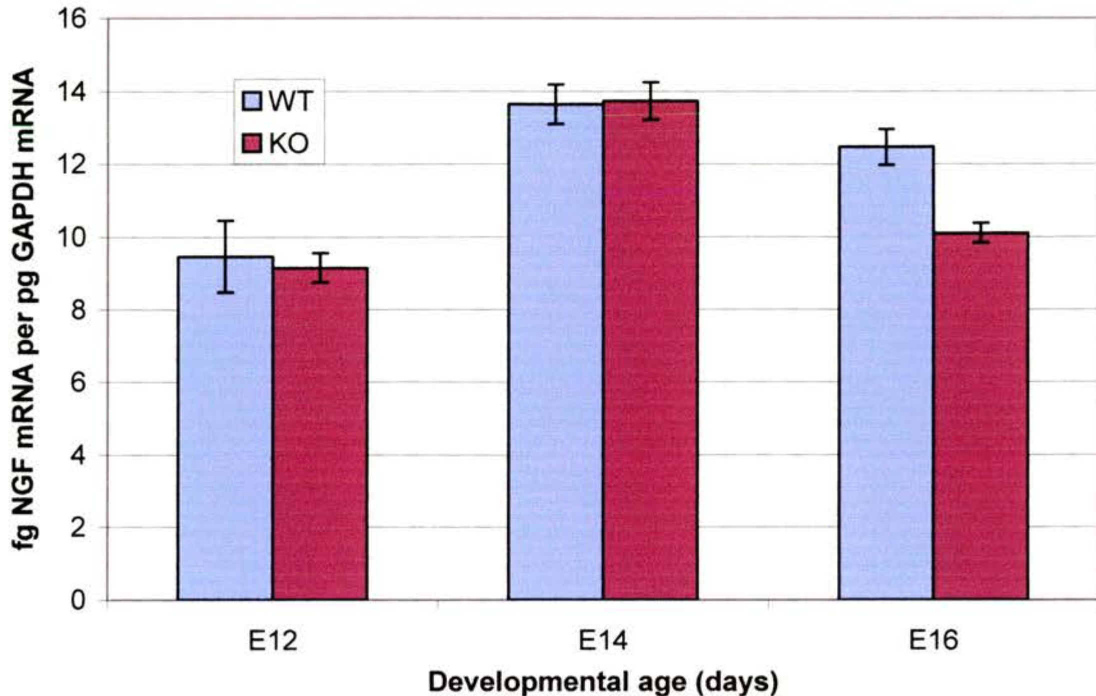


Mean relative levels of NGF mRNA relative to GAPDH mRNA in the E14 maxillary target field of wildtype mice (WT-blue) and *p75-/-* mice (KO-red). Bars represent the mean ± standard error (n=3-6 per data point).

Ablation of the *trkB* gene in the mouse (Smeyne et al., 1994) causes increased neuronal death in the developing trigeminal ganglion at the age of E11 (Piñon et al., 1996). Prior to this stage, neurons appear to survive independently of trkB signalling (Buchman and Davies, 1993). The *trkB* null mutant thus looses neurons during the main period of target field innervation. NGF mRNA expression in the maxillary target field of wildtype and trkB null mutant mice was assessed at E12, E14 and E16.

Figure 4.16 shows that there were no significant differences in NGF expression between *trkB* null mutants and wildtype embryos at E12 and E14 (t-test; $p>0.05$). Paradoxically, a difference in NGF mRNA levels emerged at E16, with the *trkB* null mutants displaying a significant 20% decrease in NGF mRNA levels in the maxillary target field compared to wildtype embryos (t-test; $p<0.001$). This effect is unlikely to be related to initial innervation density, since NGF expression at previous ages appeared normal. This may identify a novel function of trkB in regulating NGF expression in the maxillary territory later in development during the period of naturally occurring cell death. The lack of effect of the *trkB* mutation on NGF mRNA expression in the early maxillary target field, suggests that the loss of neurons during the earliest stages of innervation by the trigeminal ganglion neurons does not affect NGF expression. This does not necessarily conflict with the idea that early innervation can modulate NGF expression for at least two reasons: First, some neurons reach the trigeminal ganglion prior to this stage (Stainier and Gilbert, 1991). Second, the magnitude of neuron loss at E11 in *trkB-/-* mice may be insufficient to have a significant effect on NGF mRNA expression. Although the number of dying cells in the trigeminal ganglion of *trkB-/-* embryos increases three-fold compared with wildtype, the number of surviving neurons at this stage is not known, and by E13 when total neuron counts were made there was only a 40% reduction (Piñon et al., 1996).

Figure 4.16: Relative levels of NGF mRNA in the maxillary target field of wildtype and *trkB* null mutants

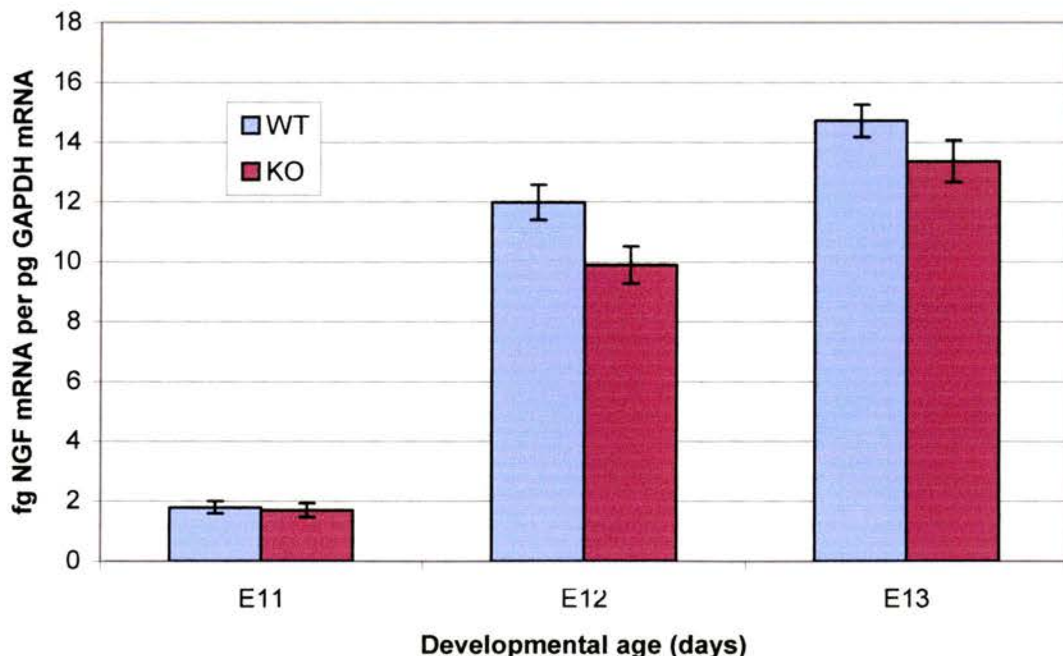


Mean relative levels of NGF mRNA relative to GAPDH mRNA in the maxillary target field of wildtype mice (WT-blue) and *trkB-/-* mice (KO-red) at E12, E14 and E16. Bars represent the mean ± standard error (n=7-17 per data point).

Ablation of the *erbB3* gene in the mouse (Riethmacher et al., 1997) causes deficits in migration and differentiation of cranial neural crest cells, resulting in a very substantial reduction in neuronal numbers in the trigeminal ganglion from the earliest stages of neurogenesis. As reported in chapter 6, the neuronal complement of the trigeminal ganglion of *erbB3-/-* is reduced by 60% at E11 and by over 90% at E13. NGF expression in the maxillary target field of wildtype and ErbB3 null mutant mice was assessed at E11, E12 and E13. Assessments could not be made later in development, because very few *erbB3-/-* embryos survive.

Figure 4.17 shows that although no decrease in NGF expression was observed in *erbB3-/-* mice at E11, NGF mRNA levels were significantly lower (18%) at E12 (t-test; $p<0.05$). No difference would necessarily be expected at E11, due to the low levels of NGF mRNA at this stage preceding the major increase in NGF expression seen between E11 and E13. A small decrease (10%) in NGF mRNA was also observed in *erbB3-/-* mice at E13 as well, although this was not statistically significant (t-test; $p>0.05$). This study suggests that changes in early innervation of the target field may, to some extent, modulate NGF mRNA expression in vivo, although the lower insignificant decrease in NGF mRNA by E13 may suggest that this effect is transient in vivo.

Figure 4.17: Relative levels of NGF mRNA in the maxillary target field of wildtype and *ErbB3-/-* embryos



Mean relative levels of NGF mRNA relative to GAPDH mRNA in the maxillary target field of wildtype (WT-blue) and *ErbB3-/-* mice (KO-red) at E11, E14 and E16. Bars represent the mean ± standard error (n=12-22 per data point).

4.3 Discussion

In vitro studies using E10.5 or E11 maxillary target fields, cultured either together or without the trigeminal ganglion neurons innervating them, revealed that early target field innervation can positively influence NGF mRNA expression in vitro. This effect was greater in earlier (E10.5) explants than in later (E11) explants, suggesting that initial early innervation is potentially more effective at inducing this change than later innervation.

The concept that innervating axons can control NGF expression is not new, with several in vivo studies suggesting such an effect in adult animals. In the adult rat, denervation of sympathetic fibres projecting to vascular tissue results in decreased levels of NGF in these targets four days after the operation (Liu et al., 1996). Further, experiments in the adult rat demonstrate that the proximity of some sympathetic and parasympathetic neurons (closely juxtaposed within a common Schwann cell sheath) maintains NGF expression in the latter, thus promoting sympathetic neuron survival through prejunctional interactions. This was demonstrated by removal of the superior cervical ganglion, which results in reduced levels of NGF expression in the pterygopalatine cranial parasympathetic ganglion in vivo (Hasan and Smith, 2000). These experiments in adult animals are the more significant for demonstrating such interactions in vivo. The explant experiments described in this chapter are the first to demonstrate such modulation of NGF expression by innervating neurons during embryonic development and initial innervation.

The findings of a descriptive study in the mouse trigeminal system are consistent with the notion that initial innervation might influence NGF expression. The main peripheral cutaneous target territories of the trigeminal ganglion (maxillary, mandibular and opthalmic territories) were found to synthesize NGF in proportion to the final innervation density they receive from neurons in the trigeminal ganglion (Harper and Davies, 1990). The relative proportions of neurons in the ganglion innervating the maxillary, mandibular and opthalmic territories are the same preceding and following the period of embryonic cell death (Davies and Lumsden, 1984). That is, the proportion of neurons lost among those subsets of neurons that innervate each territory is the same (~50%). This indicates that some sorting of neuronal innervation in relation to final innervation density of the target field occurs prior to neurons reaching their target fields, and that regional differences in NGF synthesis are not responsible for sculpting out these differences. This is consistent with the idea that the required level of NGF synthesis in each territory is regulated by the number of arriving axons (Davies et al., 1987). The in vitro observations in this section would support such a mechanism, suggesting that the initial number of axons contacting the target field may influence the level of NGF synthesis to some extent.

Many of the results in vivo can be explained by the timing of neuronal death or improved survival in the null mutants employed. In *Bax*, *Bad*, *trkA* and *p75* null mutants, changes in neuronal numbers do not occur until E13/E14, which is well beyond the early period of innervation of the maxillary process by trigeminal sensory neurons. The results obtained for *trkB-/-* embryos do not necessarily conflict with the idea that early innervation can modulate NGF expression, due to some neurons

reaching the trigeminal ganglion prior to this stage (Stainier and Gilbert, 1991), and the magnitude of neuron loss at E11 being insufficient to have a significant effect on NGF mRNA expression (Piñon et al., 1996).

ErbB3-/- embryos have a very early loss/absence of neural crest-derived neurons. They lack two-thirds of the normal neuronal complement in the trigeminal ganglion at E11. The other knockout displaying early neuronal loss, the *trkB* knockout, displays a 40% loss of neurons in the ganglion by E12. However, neuronal numbers in the trigeminal ganglion at E11 are unknown.

Some effects of neuronal numbers in the trigeminal ganglion on NGF mRNA in the maxillary process were observed in *ErbB3-/-* embryos, but they were not maintained by E13. Although neuronal numbers are greatly reduced in the trigeminal ganglion of *ErbB3-/-* mice, the outgrowth towards the maxillary process initially appears to be normal (see chapter 6), suggesting that initial innervation of the maxillary process is less affected by the large loss of neurons in this ganglion. This could explain the relatively small differences in NGF mRNA levels observed. Additionally, pioneer axons were proposed to be placode-derived (Stainier and Gilbert, 1991), a neuronal population in the trigeminal ganglion less affected in the ErbB3 null mutant (chapter 6). Together, the in vitro and in vivo data appear to suggest some effect of neuronal innervation on NGF expression in the maxillary target field.

There is evidence, however, that this may not be the case in all tissues. Rohrer and colleagues demonstrated that levels of NGF mRNA of intact chick leg skin and leg skin denervated prior to the arrival of the earliest axons were identical, which would suggest that NGF synthesis in the skin of chick embryos is independent of its

innervation (1988). Similar studies have suggested that innervating sympathetic neurons do not influence NGF mRNA production in the heart, even though the onset of NGF expression in this target coincides with innervation (Clegg et al., 1989). The aforementioned study on sensory neurons in the frog limb would also argue against such a mechanism, since the number of neurons innervating the hind-limb bud after bilateral innervation and programmed cell death on both sides added up to the neuronal numbers usually observed on one side (Lamb et al., 1989). This has led to the assumption that NGF synthesis is not dependent on nerve-target contact but appears to occur as part of an intrinsic developmental program in the target field (Davies, 1990). The above-presented findings in vitro do not contradict that increases in NGF mRNA levels are intrinsically regulated. The substantial increases in NGF expression observed in the E10.5 and E11 maxillary processes grown alone suggest that NGF expression may indeed be significantly controlled by intrinsic mechanisms. A factor to consider, however, is the difference in the sensitivity of the methods employed. Competitive RT-PCR is likely to detect more subtle differences in NGF mRNA than the northern blotting technique used in previous experiments. If one trics to integrate the in vitro results in this section with previous literature suggesting that NGF expression is regulated by intrinsic mechanisms, it could be hypothesized that, at least for the maxillary process and its innervating axons: 1. Expression of NGF in the target field develops as part of an intrinsic developmental program in the target field. 2. Initial regional differences in innervation density are intrinsically regulated. 3. This intrinsic developmental program of NGF expression in the target field may be modulated by the innervating axons, to account for developmental differences in the number of innervating axons or the timing of arrival of the innervating axons. Thus

initial innervation may not be involved in directly controlling whether the target field synthesises NGF or not, but it may be able to modulate the onset/rate of increase in NGF expression, depending on the timing of innervation and/or the numbers of innervating neurons.

Thus, early innervation could modulate the levels of NGF mRNA, ensuring adequate supply of NGF as the innervating axons arrive in greater numbers at the target field, which normally would be about E11. This might create a safety net for axonal processes arriving within a time-window where NGF can be modulated by the first arriving axons and for neurons arriving in slightly higher numbers than intrinsically 'set' in the target field. If NGF expression was strictly intrinsically set, small differences in the onset of innervation could produce dramatic differences in the numbers of neurons surviving the periods of neurogenesis before E13. If one imagines innervation occurring slightly early or at slightly increased numbers, before the intrinsically regulated increase in NGF expression, this could potentially cause a significant proportion of neurons to die due to the levels of NGF not being adequate as these early-comers arrive.

'Fine-tuning' interactions between the innervating axons and their target field(s) have previously been suggested in some placode-derived sensory neurons, which undergo an intrinsically regulated period of trophic factor independence (Vogel and Davies, 1991). Nodose neurons, for example, survive independently of trophic factors for intrinsically pre-determined periods in vitro, correlating with the distance their axons grow to their target fields before becoming dependent on the survival

factor released from their target fields in vivo (BDNF). The onset of responsiveness of early nodose neurons to BDNF, however, can be accelerated by transiently exposing them to BDNF within 24 hrs of the time they normally start responding to BDNF (Vogel and Davies, 1991). This was interpreted as an indication that the intrinsic programme in early neurons can be fine-tuned, and that the target field could provide the final adjustment by its provision of the requisite neurotrophin. In line with the results presented above, it is conceivable that such fine-tuning of intrinsic mechanisms occurs in the other direction, with the first axons reaching the target field adjusting the timing/rate of increase of the intrinsic mechanism that controls NGF synthesis. Axons could regulate the expression of NGF in the target field to be 'ready' to support the second wave of axons that follow, thus ensuring that enough NGF is available as neurons arrive at the target fields. Such a mechanism would allow for slight variations of the time point of arrival of sensory neurons at the target field, as well as slight variations in number, ensuring that optimal numbers of neurons are maintained prior to naturally occurring cell death. This hypothesis would not necessarily imply that higher numbers of neurons would be supported later in development, it may be that the modulation of NGF levels achieves an optimal level of support at the peak of NGF expression at E13/E14, an effect that may not be maintained when NGF levels decrease later in development.

An interesting observation in this context is that the difference between the E10.5 maxillary target field grown with its innervation intact, or not, was smaller after 60 hrs in culture than after 48 hrs in culture. Also, the effect observed in the ErbB3 null mutants was larger at E12 than it was at E13. This may suggest that, even though

initial innervation appears to influence NGF expression in the maxillary process during the period of NGF mRNA increase, the effect observed may not be maintained as the levels of NGF expression approach their peak levels at E13/E14.

The extent to which early arriving neurons may be able to modulate NGF mRNA expression in vitro will need to be determined using younger ages, which would also clarify the extent to which induction of NGF expression is intrinsically regulated in the maxillary process. This is made difficult by the problems encountered when growing such young ganglia in culture, however (see chapters 2 and 3). Also, it will be necessary to establish whether such differences are maintained at later stages in vivo. Finally, additional mouse mutants may become available to confirm and extend the results of the experiments reported here. It is possible that the differences in NGF mRNA levels observed in *ErbB3-/-* embryos may not be primarily due to reductions in neuronal numbers in the trigeminal ganglia of these embryos but to some unrelated effect of the mutation. Hence it would be beneficial to study NGF expression in embryos that display a marked early reduction in trigeminal neurons (such as *Sox10-/-* mice). It may also be beneficial to study the effects of mutations that affect early innervation in vivo by interfering with the early growth and branching of axons from the trigeminal ganglion to the maxillary process. Such models include semaphorin III/D (Kitsukawa et al., 1997; Taniguchi et al., 1997), Neuropilin-2 (Chen et al., 2000; Giger et al., 2000), netrin-3 (Løes et al., 2003), or Brn3a (Eng et al., 2001) null mutants, all of which display deficits in sensory axon growth to the maxillary process.

If further evidence for modulation of NGF expression by innervation could be confirmed, the main question would be to identify the mediators of such modulation. IL-1, EGF, FGF, TGF-α and TGF-β1 have all been found to increase NGF mRNA expression in astrocytes (Spranger et al., 1990; Linholm et al., 1990; 1992). Cholecystokinin-8 has recently been found to increase NGF mRNA and protein in peripheral tissue of adult mice (Manni et al., 2000). Rohrer and colleagues have also discovered an activity produced in older maxillary processes (E11.5) that was able to increase NGF mRNA levels in younger maxillary processes (E10/E10.5) far more efficiently than when these were cultured in normal medium (Rohrer et al., 1993). Thus it is possible that a developmentally regulated inducer of NGF is produced in the older maxillary process. This inducer has been suggested to be TGF-β, since it is present in the maxillary territory at the stage that NGF mRNA expression begins, and increases in vivo during the early stages of innervation. TGF-β also increases NGF mRNA levels in early maxillary territory cells in vitro (Buchman et al., 1994). During development, TGF-β is produced and released by neurons, an event that was thus far mainly linked with its function as an inhibitor of Schwann cell proliferation and myelination (Einheber et al., 1995). However, in view of the evidence presented above, it is conceivable that it may be involved in modulating NGF mRNA expression in the target field as well. Further studies in vitro, using inhibitors to substances known to increase NGF expression, may provide more insight into the substances responsible for the effect of early innervation on NGF expression in the target field.

Even though I have argued for a modulatory role for early innervation in regulating NGF mRNA expression, there are other possible explanations for the effects observed in the maxillary/trigeminal co-culture experiments.

First, it is possible that the increase in mRNA expression in the maxillary process is not intrinsically regulated, but entirely controlled by the early innervating neurons. As illustrated in the introduction, different neuronal populations or target fields may interact in different ways. This would need to be assessed using younger explants or null mutants with complete absence of early maxillary innervation.

Second, the lesion of neuronal connections involved in setting up the cultures may have caused the alterations in NGF mRNA in the maxillary process, making this more of a model of peripheral nerve injury than of the effects of early target field innervation. However, NGF has thus far generally been found to be up-regulated in non-neuronal cells upon nerve lesion (e.g. Dethleffsen et al. 2002), although this has only been studied in postnatal animals.

Third, another explanation is that the prevention of innervation of the early maxillary process somehow interfered with the survival of its NGF secreting cells.

Conclusion

The data presented in this chapter, together with previous studies, suggest a model in which the timing and rate of increase in NGF expression is regulated by two mechanisms: An intrinsic developmental programme in the target field (Rohrer et al., 1988; Clegg et al., 1989) and modulation of this programme by the earliest innervating axons. This latter modulatory influence of sensory axons can fine-tune the onset of neurotrophin synthesis upon target field contact, in a similar manner in

which the onset of neurotrophin dependency can be fine-tuned by target field contact (Vogel and Davies, 1991; Davies, 1994). The in vitro studies presented here suggest that such a mechanism may be mediated by pioneer axons, the first to reach the target field.

Figure 4.18: Modulation of NGF expression in the maxillary target field by early innervation

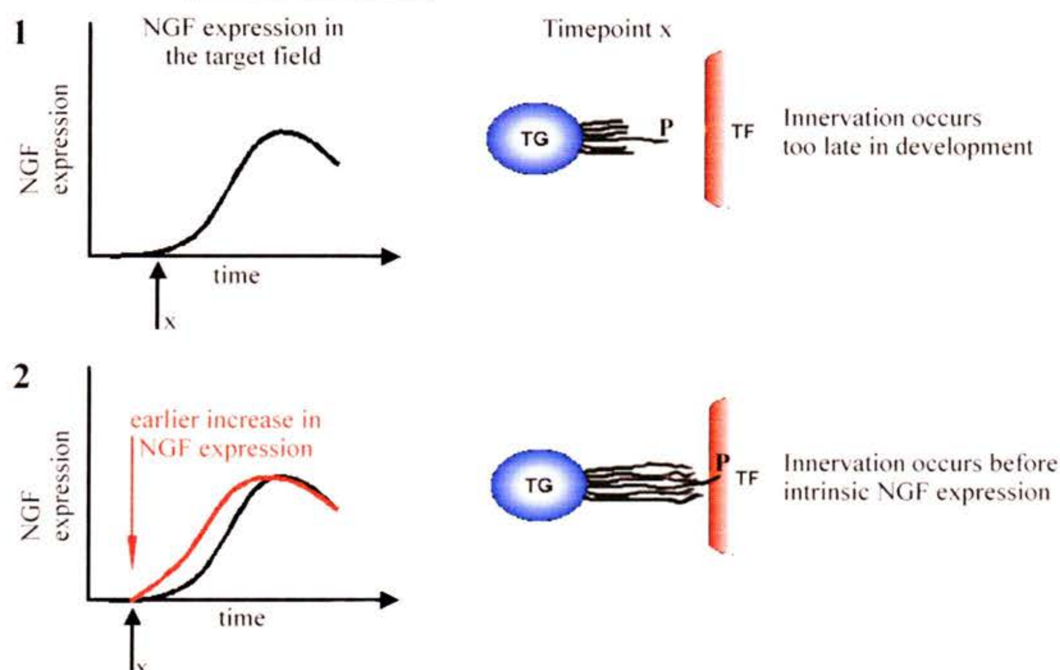


Illustration of the way in which NGF expression may be controlled by an intrinsic developmental programme in the target field, and how the modulation of this intrinsic programme by the earliest innervating axons can adapt NGF supply to differences in the timing of innervation. The right column shows two different time points in target field innervation (timepoint x), and the left column shows NGF expression over time and the effect exerted on it by different innervation timing or lack of innervation (x). (1) If neuronal processes arrive at the target field at an unusually late point in development (or not at all) and the target field has received no signal from the pioneer axons (P), an intrinsic programme of NGF expression is executed that will ensure that any neurons that are still growing towards the target field have sufficient survival factors to maintain them in the last stages of innervation (should they become survival-dependent while still growing). (2) When neurons arrive at the target on time or even too early in development, the pioneer axons signal this to the target field, and the programme of NGF expression is initiated earlier (red line, the black shows intrinsic programme that would occur with late/absent innervation). The intrinsic programme could thus be seen as a 'safety net' for late arriving neuronal processes. Neurons that reach the target field on time or before they are due need a supply of NGF earlier, which is initiated by the pioneer axons reaching the target field. This may not necessarily mean that more neurons will survive the period of naturally occurring cell death (PCD) if they arrive early. Although NGF synthesis begins earlier, the maximum level of NGF expression and the amount of NGF that neurons are supplied during PCD may be the same in both cases, resulting in similar neuronal numbers after PCD. Such an intrinsic programme modulated by early arriving axons may thus serve to maximise survival before PCD and therefore optimise competition during it. TG: Trigeminal ganglion; TF: Target field; P: Pioneer axons.

An advantage of such a system would be to provide a safety net for innervating neurons (Figure 4.18). The arrival of pioneer axons would provide an advance signal to the target of the arrival of the second, main wave of axons, enabling it to fine-tune its levels of NGF expression for their arrival. This would ensure that NGF levels are adequate for the actual timing of arrival by innervating axons. Should the main wave of axons reach the target field late, neurons may have started 'switching' their survival dependence from BDNF and NT-3 to NGF due to target unrelated cell interactions within the ganglion (Buchman and Davies, 1993; Piñon et al., 1996; Enokido et al., 1999). Should this occur for some reason, intrinsically up-regulated levels of NGF would be able to support these neurons as their axons grow the remainder of the way to the target field. Such a hypothesis would thus ensure minimal loss of neurons in circumstances of both early and late arrival.

CHAPTER V

Developmental changes in embryonic sensory neuron dependency on trkA, trkB and p75 signalling

5.1 Introduction

Neuronal survival in the developing vertebrate nervous system has been found to depend on several trophic factor families. One such family, the neurotrophins, plays a major role in the nervous system, supporting a variety of sensory and sympathetic neurons (e.g. Davies, 1994; Fariñas, 1999; Davies, 2003). The neurotrophin family members in birds and mammals are nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and –4 (NT-4). They exert their actions through the Trk tyrosine kinase receptors and another receptor, p75 (Barbacid, 1994). TrkA is the preferred receptor for NGF, TrkB for BDNF and NT-4, and TrkC for NT-3, although some signalling of NT-3 can occur through trkA and trkB. The p75 receptor has a similar affinity for all neurotrophins.

Most developing sensory neurons initially appear to be independent of neurotrophins at the early stages of outgrowth towards their targets (Davies and Lumsden, 1984; Ernsberger and Rohrer, 1988; Vogel and Davies, 1991). Many cranial ganglion neurons then become dependent on BDNF or NT-3 as they approach their target fields (Vogel and Davies, 1991). Although many neuronal populations maintain dependence on these factors at later stages of development, including the phase of naturally occurring cell death, the neurons of other ganglia subsequently become dependent on NGF during the early stages of target innervation (Buchman and Davies, 1993; Buj-Bello et al., 1994). Some populations of neurons thus appear to switch their dependence from one neurotrophic factor to another during embryonic development. This observation was termed 'neurotrophin switching' (Davies, 1994).

One of the most extensively studied populations of neurons in which this neurotrophin switching takes place is the trigeminal ganglion. At approximately E10 trigeminal neurons start growing axons towards their peripheral target fields (Davies and Lumsden, 1984). When they are removed at this point and grown in culture, they become survival-dependent on BDNF or NT-3 after 24 to 48 hours following a brief phase of neurotrophin independence, i.e. they died if neither of these neurotrophins were added to the culture medium. This coincides with the point at which the first of the axons approach their peripheral target fields in vivo. Very few neurons are dependent on NGF at this stage. However, neurons cultured at successive stages of development become more and more dependent on NGF and less on BDNF and NT-3, until almost all neurons are dependent on NGF by E13. At intermediate stages the number of surviving neurons in cultures is not significantly higher in medium containing NGF plus BDNF or NT-3 than in medium containing the factors alone, suggesting that the neurons are able to respond to all three neurotrophins at this stage (Buchman and Davies, 1993; Paul and Davies, 1995). A recent study has suggested that much of the switching effect found may be due to sequential generation of BDNF and NGF-dependent neurons (Huang et al., 1999). However, BrdU labelling experiments have shown that at least some of the neurons do switch their response form one neurotrophin to another in vivo (Enokido et al., 1999).

In vitro results are supported by expression studies of the neurotrophins and their receptors. BDNF and NT-3 mRNA are already expressed in the mesenchyme, when the earliest trigeminal axons are growing through this tissue to the periphery, whereas NGF mRNA expression begins in the target field epithelium slightly later with the arrival of the first axons (Buchman and Davies, 1993; Wilkinson et al.,

1996; Arumae et al., 1993; Davies et al., 1987). Early in development the levels of trkB and trkC mRNA are much higher than trkA, whereas later, as most neurons become NGF dependent, the level of trkA mRNA is much higher (Ernfors et al., 1992; Arumae et al., 1993; Wyatt and Davies, 1993). Also, there is a relative increase in the expression of a truncated form of trkB, which lacks the kinase domain, and which can act as a negative modulator of BDNF signalling (Ninkina et al., 1996). Another study found that mRNA for more than one of the trk receptors is present in developing rat trigeminal neurons during the switchover period (Moshnyakov et al., 1996).

Knockout studies of neurotrophin receptors also support the concept of neurotrophin switching in vivo. In *trkB*-deficient embryos, the number of early neurons undergoing apoptosis is greatly increased compared with wildtypes, whereas in *trkA*-deficient embryos, the number of apoptotic neurons is increased later in development. In *trkC*-deficient embryos, apoptosis is not increased as much as in *trkB*-deficient embryos early in development (Piñón et al., 1996). Also, it was found that the non-catalytic trkC receptors outnumber the catalytic ones in early trigeminal ganglia (Wyatt et al., 1999). In *NT-3*-deficient embryos, however, there is a decrease of neuronal numbers in the trigeminal ganglion before the peak of naturally occurring cell death, suggesting that NT-3 may be acting through a different receptor (ElShamy and Ernfors, 1996; Wilkinson et al., 1996; Huang et al., 1999). It has been demonstrated that NT-3 in *trkC*-deficient mouse trigeminal neurons acts via trkA and trkB receptors in vitro (Davies et al., 1995), and the possibility that NT-3 acts via

these two receptors in addition to trkC in vivo has recently been supported (Huang et al., 1999).

Although it is not quite clear what induces neurons to switch their responsiveness from BDNF and NT-3 to NGF, it has been hypothesised that this occurs due to a signal the neurons receive in vivo, since E10 neurons do not lose their responsiveness to BDNF in vitro (Paul and Davies, 1995). It has been demonstrated that this signal does not originate from the target field, and it is thus likely that the switch depends on cell interactions within the ganglion (Enokido et al., 1999).

The trigeminal ganglion appears to undergo further changes in its survival requirement later in development. During the late fetal period, trigeminal ganglion neurons are supported by GDNF, CNTF, LIF, OSM, CT-1 and MSP in vitro, several days after the neurons have become NGF-dependent (Buj-Bello et al., 1995; Horton et al., 1998; Forgie et al., 2003).

Since the discovery of neurotrophin switching in the trigeminal ganglion a number of other instances of trophic switching have been identified. In DRG, for example, neurons dependent on NGF or NT-3 during early development may become dependent on LIF, GDNF, NTN, ART or bFGF later in development (Molliver et al., 1997; Baudet et al., 2000; Acosta et al., 2001). Sympathetic neurons have also been reported to change their survival dependence during development. Cells from early sympathetic ganglia could be maintained alive by neurotrophin-3 (NT-3) in a serum-free medium, but not by NGF. By birth, these sympathetic neurons were supported by NGF, whereas NT-3 supported survival only weakly and at very high doses (Birren et al., 1993). This was associated with a reciprocal switch in the expression

of trkC and trkA mRNAs in vivo (e.g. Birren et al., 1993; Wyatt and Davies, 1995). In *NT3* knockout mice, however, a decrease in the number of SCG neurons was only observed at later stages in development, when the neurons were also dependent on NGF for survival (Wyatt et al., 1997; Francis et al., 1999). In addition to neurotrophins, artemin is able to enhance sympathetic neuron survival at distinct stages of embryonic and postnatal development, in this case just after terminal mitosis and just before the neurons acquire neurotrophin independence in the adult. Between these stages, no survival effect of artemin was observed. Thus some neurons may switch their survival requirement from artemin to NGF and/or NT-3 and then back to artemin at later stages before finally becoming neurotrophin independent (Andres et al., 2001).

The p75 receptor shares no sequence similarity to the trk receptors and utilises distinct neurotrophin-dependent signalling pathways (reviewed by Roux and Barker, 2002; Hempstead, 2002). The p75 receptor is expressed in the peripheral nervous system from the earliest stages in development, being already present in migrating neural crest cells (Heuer et al., 1990). Levels then increase substantially as the ganglia form and the crest cells differentiate. In the trigeminal ganglion, p75 is initially expressed at low levels, and like trkA increases simultaneously with the acquisition of the NGF survival response (Wyatt and Davies, 1993). The function of p75 is less well understood than the functions of the trk receptors. For trigeminal ganglion neurons, in vitro studies have demonstrated that the dose response of p75 null mutant neurons to NGF is shifted to higher NGF concentrations (Davies et al., 1993). A similar effect has since been observed in other ganglia of the peripheral

nervous system, such as the DRG and SCG (Lee et al., 1994). This indicates that p75 enhances the sensitivity of NGF-dependent neurons to NGF, a hypothesis reinforced by the finding that mutant NGF which binds trkA but not to p75 is less efficient at promoting the survival of sensory trigeminal and SCG neurons (Horton et al., 1997). Although it has been shown that overexpressing trkA or trkB with p75 increases the binding affinity of trkA for NGF and trkB for BDNF, NT-3 and NT4/5 (Mahadeo et al., 1994; Bibel et al., 1999), other studies suggest that the sensitising effect is restricted to NGF, since p75 deficient neurons do not display decreased sensitivity to other members of the neurotrophin family for survival (Davies et al., 1993; Lee et al., 1994). The rate at which NGF can associate with trkA has been shown to increase 25-fold when p75 is co-expressed (Mahadeo et al., 1994), which may explain the increased activation of trk receptors by low concentrations of neurotrophins when p75 is also present (Hantzopoulos et al., 1994; Verdi et al., 1994). This binding- and survival-enhancing role of p75 is confirmed by neural losses observed in p75-deficient mice in embryonic and postnatal trigeminal, SCG and DRG (Lee et al., 1992; 1994). A recent study has additionally demonstrated that p75 increases the specificity of trkA for NGF. The survival-promoting effect of NT-3 via trkA is impaired by p75 in sympathetic neurons (Brennan et al., 1999).

In addition to this role in enhancing survival, p75 has a prominent role in inducing cell death in neurons upon NGF binding in the absence of trk receptors (Frade et al., 1996; Bamji et al., 1998; Davey & Davies 1998). This mechanism is unlikely to occur in the developing trigeminal ganglion since p75 and trkA are co-expressed in development (Wyatt and Davies, 1993) and neurons expressing the p75 receptor

alone are rare during the phase of neurogenesis in the trigeminal ganglion (Moshnyakov et al., 1996).

Some experiments suggest that p75 provides a constitutive cell death signal in sensory neurons in the absence of ligands, since the death of DRG neurons following neurotrophin withdrawal can be partially prevented by reducing p75 levels (Barrett and Bartlett, 1994). The mediators of the opposing actions of the p75 receptors are poorly understood, but it is likely that different adaptor proteins within the cell provide a bifunctional switch for cell survival or cell death decisions mediated by the receptor (Khursigara et al., 2001).

The aims of the experiments in this chapter were two-fold:

First, to assess the role trkA and trkB play in the development of the trigeminal ganglion by using mice with null mutations in the genes encoding these receptors, singularly and in combination. The aim was to establish whether there are periods during development where neurons require either or both trk receptors.

Second, to establish the stage in development at which p75 begins to influence neuronal survival in the trigeminal ganglion. Previous histological studies in *p75*-deficient mice have only quantified effects on neuronal numbers late in embryonic development or postnatally. Furthermore, the original deletion mutation of the *p75* gene (Lee et al., 1992) was generated by removal of the third exon in the gene. However, it has since been demonstrated that there is a naturally occurring p75 isoform resulting from removal of exon 3 by alternative splicing, and this protein is left intact in the original mouse mutant (von Schack et al., 2001). This protein (s-(short) p75) differs from the full-length receptor in that it only possesses one of the

four cysteine-rich domains found in the extracellular domain of the full-length receptor. To assess the role of this receptor, Schack et al. (2001) targeted exon four of the *p75* receptor locus, generating null mutants missing both isoforms (referred to as *p75D*). In the present study the effect of this null mutant was compared with the mutant lacking only the full-length p75 receptor (referred to as *p75L*).

5.2 Results

trkA/trkB double mutant embryos and *p75* mutant embryos were collected at E12, E14, E16 and E18. No *trkA/trkB* double mutants could be obtained from E18 litters, suggesting that the double mutation is lethal past the E16 developmental stage. The embryos were processed and the neuronal and pyknotic numbers quantified as described in chapter 2.

5.2.1 Neuronal counts in the trigeminal ganglion of *trkA/trkB* double knockouts

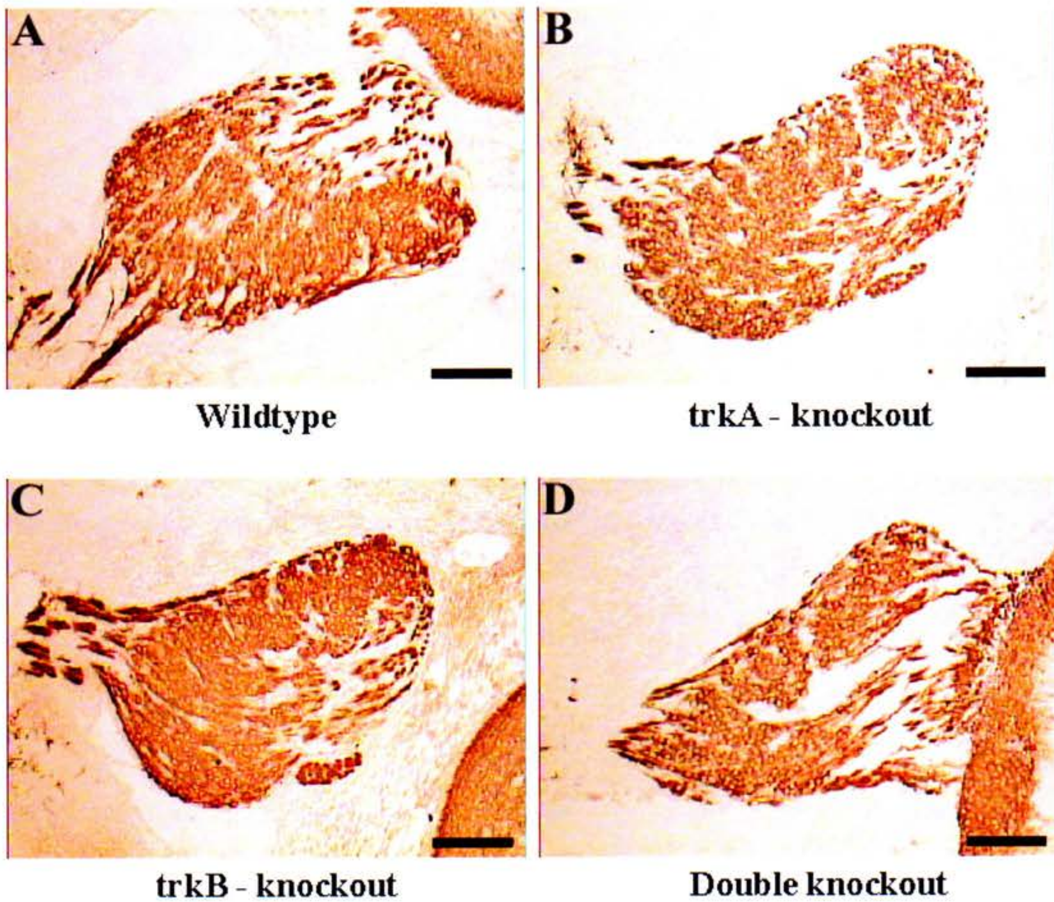
Neurons were counted on βIII-Tubulin and CFV-stained sections using the criteria outlined in chapter 2. The appearance of trigeminal neurons in sections of the knockouts at E12 and E14 are shown in Figures 5.1 and 5.2.

Neuronal loss in the trigeminal ganglion was observed in trkB and double null mutant embryos at E12 (Figure 5.3) (ANOVA; $p<0.01$). The neuronal numbers in the wildtype and *trkA*-knockout embryos were not significantly different, whereas the *trkB*-knockouts (Fisher's LSD; $p<0.005$) and double knockouts (Fisher's LSD; $p<0.005$) had significantly less neurons than wildtype embryos (38% and 42% less respectively). There was no significant difference between the *trkB*-knockout and the double knockout embryos. Taken together, the findings suggest that trkA has no effect on survival at this early stage in development. Also, it appears that at this early stage 58% of neurons were independent of either trk receptor for their survival. By E14 (ANOVA; $p<0.0001$), there was a significant 38% reduction in the neuronal numbers in the trigeminal ganglion of *trkA*-knockout embryos compared to wildtype (Fisher's LSD; $p<0.0001$). Interestingly, the neuronal numbers in *trkB*-knockout

embryos were now not significantly different to the ones in wildtypes (5% less neurons; not significant), suggesting that trkB had no more significant effect on neuronal survival at this age. It is possible that more neurons were generated in the *trkB*-knockouts since the neuronal deficit observed at E12 was not present by E14. However, double mutant embryos displayed a significant 68% reduction in neuronal numbers compared to wildtype (Fisher's LSD; $p<0.0001$), which, when compared with the 38% reduction observed in the single *trkA* mutant, suggests that some neurons rely on either trkA or trkB signalling for survival between E12 and E14 in vivo. The difference in neuronal numbers between *trkA*-knockout embryos and double mutant embryos was significant (Fisher's LSD; $p<0.0001$). At this stage in development it appears that just 32% of neurons survive independently of these two receptors.

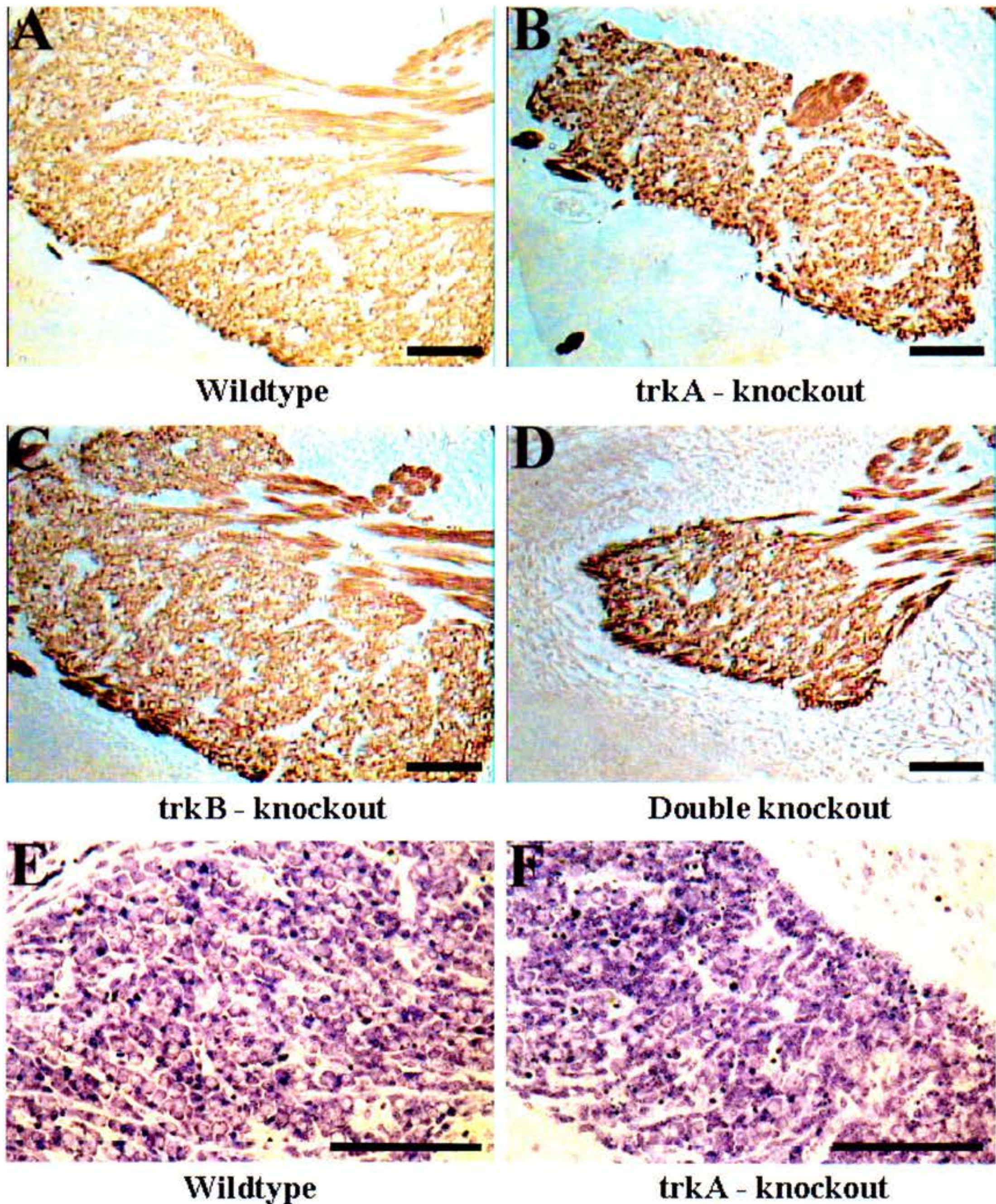
By E16 (ANOVA; $p<0.0001$) the neuronal numbers in the trigeminal ganglion of *trkA*-knockout embryos had further decreased by 79% compared to wildtype numbers (Fisher's LSD; $p<0.0001$). Interestingly, *trkB*-knockout embryos displayed a 21% reduction in neuronal numbers compared to wildtype embryos (Fisher's LSD; $p<0.0001$). This raises the possibility that some neurons that were supported by either trkA or trkB receptor signalling (singularly or in combination) at E14, depend solely on trkB signalling at E16. The neuronal loss occurring in the double knockouts (81%) was less than the combined loss of neurons in the single knockouts (79% for *trkA* and 21% for *trkB*). This suggests that approximately 10% of the neurons require both trkA and trkB signalling between E14 and E16 in vivo.

Figure 5.1: E12 trigeminal ganglia of wildtype, single and double *trkA/trkB* knockout embryos



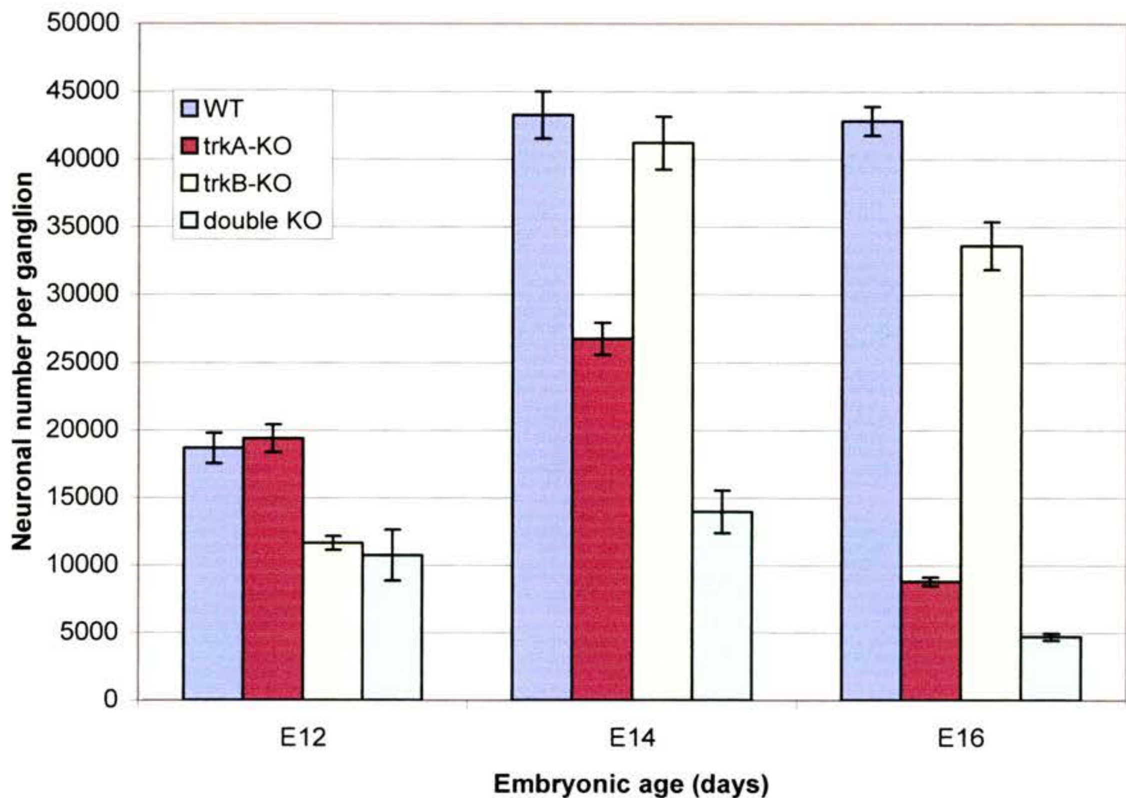
Bright-field view of E12 trigeminal ganglia of wildtype (A), single (trkA (B) and trkB (C)) and double null mutant (D) embryos clearly identified by cytoplasmic staining for βIII-Tubulin (Promega) and detected using DAB-peroxidase substrate (see chapter 2). *TrkB* – knockout and double knockout ganglia were found to be slightly smaller than wildtype and *trkA* – knockout ganglia. Scale bar: 100μm.

Figure 5.2: E14 trigeminal ganglia of wildtype, single and double *trkA/trkB* knockout embryos



Bright-field view of E14 trigeminal ganglia of wildtype (A), single (trkA (B) and trkB (C)) and double null mutant (D) embryos clearly identified by cytoplasmic staining for βIII-Tubulin (Promega) and detected using DAB-peroxidase substrate (see chapter 2). As can be seen, whereas there appears to be no difference in the size of the trigeminal ganglion between wildtypes (A) and trkB-knockouts (C), both the trkA- (B) and the trA/trkB double (D) knockouts are significantly reduced in size. The bottom row illustrates the differences in the number of pyknotic nuclei that can be seen between wildtype (E) and trkA-knockouts (F) on CFV-stained sections, with more pyknotic nuclei observed in trkA and trkA/trkB knockout animals at E14. Refer to Figure 2.13 for a high power image of a pyknotic neuron. Scale bar: 100μm.

Figure 5.3: Mean neuronal numbers in the trigeminal ganglia of single and double *trkA* and *trkB* null mutant mice during embryonic development



Mean neuronal numbers in trigeminal ganglia of E12, E14 and E16 embryos with no mutations (blue), a homozygous null mutation for trkA (red), a homozygous null mutation for trkB (yellow), or homozygous null mutations for both trkA and trkB (turquoise). Bars represent the mean ± standard error (n=6-12 per data point).

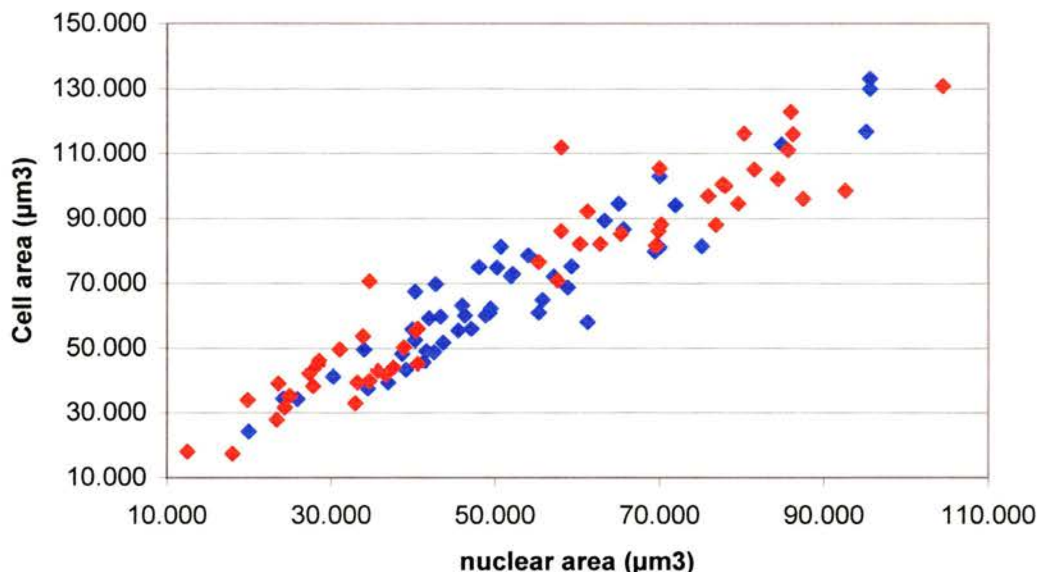
5.2.2 Mean nuclear areas in the trigeminal ganglion of *trkA/trkB* double knockouts

As described in chapter 1, the trigeminal ganglion is composed of different subpopulations of neurons. There is a strong positive correlation between the size of the nucleus and the size of the neuronal body in the trigeminal ganglion at both E14 and E16 in both wildtype and *trkA* knockout animals (Figure 5.4 and 5.5; correlation coefficients of 0.95 and 0.91 respectively). Hence the nuclear area measurements,

that were a by-product of the Abercrombie corrections used to correct neuronal numbers, can be seen as an indicator of cell size. To investigate whether the populations of trigeminal neurons that depend on particular trks for their survival have distinct cell sizes, the mean nuclear area of trigeminal ganglion neurons obtained from Abercrombie corrections was examined. The mean nuclear areas for the trigeminal neurons of wildtype, single and double mutant embryos can be seen in Figure 5.6.

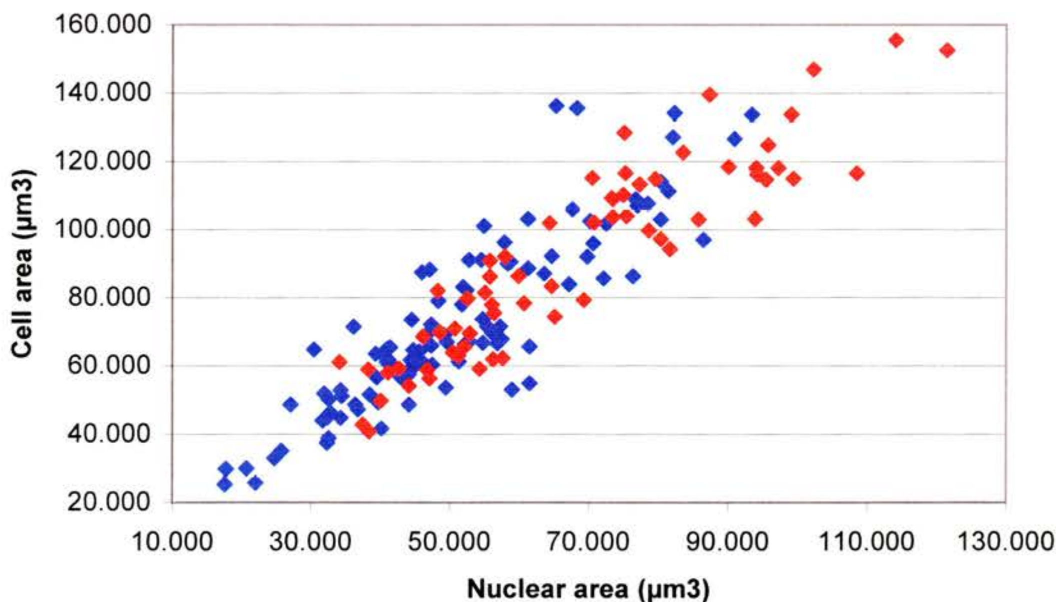
An ANOVA revealed that there were no significant differences in the mean nuclear areas of the different knockouts at E12. Nuclear areas of neurons remaining in the trigeminal ganglion of *trkA* and *trkA/trkB* knockout embryos at E14 however (ANOVA; $p<0.01$) were found to be significantly smaller on average (-28% and –17% respectively) than the nuclear areas in the neurons of wildtype embryos (*trkA*: Fisher's LSD; $p<0.001$; *trkA/trkB*: Fisher's LSD; $p<0.05$). The mean nuclear areas in the trigeminal ganglia of the *trkB*- knockouts were not significantly different to the wildtypes. The nuclear areas of neurons in the trigeminal ganglia of *trkA*-knockout embryos at E16 (ANOVA; $p<0.05$) were significantly larger than the ones in wildtype embryos (+23%) (Fisher's LSD; $p<0.01$), whereas the nuclear areas of neurons in the trigeminal ganglion of *trkB* and *trkA/trkB* double knockout embryos were not significantly different.

Figure 5.4: Correlation between nuclear area and cell area of neurons in the trigeminal ganglion at E14



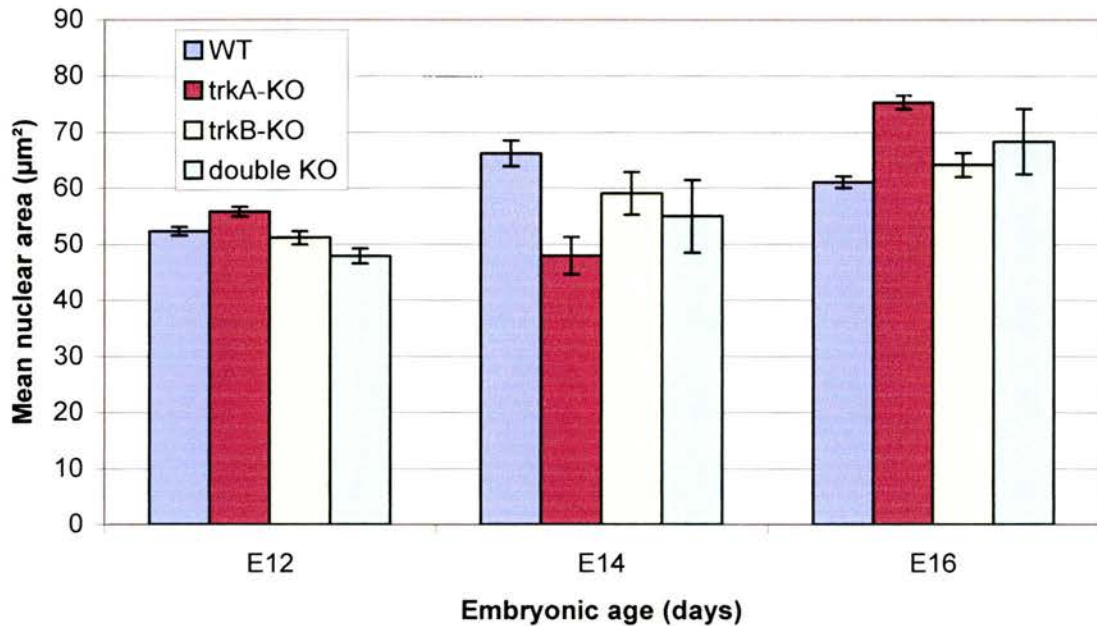
Nuclear area and cell area measurements of 100 neurons in the trigeminal ganglion at E14. Area measurements were obtained by randomly drawing neurons from wildtype (blue) and trkA-/- (red) animals and analysing the drawings using NIH Imager.

Figure 5.5: Correlation between nuclear area and cell area of neurons in the trigeminal ganglion at E16



Nuclear area and cell area measurements of 164 neurons in the trigeminal ganglion at E16. Area measurements were obtained by randomly drawing neurons from wildtype (blue) and trkA-/- (red) animals and analysing the drawings using NIH Imager.

Figure 5.6: Mean nuclear areas of neurons in the trigeminal ganglion of single and double trkA and trkB null mutant mice during embryonic development:



Mean nuclear area of neurons in the trigeminal ganglia of E12, E14 and E16 embryos with no mutations (blue), a homozygous null mutation for trkA (red), a homozygous null mutation for trkB (yellow), or homozygous null mutations for both trkA and trkB (turquoise). Bars represent the mean ± standard error (n=6-12 per data point).

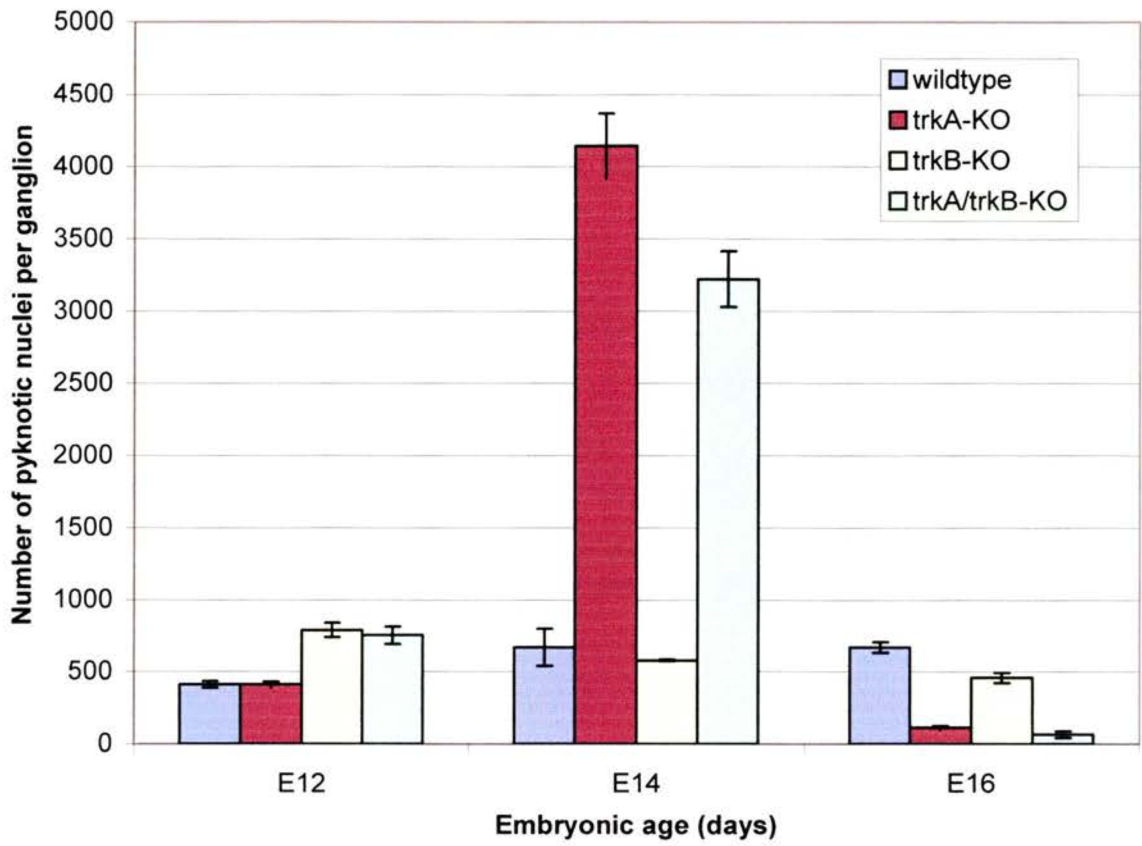
5.2.3 Mean numbers of pyknotic nuclei in the trigeminal ganglion of *trkA/trkB* double knockouts

Pyknotic nuclei were identified and counted on CFV-stained sections as specified in chapter 2. As can be seen in Figure 5.7, consistent with the reduced number of neurons observed in the trigeminal ganglion of *trkB* and double knockout embryos at E12, these ganglia also had significantly increased numbers of pyknotic neurons (Kruskal-Wallis; $p<0.005$) at that age (91 and 82% more respectively; trkB knockout: Dunn's; $p<0.001$; double knockout: Dunn's; $p<0.005$). *trkA*-knockout ganglia in contrast displayed no difference in the number of pyknotic neurons

compared to wildtype ganglia. In E14 ganglia the change in neuronal number was again reflected in the number of pyknotic neurons counted in the trigeminal ganglion Kruskal-Wallis; $p<0.005$). Pyknotic neurons in *trkA*-deficient embryos and double mutants now showed a drastic increase (~600% and ~500% respectively) compared to wildtype counts (Dunn's; both $p<0.05$). There was no significant difference in the number of pyknotic neurons in trkB trigeminal ganglia compared to wildtype embryos. By E16 (Kruskal-Wallis; $p<0.005$), the number of pyknotic neurons was actually significantly lower in *trkA* and *trkA/trkB* double knockouts (83% and 90% less pyknotic nuclei respectively; Dunn's; $p<0.05$), which is most likely due to most neurons in the trigeminal ganglion having already undergone apoptosis in these animals. In the *trkB* knockout, less pyknotic neurons (32%) than in the wildtype trigeminal ganglion were observed at E16 (Dunn's; $p<0.01$).

There were no obvious differences in the appearance of pyknotic nuclei in the trigeminal ganglia of the different genotypes examined in this experiment (Figure 5.2).

Figure 5.7: Mean numbers of pyknotic neurons in the trigeminal ganglion of single and double trkA and trkB null mutant mice during embryonic development



Mean number of pyknotic neurons in trigeminal ganglia of E12, E14 and E16 embryos with no mutations (blue), a homozygous null mutation for trkA (red), a homozygous null mutation for trkB (yellow), or homozygous null mutations for both trkA and trkB (turquoise). Bars represent the mean ± standard error (n=4-8 per data point).

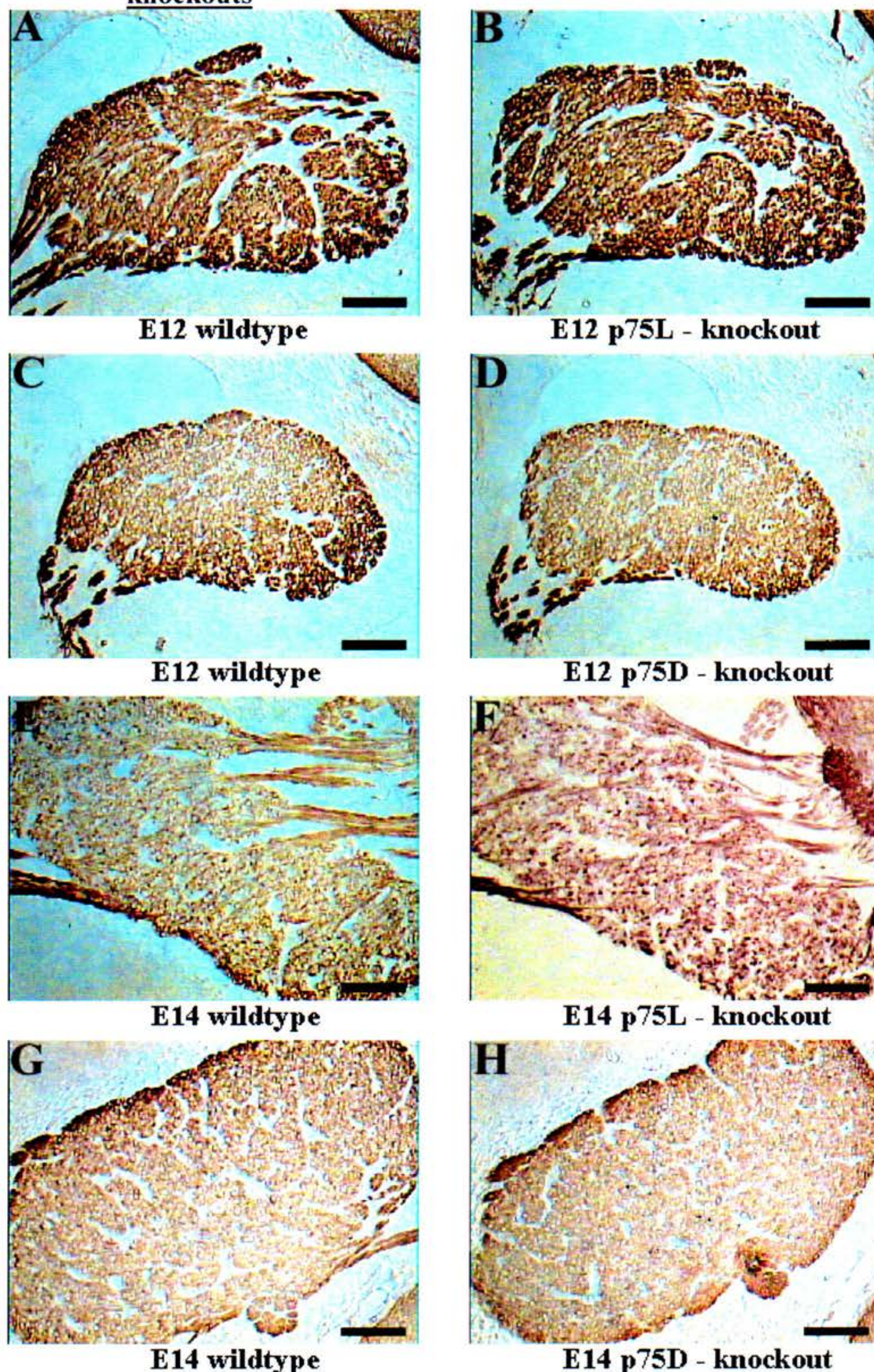
5.2.4 Neuronal counts in *p75L-/-* and *p75D-/-* mutant embryos

Neurons were counted on βIII-Tubulin and CFV-stained sections following the criteria laid out in chapter 2. Figure 5.8 shows representative micrographs of wildtype, *p75L-/-* and *p75D-/-* embryos at E12 and E14. Whereas at E12 and E14, neuronal numbers in the trigeminal ganglion appeared normal in *p75L-/-* embryos (Figure 5.9) and were not significantly different from wildtype numbers, by E16 a 22% loss in neurons was observed in the *p75L-/-* embryos compared to wildtype (t-test; $p<0.0001$). This loss was not further increased between E16 and E18 (16% loss compared to wildtype, t-test; $p<0.005$). This suggests that p75 has a transient effect on supporting the embryonic survival of trigeminal sensory neurons.

In contrast to *p75L-/-* embryos, *p75D-/-* embryos displayed a reduced number of neurons in the trigeminal ganglion at E14 (t-test; $p<0.005$; Figure 5.10). This loss of 14% of neurons occurred between E12 and E14 since no differences between mutants and wildtypes was observed at E12. This loss increased further to 22% at E16 (t-test; $p<0.0001$) and 24% at E18 (t-test; $p<0.0005$) compared to wildtypes. The difference between the two *p75* mutants was significant at E14 (t-test; $p<0.01$), but not at other developmental ages.

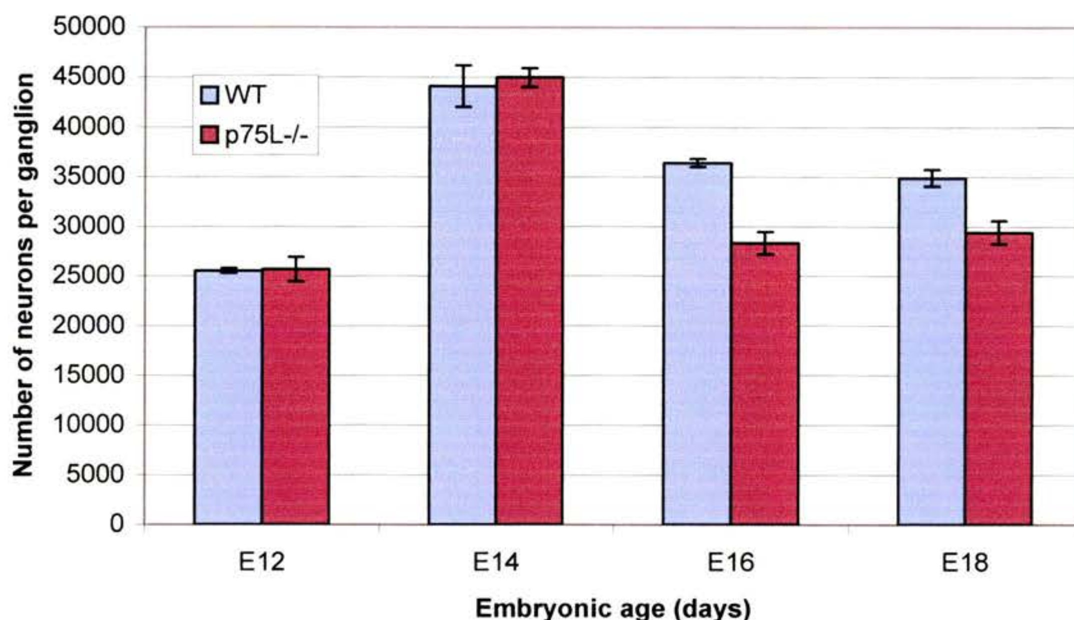
The nuclear areas of neurons in the trigeminal ganglion of these embryos were also assessed. However, no major differences in the mean nuclear area were found in either the *p75L-/-* or *p75D-/-* embryos compared to wildtype embryos, apart from a slight, 8% increase in the mean nuclear area of *p75D-/-* embryos compared to wildtypes late in embryonic development at E18 (t-test; $p<0.005$).

Figure 5.8: E12 and E14 trigeminal ganglia of wildtype, p75L and p75D knockouts



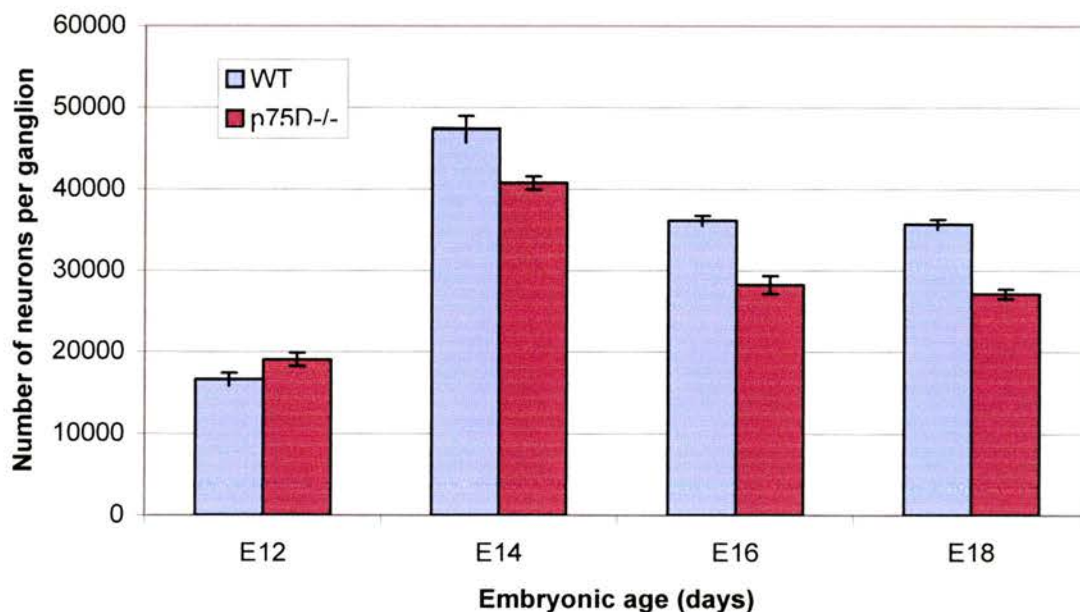
Bright-field view of E12 and E14 trigeminal ganglia of wildtype (A, C, E, G) and mutant embryos (p75: B, F; p75D: D, H) clearly identified by cytoplasmic staining for βIII-Tubulin (Promega) and detected using DAB-peroxidase substrate (see chapter 2). Whereas there appears to be no difference in the size of the trigeminal ganglion between wildtypes and knockouts at E12, at E14 there is a slight decrease in neuronal number, but it is too slight to show up on these images. Scale bar: 100μm.

Figure 5.9: Mean neuronal numbers in the trigeminal ganglion of wildtype and full-length *p75L-/-* mice during embryonic development



Mean neuronal numbers in trigeminal ganglia of E12, E14 and E16 embryos with no mutations (blue) or homozygous for the p75 receptor mutation (red). Bars represent the mean ± standard error (n=6-8 per data point).

Figure 5.10: Mean neuronal numbers in the trigeminal ganglion of wildtype and *p75D-/-* mice during embryonic development



Mean neuronal numbers in trigeminal ganglia of E12, E14 and E16 embryos with no mutations (blue) or homozygous for the p75 receptor null mutation (red). Bars represent the mean ± standard error (n=4-8 per data point).

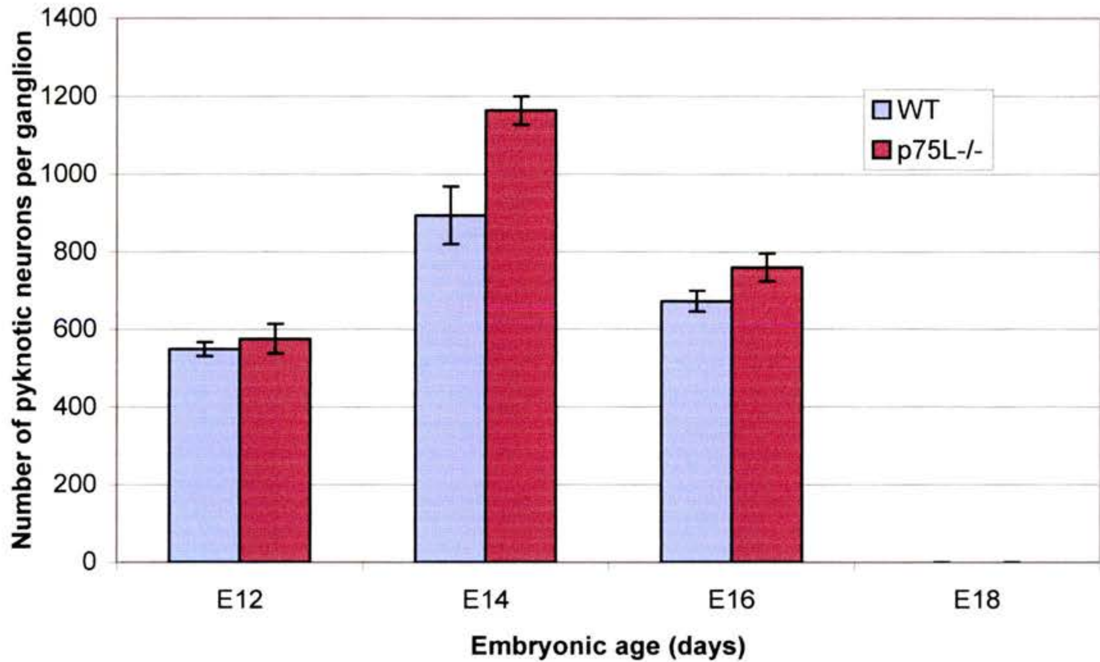
5.2.5 Mean numbers of pyknotic nuclei in the trigeminal ganglion of *p75L* and *p75D* mutants

Pyknotic nuclei were identified and counted on CFV-stained sections as specified in chapter 2. It should be noted that, because of the strict criteria for classifying pyknotic cells as neurons, the actual proportion of dying cells is likely to be greater than the estimates suggest. Counts of pyknotic neurons have previously been shown to be comparable to the numbers achieved by double-labelling neuron markers and apoptotic nuclei immunohistochemically (Piñón et al., 1996).

Although there was no difference in the number of pyknotic neurons in *p75L-/-* embryos at E12, by E14 30% more pyknotic neurons could be observed in the *p75L-/-* embryos (Mann-Whitney; $p<0.05$), suggesting that enhancement of neuronal survival by full-length p75 begins at this stage (Figure 5.11). By E16 there were still slightly more pyknotic neurons in *p75L-/-* embryos than in wildtypes, although this difference was not statistically significant (Mann-Whitney; $p>0.05$). By E18, hardly any pyknotic neurons could be found in either wildtype or mutant animals.

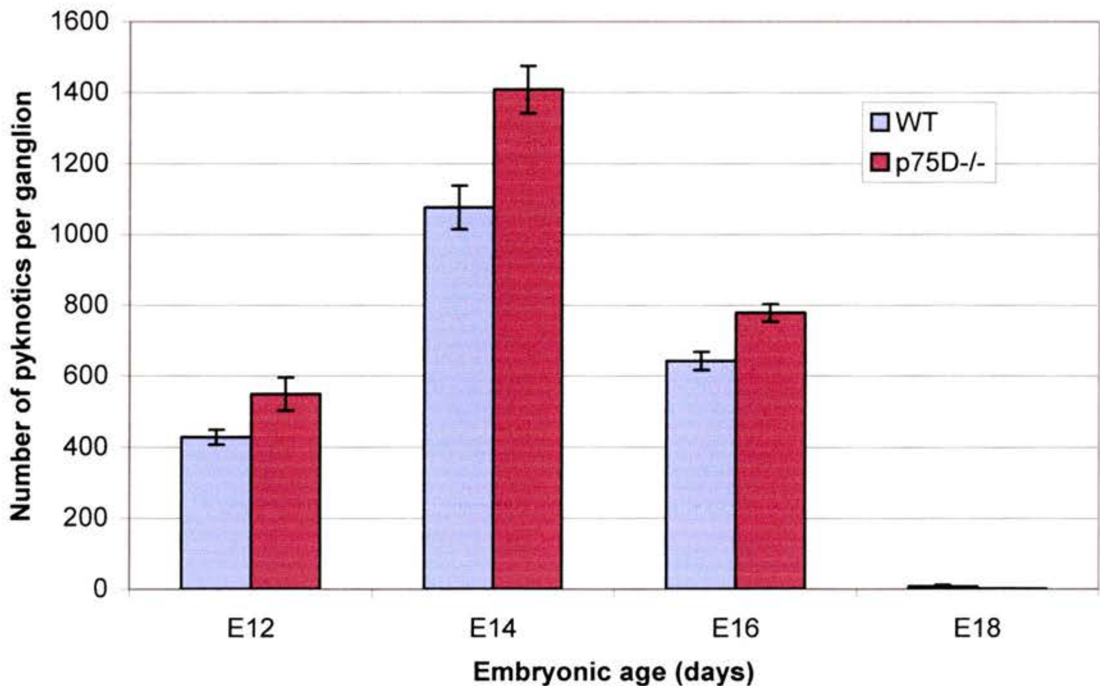
In agreement with the earlier onset of neuronal loss in *p75D-/-* embryos, there was a slightly significant increase (28%) in pyknotic neurons observed at E12 (Mann-Whitney; $p<0.05$, Figure 5.12), i.e. before significant increases in pyknotic neurons were observed in *p75L-/-* mutants. Similar percentage increases in the number of pyknotics, relative to wildtype numbers, were observed in *p75L-/-* and *p75D-/-* embryos at E14 (30% and 31% respectively; Mann-Whitney; $p<0.05$). At E16, however, there were still significantly more (21%) more pyknotic neurons in *p75D-/-* embryos compared to the number of pyknotics in wildtype embryos (Mann-Whitney; $p<0.005$). By E18, hardly any pyknotic neurons could be found in either wildtype or *p75D-/-* embryos.

Figure 5.11: Mean numbers of pyknotic neurons in the trigeminal ganglion of wildtype and *p75L-/-* mice during embryonic development



Mean number of pyknotic neurons in trigeminal ganglia of E12, E14 and E16 embryos with no mutations (blue) or homozygous for the *p75L* mutation (red). Bars represent the mean ± standard error (n=4-6 per data point).

Figure 5.12: Mean numbers of pyknotic neurons in the trigeminal ganglion of wildtype and *p75D-/-* mice during embryonic development



Mean number of pyknotic neurons in trigeminal ganglia of E12, E14 and E16 embryos with no mutations (blue) or *p75D-/-* (red). Bars represent the mean ± standard error (n=4-6 per data point).

5.3 Discussion

The use of combined null mutants lacking both the trkA and trkB receptor genes, and their comparison with single null mutants, has for the first time enabled a direct comparison of the effects the combined signalling of these receptors has in relation to their individual survival-promoting actions in the mouse trigeminal ganglion in vivo. Stages have been identified at which trigeminal neurons are predominantly dependent on trkB signalling or trkA signalling for survival as well as a period when some neurons can signal via either receptor and some neurons require both receptors. Although initial studies of p75 mutant embryos have established the importance of this receptor for the survival of trigeminal neurons, the onset of p75 dependence in this neuronal population has been unknown thus far. The present study shows that a subset of trigeminal neurons becomes dependent on p75 signalling between E12 and E14 (p75D). This dependence is delayed slightly in embryos that express a truncated p75 that lacks most of the extracellular domain of the receptor (p75L).

The data in this chapter replicate earlier evidence that trigeminal neurons require functional trkB receptors at an early stage in their development in vivo (Piñón et al., 1996). This is suggested by the reduced numbers of trigeminal neurons and increased number of pyknotic neurons in the ganglion of *trkB*-deficient mice at E12. It is likely that the trkB ligands important for mediating this survival effect in vivo are BDNF and NT-3 because embryos lacking these neurotrophins display reduced numbers of neurons in the trigeminal ganglion during this period of development (Ernfors et al., 1994; Davies et al., Huang et al., 1999) and the majority of early trigeminal neurons are supported by BDNF and NT-3 *in vitro* (Buchman and Davies, 1993; Davies,

1993; Paul and Davies, 1995). The present study also confirmed the previous finding (Piñón et al., 1996) that the survival of trigeminal neurons depends on functional trkA receptors at later stages in development. Accordingly, I found no difference in the number of surviving or pyknotic neurons in the trigeminal ganglion of *trkA*-deficient embryos at E12 compared with wildtypes, whereas by E14, *trkA*-deficient mice displayed a 38% decrease in neurons and a six-fold increase in the number of pyknotic neurons.

The results presented above thus replicate the genetic proof for the switch in survival dependence of early trigeminal ganglion neurons (Piñón et al., 1996). However, in addition to replicating previous findings, this study used double null mutants for trkA and trkB. These double knockouts confirm in vivo that neurons can be simultaneously dependent on either trkA or trkB-mediated trophic support.

The ratio of neurons missing in trkA and trkB mutant embryos at E14 (38% and 5% respectively) compared to the ratio missing in double mutants (68%) suggests that in the absence of trkA 25% of neurons could utilize trkB-signalling for their survival and, similarly, in the absence of trkB signalling 25% of neurons could use trkA/NGF signalling for survival. This indicates that at least 25% of neurons before E14 not only expressed both receptors simultaneously at some stage of development, but were able to respond through either receptor to induce survival. The sequential action of distinct neurotrophic factors is a recurring theme for many neuronal populations (Verdi and Anderson, 1994□; Davies, 1997). It is hence important to identify the proportion of neurons that undergo such a developmental switch in order to assess its importance for the continued development of particular neuronal populations. This

experiment has enabled the quantification of the proportion of neurons that are able to respond to trkA- or trkB-signalling in vivo in the embryonic mouse trigeminal ganglion. Quantifying the number of neurons 'switching' from complete dependence on trkB-signalling to complete dependence on trkA-signalling early in development is not possible, however, since these die before E12 due to the trkB-deficiency. The neurons detected here are thus the ones generated at intermediate stages that can respond to both NGF and BDNF (Enokido et al., 1999). The losses in the trigeminal ganglion seen in the double mutant embryos most likely account for all of the survival-promoting effects of the three major neurotrophins, since most of the survival mediated by NT-3 is thought to occur through trkA and trkB. It is unlikely that trkC plays a major role at these early stages, since trkC knockouts do not display a loss of sensory neurons in the trigeminal ganglion, at least not until later in development (Piñón et al., 1996).

The effect of the double mutation on neuronal survival in the ganglion at E14 most likely requires both sets of receptors to be expressed by the same cell. This contradicts a previous study finding little such co-expression, with each neuron expressing only one receptor type by E13.5 (Huang et al., 1999). However, the immunohistochemical technique used in that study may not be sensitive enough to detect low, albeit functionally significant levels of trk receptors. In support of my findings, neurons generated at intermediate stages have been shown to respond to both NGF and BDNF *in vitro* (Enokido et al., 1999). Also, the more sensitive method of single-cell RT-PCR found mRNA expression for two or all three trk receptors in neurons of the developing rat trigeminal ganglion (Moshnyakov et al., 1996).

The additive effect of trkA- and trkB-deficiency induced cell loss at E16 (79% and 21% respectively) ought to account for 100% of neurons in the trigeminal ganglion at this stage. However, 11% of neurons remained in *trkA/trkB* double mutants. Since the sum of the single trk knockout neuron losses (100%) was higher than the loss in the double mutants (89%), it would appear that approximately 11% of neurons in the ganglion require combined trkA- and trkB-signaling for their survival. Such a dual support requirement has been found in a variety of neuronal populations. For example, many sympathetic neurons appear to require both NGF and NT-3, as almost all sympathetic neurons are eliminated in the absence of NGF and around 50% are eliminated in the absence of NT-3 (Francis et al., 1999). Also, in the nodose-petrosal complex, neuronal losses in BDNF/GDNF double mutants are not additive to losses in single BDNF or GDNF null mutants, indicating that many cells require both for survival in vivo (Erickson et al., 2001).
Although it has previously been shown that trigeminal neurons are able to survive in the presence of different trophic factors, this is the first time that such a dual embryonic requirement for two simultaneous trophic factors has been found in this neuronal population. This further contradicts previous evidence suggesting that trigeminal neurons express only one trk type (Huang et al., 1999), as some neurons require both receptors to be expressed to survive between E14 and E16.

Furthermore, the results show that the number of neurons surviving independently of trk receptor signalling is reduced with development. At E12, 58% of neurons in the trigeminal ganglion survive independently of neuronal support through the trk receptors. This proportion of trk-receptor signalling independent neurons decreases

with age (32% at E14 and 19% by E16). The importance of trk-receptor signalling for trigeminal neuron survival thus increases with age. Since NT-3/trkC signalling appears to be required for survival by a subset of trigeminal neurons at E16 (Piñón et al., 1996), some of the neurons still present in the *trkA/trkB* double knockouts may be NT-3/trkC dependent. It would be interesting to compare NT-3 and trkC mRNA levels in the mutant embryos as opposed to wildtype embryos, to ascertain whether there is compensation of NT-3 signalling via trkC for trkA and trkB signalling loss in vivo. Culturing *trkA/trkB* trigeminal neurons with NT-3 may also enable the assessment of survival mediated via the trkC receptor alone.
More information is emerging on the factors able to support/enhance neuronal survival in the trigeminal ganglion at later stages in development (Horton et al., 1998; Forgie et al., 2003). However, it is currently not well known what alternative factors may be involved in the survival of earlier trigeminal neurons.

It is noteworthy that the numbers of neurons in the trigeminal ganglion of the trkB knockout studied in this experiment were reduced by 38% at E12 and an insignificant 5% at E14, which is in contrast to the 42% reduction in neuronal numbers at E13 in previous studies, with ~33% neuronal deficit at E15 (Piñón et al., 1996). At E17 the previous study still found a 33% deficiency in *trkB-/-* ganglia, whereas I found a 21% decrease at E16. Part of the reason for the different proportion of loss seen in this experiment may be due to the previous study using mice in a 129xC57Bl/6 background, whereas mice in a CD1 background were used here. Differences in background have previously been reported, with neuronal numbers in the DRG of BDNF-deficient mice, for example, being normal prenatally in one background

(Fariñas, 1999), and decreased in a different background (Liebl et al., 1997). This, however, still does not explain the recovery in the proportional numbers of trigeminal neurons at E14 in this study.

Something appears to cause a proportional increase in neuronal numbers of *trkB*-knockouts between the developmental stages of E12 and E14. Similar observations to the ones made in this experiment were made in the dorsal root ganglion, where there is a significant loss of DRG neurons of *NT-3*-deficient mice at E11. However, neuronal numbers at E12 appear to be normal compared to wildtype embryos (Fariñas et al., 1996). This study also found that *NT-3*-deficient ganglia have fewer precursor cells by E12, suggesting that neurogenesis occurs at an abnormally higher rate in mutant embryos during the E11-E12 interval and that precursors differentiate into neurons prematurely. Neurons in the trigeminal ganglion are still generated at ~E13. It is possible that the normal neuronal numbers observed in *trkB*-deficient mice at E14 are due to a depletion of the precursor pool and premature neuronal differentiation between E12 and E14. Alternatively enhanced precursor proliferation with trkB knockouts between these ages may yield a bigger number of precursors that can differentiate into neurons at a normal rate. Further experiments would necessitate checking the number of precursor cells in the trigeminal ganglion of *trkB*-deficient mice, to ascertain whether such events may take place in these embryos.

As demonstrated above, there is a strong positive correlation between the size of the nucleus and the size of the neuronal body in the trigeminal ganglion at the ages studied. Nuclei in the trigeminal ganglia of *trkA*-deficient mice are smaller than in wildtype ganglia at E14. However, by E16 they are significantly larger. The smaller

nuclear size of neurons at E14 may be explained by the incredibly high amount of apoptotic death occurring in the ganglia of *trkA*-deficient mice at this stage. The decrease in nuclear area observed in *trkA*-deficient mice at E14 may be due to the high proportion of apoptotic neurons. Apoptosis has cell shrinkage as one of its symptoms. Neurons of *trkA/trkB* double null mutants also have smaller nuclei, but the difference is less than that observed for *trkA* mutants.

The larger nuclear area in trigeminal neurons of trkA-deficient mice at E16 may be due to the selective loss of small nociceptive neurons. These are mainly trkA-dependent in the DRG, whereas larger diameter neurons tend to depend on BDNF and NT-3 (Crowley et al., 1994; Silos-Santiago et al., 1995; Jones et al., 1994; Fariñas et al., 1994). A similar selective loss could be occurring in the trigeminal ganglion. No difference was seen in *trkB*-deficient embryos, which would not be expected, since the trkA-dependent neurons outweigh trkB-dependent neurons by far at this stage. Thus a loss in the relatively small proportion of larger neurons may not significantly alter the average nuclear size, predominantly determined by the larger proportion of trkA-responsive neurons. Mean nuclear size in trigeminal neurons of the double mutant was not much different from wildtypes. One would not expect as strong an effect as for trkA-deficient trigeminal neurons in these embryos, since they loose neuronal subpopulations dependent on both receptors. It will be interesting to use specific markers for neurotransmitters and neuropeptides found in the trigeminal ganglion (reviewed by Lazarov, 2004), to establish if there are neuronal subpopulations lost preferentially in the different knockout mice.

Although numerous studies have indicated neuronal losses in the trigeminal ganglion of *p75*-deficient mice in vivo, it is not known when in development this deficit develops. It has not been shown in vivo whether this loss is due to decreased proliferation and/or differentiation of these neurons or simply due to dependency on p75-signalling later in development. Analysis of neuronal numbers in the trigeminal ganglion of the well-studied *p75L-/-* mutant (Lee et al., 1992) indicates that neuronal loss occurs between E14 and E16, thus after the phase of neurogenesis. This onset correlates with in vitro evidence, with dose-responses of *p75*-deficient trigeminal neurons to NGF shifting to higher concentrations at E14, with less of a shift at E12 (Davies et al., 1994). Although the neuronal complement in the trigeminal ganglion of *p75L-/-* embryos is not reduced at E14, the significantly higher number of pyknotic neurons indicates that increased neuronal death is initiated at this stage. E14 is also the stage at which a shift in dose-response is observed in mouse trigeminal neurons in response to an NGF mutant able to bind only trkA (Horton et al., 1997).
Neuronal losses in *p75D-/-* null mutant embryos occur earlier between E12 and E14, and relative pyknotic counts at E14 are higher. This would indicate that the short-p75 receptor isoform expressed by *p75L-/-* mice mediates improved survival early in development in vivo. This would correlate with the slightly higher loss of lumbar DRG neurons found in the *p75D-/-* embryos compared to *p75L-/-* embryos (von Schack et al., 2001).
High affinity binding sites have been shown to be created by the interaction of the transmembrane and the cytoplasmic domain of p75, indicating that these are sufficient to convert trkA to a higher affinity (Esposito et al., 2001). Also, a p75 mutant deficient in neurotrophin binding increases the affinity of NGF for trkA,

showing that direct interaction between neurotrophins and p75 is not essential for the formation of high affinity binding sites. Because short-p75 possesses the transmembrane and cytoplasmic domains of the full-length receptor, it could still enhance NGF binding to trkA with the result that *p75L-/-* may be more sensitive to NGF than *p75D-/-*. This would need to be assessed by examining the dose-response of the *p75D-/-* versus *p75L-/-* trigeminal neurons in culture, which may give a clearer indication whether such a mechanism may occur. An alternative explanation is that the short p75 isoform may be able to mediate survival independently of neurotrophin binding (as it cannot bind due to its truncated extracellular domain, von Schack et al., 2001). The p75 intracellular domain is able to enhance neuronal survival in a variety of cells (Roux et al., 2001). Also, a recent study demonstrated that proteolytic cleavage of p75 receptors results in the release of the cytoplasmic domain into the cell cytoplasm (Kanning et al., 2003). Since such soluble intracellular domains of p75 were shown to activate NFκB, this may identify an additional pathway through which the short p75 receptor induces survival in the absence of neurotrophin binding. At later stages in development there appears to be no additional loss in neuronal numbers of *p75D-/-* compared to *p75L-/-* trigeminal ganglia, which may suggest that the role of the short p75 receptor isoform at later stages of development is negligible. This is in contrast to the DRG, where further neuronal losses are still observed at P3/4 in null mutants (von Schack et al., 2001). An in depth study of the full- and short-length forms of p75 and of trkA in the trigeminal ganglion and DRG, along with NGF expression in the target field of both neuronal populations in wildtype and *p75* mutants, may help resolve the reasons behind this difference.

The beginning of survival dependence on p75-signalling coincides with the beginning of survival dependency on trkA-signalling. This is in line with the p75 mutation causing a selective decrease in the sensitivity of sensory neurons to NGF, but not apparently affecting the response of sensory neurons to either BDNF or NT-4/5 (Davies et al., 1993; Lee et al., 1994). The present study does not indicate whether particular subtypes of neurons are lost in the trigeminal ganglion is response to a *p75* mutation. The significantly larger neurons present in this ganglion at E18, however, may suggest that the neurons lost are the small trkA-dependent nociceptive neurons.

Conclusions

Trigeminal neurons can thus potentially undergo five stages in their trk-signalling dependent survival during development. Firstly, they are mostly independent of trk-signalling for their survival (Davies and Lumsden, 1984). Secondly, some neurons become critically dependent on trkB-signalling for their survival. Thirdly, some of these neurons become able to survive in response to either trkA or trkB signalling, while new neurons are generated that are able to signal via either receptor as well. Further, some neurons loose their dependency on trkB and become entirely trkA-signalling dependent, while new neurons being generated respond solely to trkA-signalling. Finally, some neurons later in development necessitate combined trkA- and trkB-signalling for their survival. It is not clear from this study whether these are neurons that were able to respond to either trophic factor at earlier stages of development, or whether there may be a conversion of neurons solely dependent on trkA-signalling to neurons requiring additional trkB-signalling.

For neurons to switch survival-dependence, there must be a biological significance to this process. One of the explanations suggested for the intermediate dependence of trigeminal neurons on trkB ligands during the early stages of ganglion development, is that this sustains the survival of early generated neurons until the majority of late-born neurons are generated, and all the generated neurons can compete for NGF released by the target during the same period of development (Davies, 1994). The survival responses of trigeminal ganglia to the 'early' survival factors BDNF and NT-3 have almost disappeared by E13-E14, the time of onset of naturally occurring cell death, mediated by the limited supply of NGF (Buchman and Davies, 1993). This is supported by the observation that trigeminal neurons are generated up to E13 (Davies and Lumsden, 1984; Enokido et al., 1999), consistent with the recruitment of axons to the trigeminal nerve up to this time point (Davies, 1987). 'Early' trophic factors may thus assist in maintaining survival before NGF dependency and the onset of developmental cell death. All the neurons competing for NGF at the same stage in development would optimise innervation to the specific requirements of the target fields, maintaining the neurons with the best axon terminations at the target (Davies, 1994). Later in development (as seen at E16 in this study), trigeminal neurons appear to regain dependency on additional trophic factors apart from NGF. By analogy with the differences in trophic factor requirements of postnatal subpopulations of DRG neurons (e.g. Smeyne et al., 1994; Crowley et al., 1994; Ernfors et al., 1994; Molliver et al., 1997; Acosta et al., 2001), it is conceivable that the different trophic requirements of trigeminal neurons at these later stages of development serve to support distinct populations of trigeminal neurons which will develop distinct sensory modalities. Further study may reveal that a subpopulation of postnatal

trigeminal neurons do indeed become dependent on NGF, BDNF, NT-3, GDNF, FGF, a LIF/cytokine or a combination of many factors.

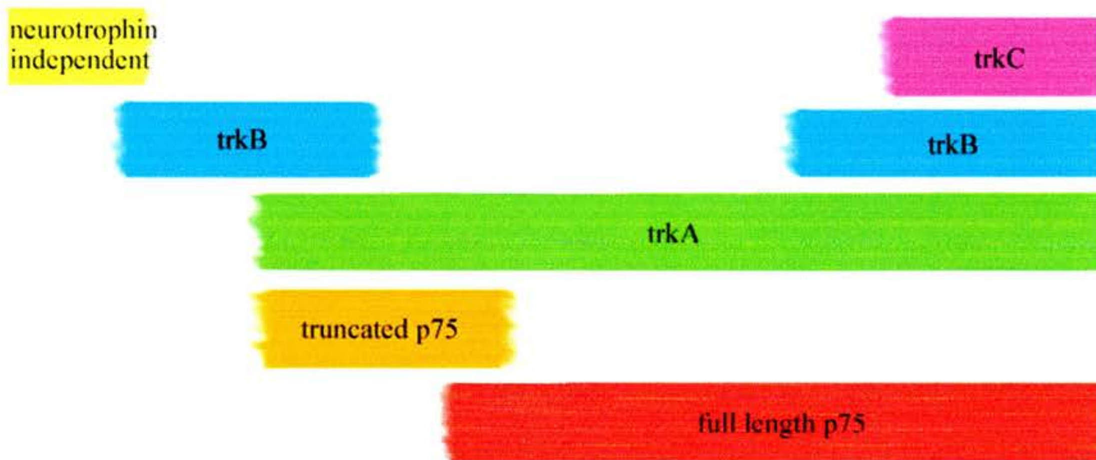
The studies examining the effect of p75 receptor mutations revealed that, in support of in vitro evidence, the stage at which neurons begin to respond to NGF signalling via p75 for improved survival is approximately E14. Further, this study revealed some significance of p75-signalling prior to E14, with truncated p75 receptors being able to improve survival compared to a complete null *p75* mutation, possible utilising mechanisms of intracellular cleavage and NFκB activation (Kanning et al., 2003) or the formation of high-affinity binding sites (Esposito et al., 2001).

The exact variations in neuronal development in vivo due to the actions of isoforms of trk and p75 receptors are still poorly understood. The generation of knockout mice including these 'missed' receptors is starting to clarify the functions of some of them, as seen in the p75 study presented in this chapter. A recent study has observed functional significance for a truncated form of trkB for example: the presence of truncated trkB receptors in the original full-length trkB knockouts (Klein et al., 1993) results in more severe sensory neuronal losses in sensory ganglia than seen when this isoform is eliminated as well (Luikart et al., 2003). These results suggest that truncated trkB receptors negatively influence neuronal survival in these ganglia. In vivo, the truncated receptor may thus bind BDNF and NT-3, and reduce the amount of ligand available for binding to trkB (BDNF-receptor) and trkA, trkB, and trkC (NT-3 receptors). *TrkB* mice used in these experiments presented above were the mutants lacking only the full-length trkB receptor (Klein et al., 1993). However, the above-mentioned study found no differences in survival between these different trkB

knockouts in the trigeminal ganglion at P0 (Luikart et al., 2003), suggesting that as far as the trigeminal ganglion is concerned, the function of this particular isoform of trkB receptor is negligible during its development.

The sequential nature of the survival-dependence of trigeminal sensory neurons on trk and p75 receptors is summarised in Figure 5.13.

Figure 5.13 Changing survival requirements of embryonic trigeminal ganglion neurons



Developmental sequence, from left to right, of the changing requirements of embryonic mouse trigeminal ganglion neurons on neurotrophin receptor signalling. After a period of independence (yellow), many trigeminal neurons become dependent on trkB-signalling for their survival (blue). This is followed by a period in which many neurons can be supported by either trkB- or trkA-signalling, with truncated p75 improving the survival response (orange), probably by forming high affinity binding sites with trkA. The full-length p75 receptor performs a similar function later in development (red), with most of the neurons dependent on trkA-signalling (green). After programmed cell death there seems to be a diversification in the survival-dependence of trigeminal ganglion subpopulations, with some neurons becoming dependent on trkB-signalling again (blue), on trkC-signalling (purple), with others remaining trkA-dependent. For some subpopulations, signalling through two or more receptors may be required for survival, as has been shown for trkA and trkB in this study. The different trophic requirements of trigeminal neurons at these later stages of development may serve to support neurons that will develop distinct sensory modalities. At early stages in development the different requirements would serve to sustain the survival of neurons until the majority of later-born neurons are generated, so that all neurons can compete for NGF released by the target during the same period of development (adapted from Davies, 1997).

CHAPTER VI

Variations in the trophic requirement of neuronal populations on Schwann cells

6.1 Introduction

The timing of neuronal dependency on trophic factors for survival has traditionally been associated with target-derived factors, which are secreted by the target fields of neurons immediately prior to, or upon innervation, with the neurons being neurotrophin independent prior to this. Whilst some neuronal populations are indeed independent during the period of growth to their target fields (Vogel and Davies, 1991), this traditional view is increasingly challenged by the discovery that other neuronal populations need to be supported by factors released from intermediate tissue en route to their target fields (Buchman and Davies, 1993).

One such mode of intermediate trophic support is believed to arise from glial cells. In the peripheral nervous system, both sensory and motor neurons are in an intimate relationship with glial cells, with myelinating and non-myelinating Schwann cells as well as satellite cells in close contact or surrounding neurons and their processes. Various glial cell types have been implicated in the maintenance of neurons, since these have been found to synthesize trophic factors (Davies, 1998; Saarma and Sariola, 1999; Lemke, 2001). Indeed, "the development of neurons is likely to depend equally on factors from the target fields they innervate and on factors from the glia that surround them" (Mirsky et al., 2002). The importance of Schwann cells for neuronal development was first identified by the discovery that they mediated the morphological transformation of embryonic sensory neurons, from an immature bipolar form to a mature pseudounipolar form (Mudge, 1984). Schwann cells have since been reported to synthesize neurotrophic factors important for neuronal survival, including NGF (Matsuoka et al., 1991), BDNF (Acheson et al., 1991), NT-3

(Cai et al., 1999), CNTF (Sendtner et al., 1991), LIF (Matsuoka et al., 1997), GDNF (Henderson et al., 1994), FGF (McGeachie et al., 2001), PDGF (Oya et al., 2002), and TGF (Eccleston et al., 1989) (Bunge, 1993 for review). Schwann cells are ideal candidates for intermediate trophic support, as their progenitors can be observed very early in development, associating with outgrowing axons (Riethmacher et al., 1997). In embryos lacking Schwann cell precursors due to mutations in neuregulin or one of its receptors, there is widespread loss of sensory and motor neurons during development (Meyer and Birchmeier, 1995; Gasmann et al., 1995; Lee et al., 1995; Riethmacher et al., 1997).

The pattern of generation and migration of neural crest cells giving rise to Schwann cells has been covered by in Chapter 1. After this migratory phase, Schwann cells are generated in three phases: transition of neural crest to precursors (E12 to E13 in the mouse), transition of precursors to immature Schwann cells (~E15), and the formation of mature myelinating and non-myelinating Schwann cells (postnatal). Neuronal derived neuregulins play a major role in controlling the development and survival of satellite and Schwann cell precursors (Riethmacher et al., 1997; Hagedorn et al. 2000; Mirsky et al., 2002). Neuregulins signal through members of the ErbB family of receptor tyrosine kinases, namely ErbB 1, 2, 3 and 4 (Gassman and Lemke, 1997; Garratt et al., 2000). Since ErbB 1 and 2 are not as efficient at binding neuregulins as ErbB 3 and 4, they rely on these other receptors to form heterodimers with functional signalling capacity. As well as these heterodimers, ErbB4 can signal by forming homodimers. ErbB3 can form homodimers as well, but since ErbB3 lacks kinase activity, it cannot signal neuregulin binding without heterodimerisation.

Among other functions, β neuregulin released from axons has been found to act via ErB2/ErbB3 heterodimer receptors to control the survival of Schwann cell precursors (Neuregulins and receptors reviewed in Garratt et al., 2000).

Elucidating the influence of neuregulins on Schwann cell survival in vivo was made difficult by the finding that *neuregulin-1*, *ErbB2* and *ErbB4* null mutants have an embryonic-lethal cardiac phenotype at ~E10. Some evidence from these short-lived embryos suggested an important role of neuregulin-1 and ErbB2 for the development of Schwann cell precursors and cranial ganglia as well as in heart development (Meyer and Birchmeier, 1995; Lee et al., 1995). *ErbB4* mutant embryos, albeit having generally normal cranial ganglia, displayed aberrant targeting of cranial sensory and motor axons (Gassman et al., 1995). The early lethality of these null mutants made further analysis of the importance of Schwann cell precursors difficult. However, the phenotype observed in *ErbB3* null mutant mice, which interestingly exhibit satellite glia but not Schwann cell precursors along their peripheral nerves, enabled the examination of the role of Schwann cells in neuronal development (Riethmacher et al., 1997). Dorsal root ganglion neurons in these mutant mice appeared to develop normally up to E12.5, before losing over 70% of their neuronal complement by E14.5 and 82% by E18.5. Most of this loss was due to apoptosis of post-mitotic neurons, as demonstrated by TUNEL staining at E13.5. In the DRG of the mouse, sensory neurons are the first detectable population of differentiated cells, differentiating slightly before satellite glia and Schwann cells are detectable (Lawson and Biscoe, 1979). This, together with the above data, indicates that Schwann cells and their precursors have no immediate effect on the differentiation and initial

development of DRG neurons. Other neuronal population in the *ErbB3* null mutant embryos, such as spinal motoneurons, were also found to initially develop normally before undergoing cell death. Riethmacher and colleagues demonstrated that the loss of neurons was due to the lack of Schwann cell precursors, and not due to a direct effect on neuronal survival. *ErbB3* null mutant stem cells injected into blastocysts of wildtype animals resulted in normal distribution of the mutant cells in neuronal populations and other tissues, but lack of them in Schwann cell precursors. This clearly suggests that the effect observed is related to the lack of Schwann cell precursors, and not to a direct effect on neuronal survival, implicating trophic support of neuronal populations by Schwann cell precursors. This effect of Schwann cell precursors does not necessarily precede other modes of trophic support, since neuronal loss in the DRG of *ErbB3*-deficient embryos occurs later in development than the one observed in *trkB* and *NT-3* knockouts. However, the death observed in the DRG of *ErbB3* null mutants still occurs in a period where axons are growing towards their target fields, suggesting that Schwann cell precursors and Schwann cells provide trophic support prior to the stage of target field innervation. The survival of motoneurons is influenced by the absence of Schwann cell precursors post-innervation, suggesting that Schwann cells play an important role after muscle innervation as well. The scale of cell death in dorsal root ganglia and motoneurons pools of *ErbB3* null mutants raises the possibility that Schwann cell precursors are crucial in providing multiple essential trophic factors to the neurons early in their development, since neuronal losses in the DRG and in spinal motoneurons far outweigh those observed in any single neurotrophic factor knockout (Davies, 1998).

Another interesting observation in *ErbB3*-deficient mice was the abnormal appearance of neural crest-derived cranial ganglia and nerves as early as E10, when these structures form. This mirrored observations in *neuregulin-1* (Meyer and Birchmeier, 1995) and *ErbB2* null mutants (Gassman et al., 1995) and was attributed to an almost complete absence of neuronal migration and differentiation from the neural crest rather than the death of post-mitotic neurons. *ErbB2* null mutants were found to have greatly reduced numbers of cranial neural crest derived neurons and lacked both sensory and motor innervation connecting cranial ganglia to their corresponding rhombomeres. Indeed, studies of the ErbB3 expression pattern in the cranial ganglia suggested that neuregulin might be required as a survival factor for neurogenic cells of the cranial crest (Meyer and Birchmeier, 1995). In summary, in the cranial neural crest neuregulin signalling via ErbB3/ErbB2 appears to be essential for the neurogenic lineage, whereas in the trunk neural crest it affects the early steps in Schwann cell development and consequently neuronal survival in the trunk. Signalling via the ErbB4 receptor appears to be more concerned with axonal guidance.

Many neural crest-derived neurons require intermediate trophic support both in vivo and in vitro at stages when they are growing towards their target fields. NT-3 has been found in tissues on the trajectory of DRG axons during development as an example (Fariñas et al., 1996; White et al., 1996; Liebl et al., 1997).

In contrast, placode-derived neurons at similar stages in their development survive in vitro for varying lengths of time without neurotrophins, a neurotrophic factor independent period varying according to the distances the axons of these populations

have to grow to their targets in vivo (Vogel and Davies, 1991). Neurotrophin independence and the timing of the neurotrophin survival response in these neurons appear to be controlled by an intrinsic timing mechanism in the neurons (Vogel and Davies, 1991). An interesting issue arising from this study is that these populations of neurons may not depend on intermediate support before reaching their targets and may therefore not be lost in *ErbB3* deficient embryos.

To assess this, the study presented in this chapter was aimed at analysing the interesting question of Schwann cell derived support for placode-derived neurons, in comparison with neural crest-derived neurons like the dorsal root ganglion. *ErbB3*-deficient mice were used to assess the differences between placode-derived neuronal populations (nodose ganglion), trunk neural crest-derived neuronal populations (dorsal root ganglion) and cranial neural crest-derived neurons (trigeminal ganglion). Neuronal numbers in cranial dorsal root ganglia were slightly decreased at E12, with a drastic reduction of neuronal numbers by E13, confirming previous findings in lumbar DRGs of *ErbB3*-deficient mice (Riethmacher et al., 1997). In contrast to these neural crest derived neuronal populations (which are believed to require intermediate trophic support), the placode-derived neurons of the nodose ganglion appeared to undergo little cell death, with only a slight reduction in neuronal numbers of *ErbB3*-deficient mice by E14.

As expected from the phenotype of the *neuregulin* and *ErbB2* null mutant mice, the trigeminal ganglion was greatly reduced in the *ErbB3* null mutants at the first age studied (E11) with the remaining cells presumed placode-derived cells due to their spatial location in the ganglion. These remaining cells, however, although displaying

normal outgrowth of their ophthalmic and maxillary branches, appeared to undergo a higher rate of apoptosis between E12 and E14 compared to wildtype neurons, suggesting they required intermediate support.

Rather than being related to differences between placode-derived neurons and neural crest-derived neurons, the effect of Schwann cell precursors appears to be correlated to the requirement of neurons on intermediate trophic support from surrounding tissues, of which Schwann cell precursors are a part. An interesting observation was that the onset of neuronal dependence on intermediate trophic support by Schwann cell precursors appears to be intrinsic to different neuronal populations, with all cell losses observed in this study coinciding with the time point of Schwann cell precursor generation, but not preceding it. A further observation suggests an excessive loss of non-neuronal cells in the nodose ganglia of ErbB3-deficient mice (compared to that observed in other ganglia), although the identity of the cells lost and the time point at which the loss occurs remains to be determined. Results further suggest that the proportional reliance of neurons on trophic support from Schwann cell precursors compared to other trophic support modes may be dependent on the particular mouse strain examined. This is related to the magnitude of trophic support by Schwann cells, and not the presence or absence of trophic support itself.

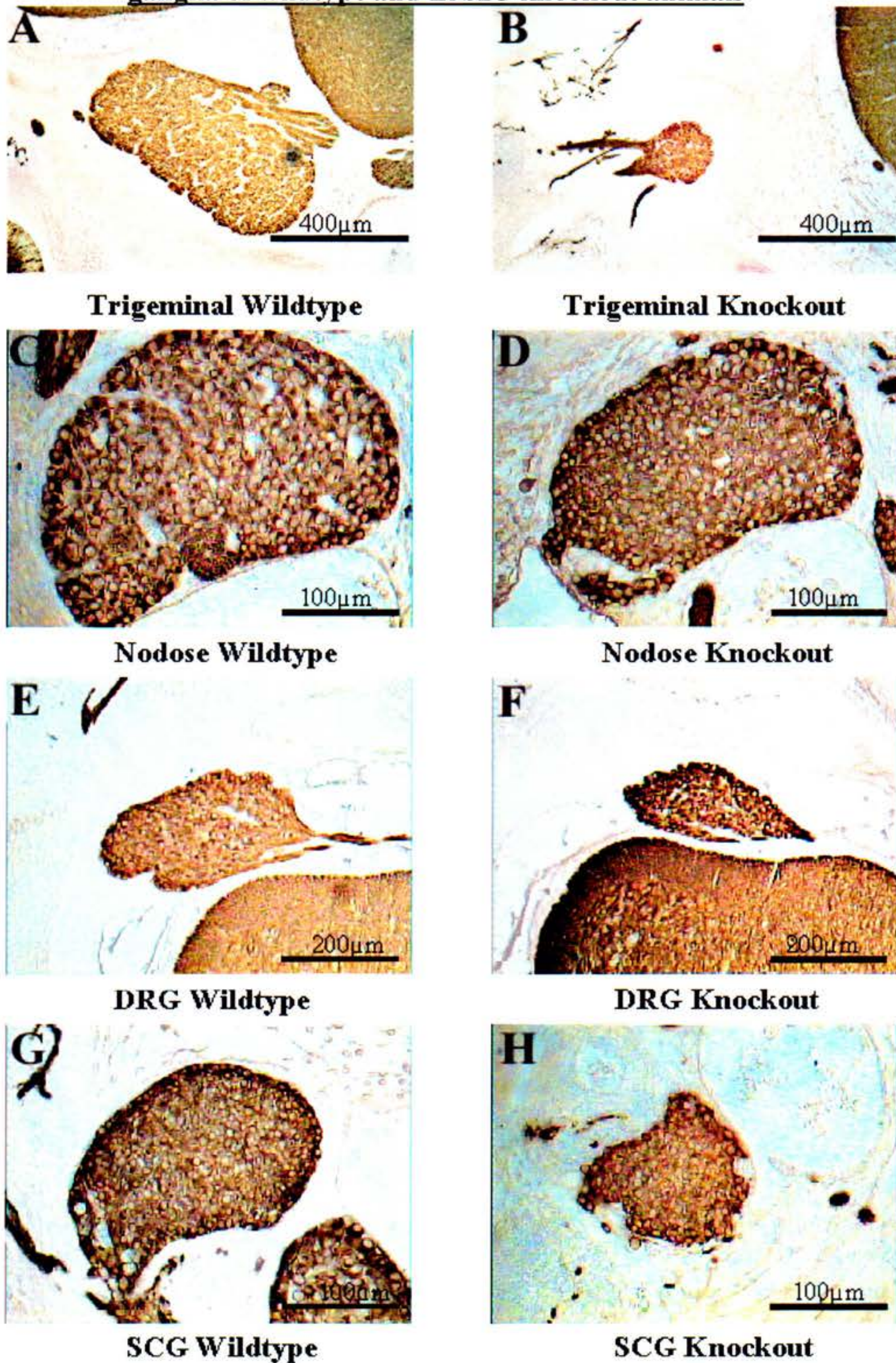
6.2 Results

ErbB3 mutant embryos were collected at daily intervals from E11 to E14. The proportion of null mutant embryos obtained from heterozygous matings was in agreement with previously found ratios, with a drastic reduction in frequency of homozygous null mice from E13 onwards. The embryos were processed for histology and the neuronal and pyknotic numbers in sensory ganglia quantified as described in chapter 2. Because of the strict criteria for classifying pyknotic cells as neurons, the actual proportion of dying cells shown in this results chapter is likely to be greater than the estimates suggest. Differences in counts of pyknotic neurons have previously been shown to be comparable to differences observed by double-labelling neuron markers and apoptotic nuclei immunohistochemically (Piñón et al., 1996). The phenotype of the sectioned trigeminal, nodose, DRG and SCG ganglia in ErbB3 knockout embryos at E14 is shown in Figure 6.1. Figure 6.2 shows the trigeminal ganglion at the stages of E11, E12 and E13.

6.2.1 The nodose ganglion

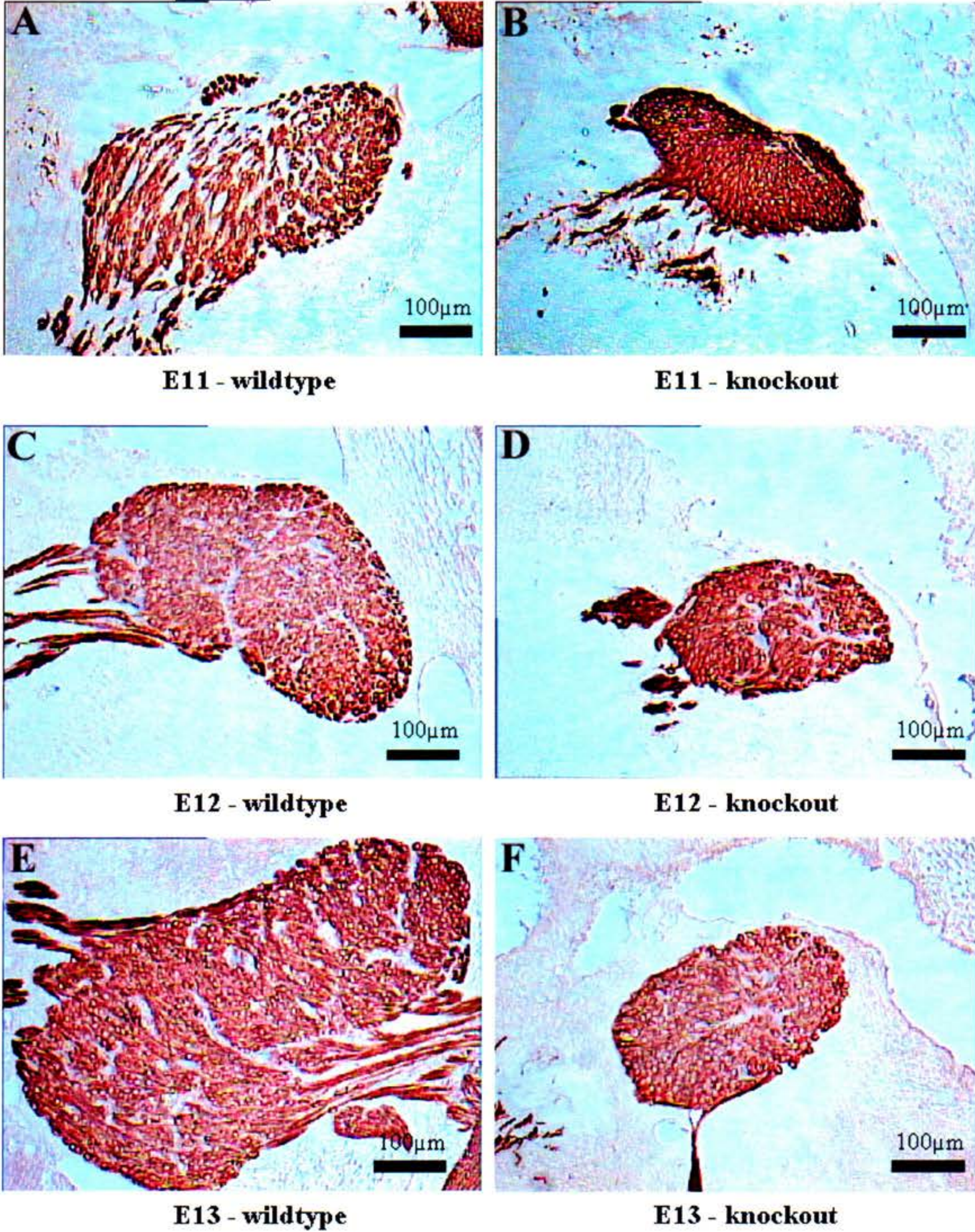
The phenotype of the nodose ganglion in *ErbB3* knockout embryos at E14 is shown in Figure 6.1. Analysis of neuronal numbers revealed no significant differences in neuronal numbers of the nodose ganglion of *ErbB3-/-* embryos compared with wildtype embryos early in development (E11 and E12), with slight differences emerging at E13 and E14 (20% and 14% reductions compared to wildtype respectively, t-tests: $p<0.005$ and $p<0.0005$) (Figure 6.3). Neurons of ErbB3 knockouts were tightly packed during the initial phase of development.

Figure 6.1: Comparison between E14 trigeminal, nodose, DRG and SCG ganglia of wildtype and ErbB3 knockout animals



Bright-field view of E14 ganglia of the peripheral nervous system in wildtype or ErbB3 receptor null mutants. The trigeminal (A–wildtype, B–knockout), nodose (C-wildtype, D-knockout), dorsal root (E-wildtype, F-knockout) and superior cervical (G-wildtype, H-knockout) ganglia are depicted. The massive neuronal loss observed in the trigeminal ganglion (B) can clearly be seen, in stark contrast to the just slightly smaller than normal nodose ganglion (D). The almost halved size of the dorsal root ganglion (F) and more than halved superior cervical ganglion (H) can also be seen.

Figure 6.2: E11, E12 and E13 trigeminal ganglia of wildtype and *ErbB3-/-* animals

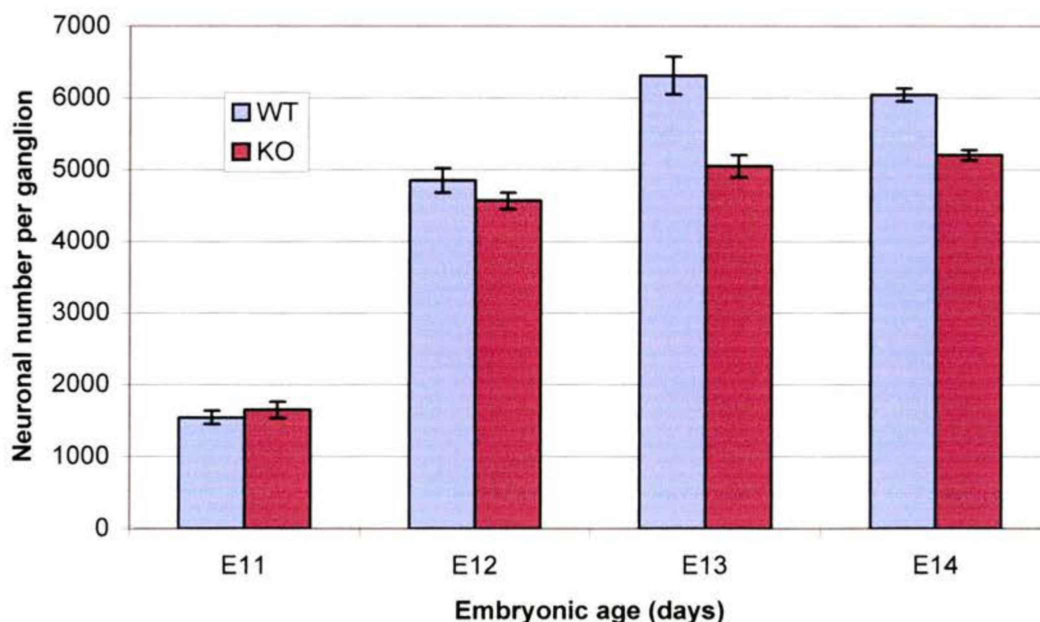


Bright-field view of E11-E13 trigeminal ganglia of wildtype (A-E11, C-E12, E-E13) and *ErbB3-/-* (B-E11, D-E12, F-E13) embryos clearly identified by cytoplasmic staining for βIII-Tubulin (Promega) and detected using DAB-peroxidase substrate (see chapter 2). The difference in size of *ErbB3-/-* ganglia can be seen clearly (B, D and F), as well as the very densely packed neurons in the knockout at E11 (B).

Neuronal density in the nodose was only restored to the equivalent of wildtype animals by E14, being significantly denser before. Although the mean size of the nodose is reduced from E12 onwards, this does not affect neuronal numbers due to the increased neuronal density at this age.

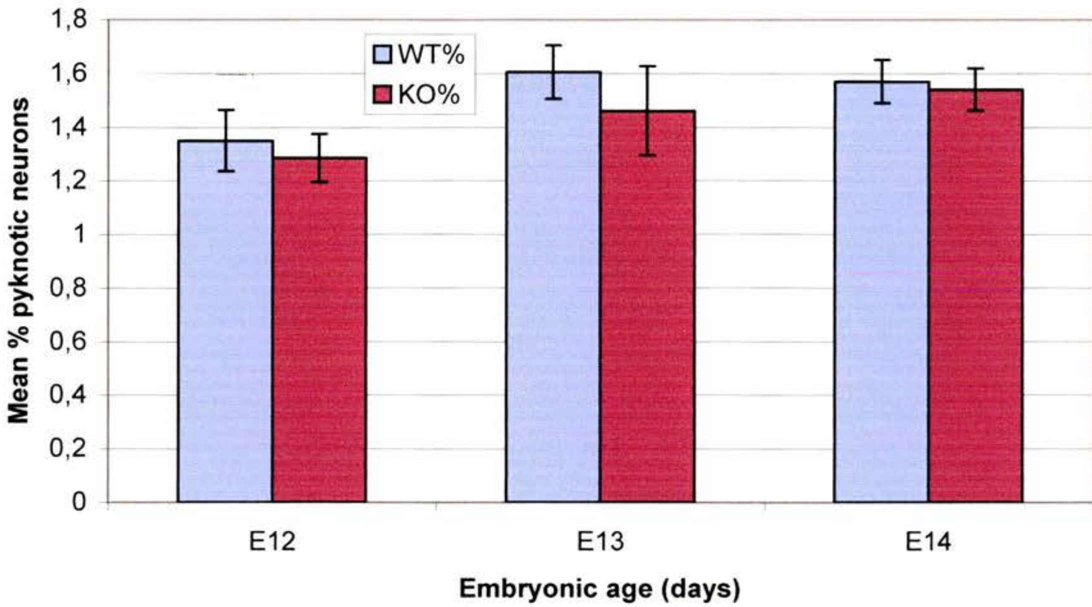
The number of pyknotic neurons in the nodose ganglion as a proportion of the total number is shown in Figure 6.4. The proportion of pyknotic neurons in the nodose ganglion of *ErbB3*-deficient embryos was not significantly different from the proportion seen in wildtypes at all ages studied. Although there may well be subtle differences in apoptosis, these were not large enough to be detected. Alternatively, it may be that neurogenesis in the nodose ganglion is slightly affected, giving rise to the neuronal complement of the nodose ganglia of *ErbB3-/-* mice at E13 and E14.

Figure 6.3: Mean neuronal numbers in the nodose ganglion of wildtype and *ErbB3-/-* embryos during development



Mean neuronal numbers in nodose ganglia of E11 – E14 embryos with no mutations (blue) or a homozygous null mutation for the ErbB3 receptor (red). Bars represent the mean ± standard error (n=6 for each data point).

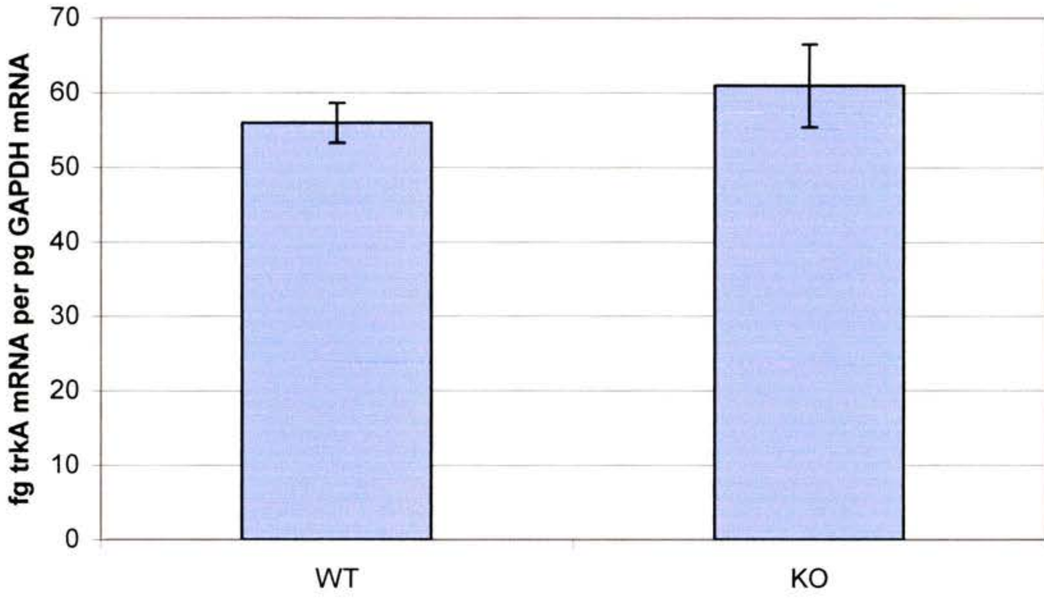
Figure 6.4: Mean percentage of pyknotic neurons as a proportion of total neuronal number in the nodose ganglion of wildtype and *ErbB3-/-* embryos during development



Mean percentage of pyknotic neurons in nodose ganglia of E12-E14 embryos with no mutations (blue) or a homozygous null mutation for the ErbB3 receptor (red). Bars represent the mean ± standard error (n=6 for each data point).

Figure 6.5 illustrates the trkA mRNA levels relative to GAPDH mRNA in the nodose ganglion of E12 *ErbB3-/-* embryos and wildtype littermates. Consistent with the normal numbers of neurons at this stage of nodose development, relative trkA mRNA expression in *ErbB3* null mutants was not significantly different from wildtype littermates.

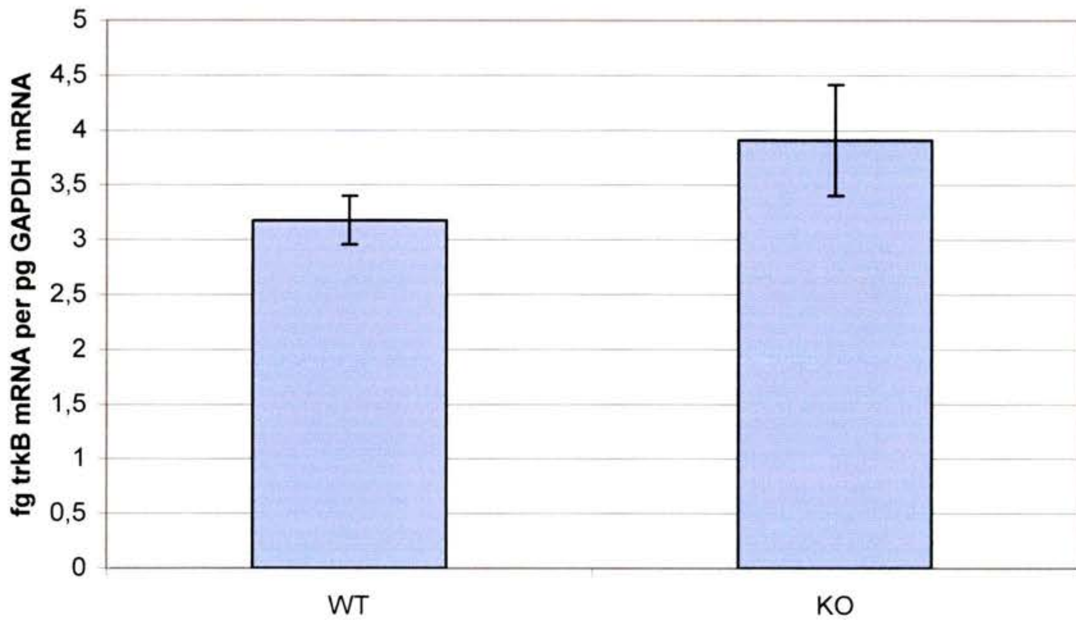
Figure 6.5: Relative levels of trkA mRNA in the nodose ganglion of wildtype and *ErbB3-/-* embryos at E12



Mean relative levels of trkA mRNA relative to GAPDH mRNA in the nodose ganglion of wildtype (WT) and *ErbB3-/-* embryos (KO) at E12. Bars represent the mean ± standard error (n=6 for each data point).

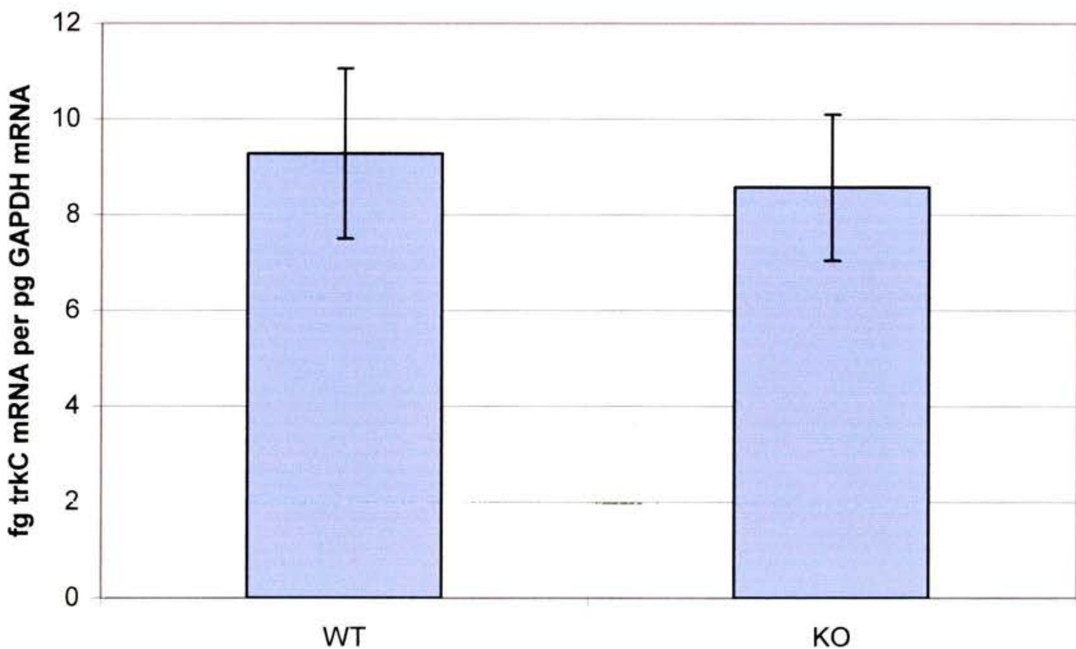
Figure 6.6 and 6.7 illustrate the trkB and trkC mRNA levels relative to GAPDH mRNA in the nodose ganglion of E12 *ErbB3-/-* embryos and wildtype littermates. Consistent with the normal numbers of neurons at this stage of nodose development, relative trkB and trkC mRNA expression in *ErbB3-/-* mice was not significantly different from wildtype littermates.

Figure 6.6: Relative levels of trkB mRNA in the nodose ganglion of wildtype and *ErbB3-/-* embryos at E12



Mean relative levels of trkB mRNA relative to GAPDH mRNA in the nodose ganglion of wildtype (WT) and *ErbB3-/-* embryos (KO) at E12. Bars represent the mean ± standard error (n=6 for each data point).

Figure 6.7: Relative levels of trkC mRNA in the nodose ganglion of wildtype and *ErbB3-/-* embryos at E12

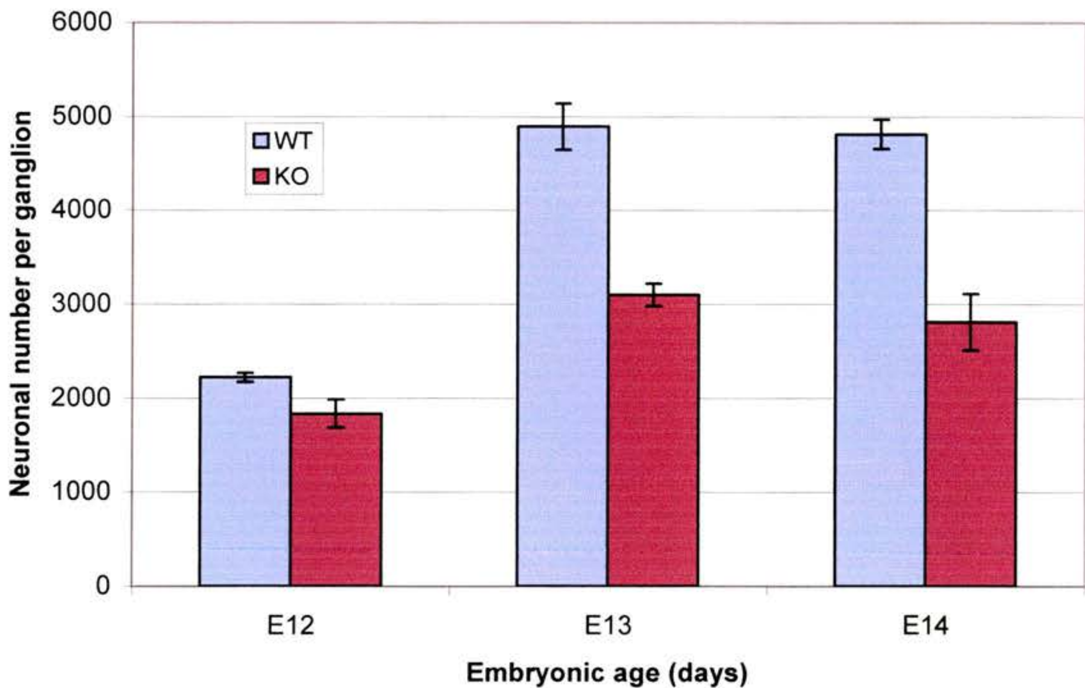


Mean relative levels of trkC mRNA relative to GAPDH mRNA in the nodose ganglion of wildtype (WT) and *ErbB3-/-* embryos (KO) at E12. Bars represent the mean ± standard error (n=6 for each data point).

6.2.2 The Dorsal root ganglia

The phenotype of the dorsal root ganglion in *ErbB3* knockout embryos at E14 is shown in Figure 6.1. Analysis of neuronal numbers revealed a small but significant 17% decrease in the neuronal complement of *ErbB3*-deficient C2 and C3 cranial dorsal root ganglia at E12 (t-test; $p<0.05$) (Figure 6.8). There was an increase in neuronal numbers between E12 and E13, but the increase in wildtypes was much greater than in *ErbB3*-deficient mice. Neuronal numbers in the mutant were 37% of wildtype by E13 in *ErbB3*-deficient mice (t-test; $p<0.00001$). Between E13 and E14 there was little additional loss with a difference of 42% between knockout and wildtype by E14 (t-test; $p<0.00001$).

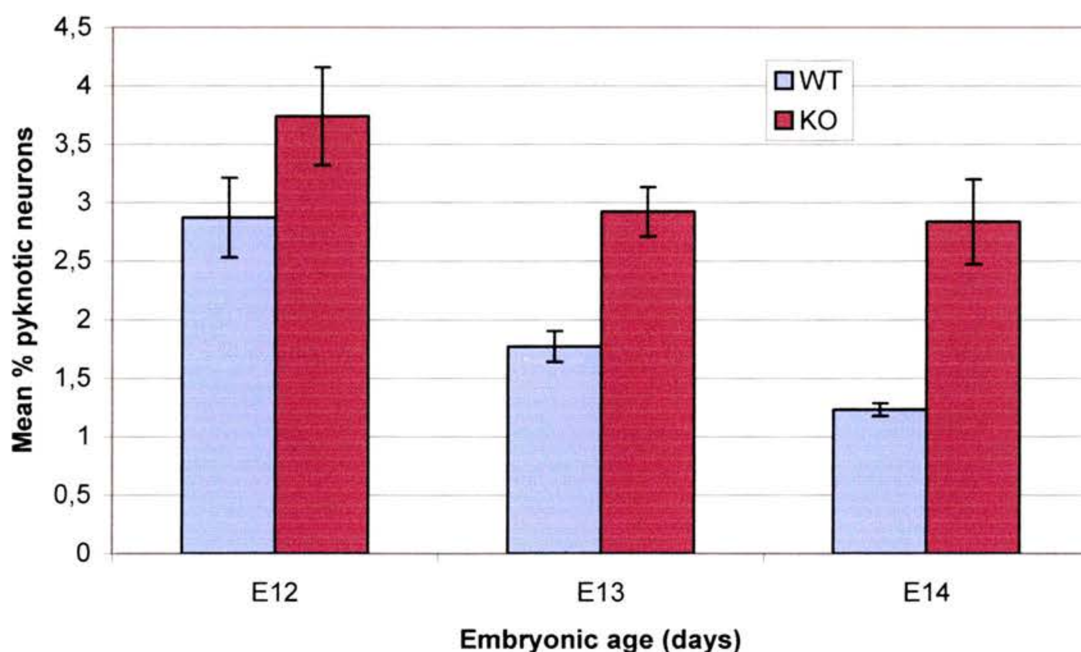
Figure 6.8: Mean neuronal numbers in the dorsal root ganglion of wildtype and *ErbB3-/-* embryos during development



Mean neuronal numbers in dorsal root ganglia of E12 – E14 embryos with no mutations (blue) or a homozygous null mutation for the ErbB3 receptor (red). Bars represent the mean ± standard error (n=6-12 for each data point).

The number of pyknotic neurons in the dorsal root ganglion as a proportion of the total number is shown in Figure 6.9. Early in development at E12 there was no significant difference in the number of pyknotic neurons in *ErbB3-/-* mice (Mann-Whitney; p>0.05). At E13 and E14, the number of pyknotic neurons decreased in both wildtype and knockout, but the proportion of pyknotic neurons in *ErbB3-/-* mice was significantly increased compared to wildtype numbers, by 65% and 165% respectively (Mann-Whitney; both p<0.0001).

Figure 6.9: Mean percentage of pyknotic neurons as a proportion of total neuronal number in the dorsal root ganglion of wildtype and *ErbB3-/-* embryos during development



Mean percentage of pyknotic neurons in dorsal root ganglia of E12-E14 embryos with no mutations (blue) or a homozygous null mutation for the ErbB3 receptor (red). Bars represent the mean ± standard error (n=4-12 for each data point).

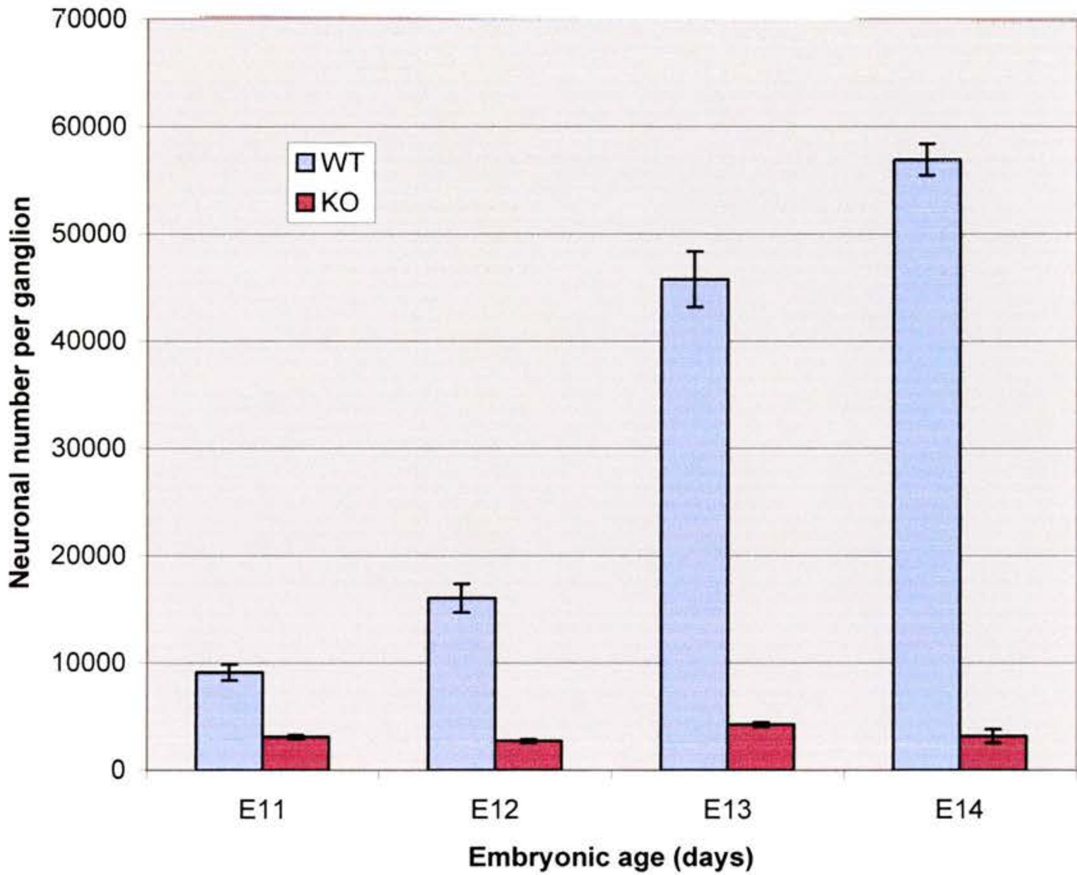
6.2.3 The trigeminal ganglion

Analysis of neuronal numbers revealed a very marked reduction in the trigeminal ganglion of *ErbB3-/-* mice from E11, the first age studied onwards (Figure 6.10). The lack of neurons in the trigeminal ganglia of *ErbB3* knockout embryos throughout early development is shown in Figure 6.2. The difference between the knockout animals and the wildtype animals grew with age, up to the last studied age of E14. Neurogenesis in the trigeminal ganglion was evident in wildtype animals, with increases in βIII-Tubulin positive cells by 76% from E11 to E12, 185% from E12 to E13 and 24% from E13 to E14. In the ErbB3 null mutant, however, there was only one obvious stage of neurogenesis in the ganglion (ANOVA; $p<0.05$), with a 56% increase in neurons between E12 and E13 (Fisher's LSD; $p<0.005$) and no significant differences between the other stages assessed.

Figure 6.2 demonstrates how the size of the trigeminal ganglion reflects the differences between the neuronal numbers in *ErbB3-/-* and wildtype mice. At E11 the density of the trigeminal ganglion was greatly increased (t-test; $p<0.0001$; Figure 6.11) making up for some of the reduced size observed. At subsequent stages in development the size of the ganglion increased, at the expense of the density, which restored itself to the equivalent of wildtype animals by E12. The significance of these events is not currently understood, but may be related to the absence of Schwann cell precursors. Tissue shrinkage may be another explanation for the differences in cell density, but as wildtypes and ErbB3-/- littermates were processed and stained at the same time and in the same solutions, this is unlikely. As can be observed in the slightly more distanced view of the trigeminal ganglion (E14) in figure 6.1, the

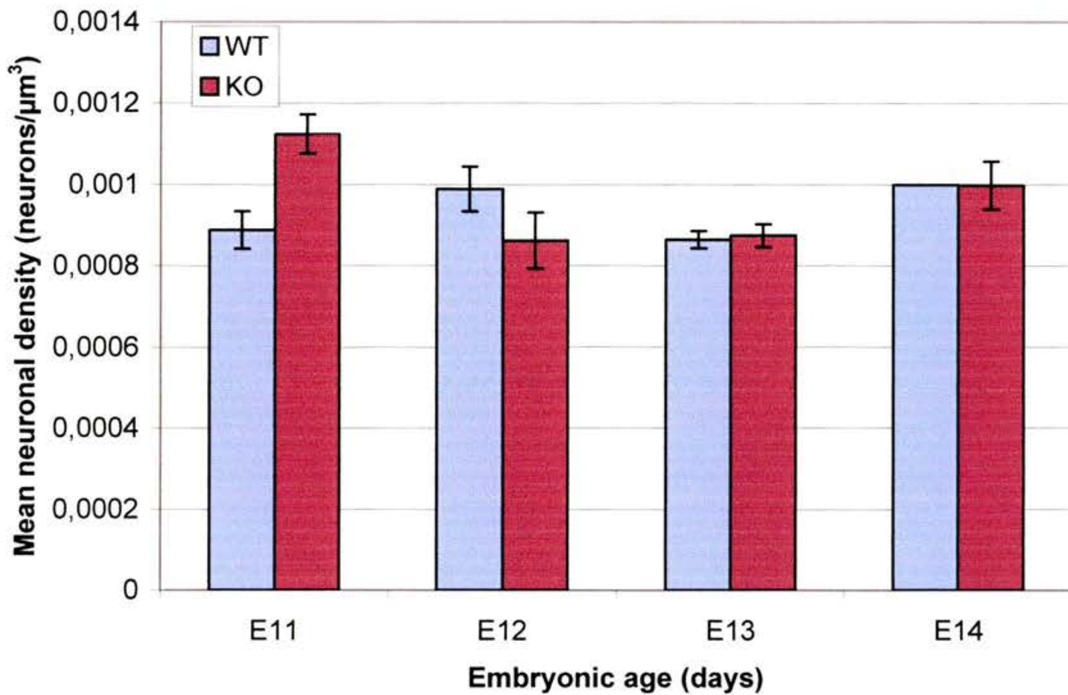
position of the neurons present in the ganglion tended to be in the ventrolateral region of the trigeminal ganglion. Neurons at prior ages appeared to be localised in the same region.

Figure 6.10: Mean neuronal numbers in the trigeminal ganglion of wildtype and *ErbB3-/-* embryos during development



Mean neuronal numbers in trigeminal ganglia of E11 – E14 embryos with no mutations (blue) or a homozygous null mutation for the ErbB3 receptor (red). Bars represent the mean ± standard error (n=6-8 for each data point).

Figure 6.11: Mean neuronal density of the trigeminal ganglion of wildtype and *ErbB3-/-* embryos during development

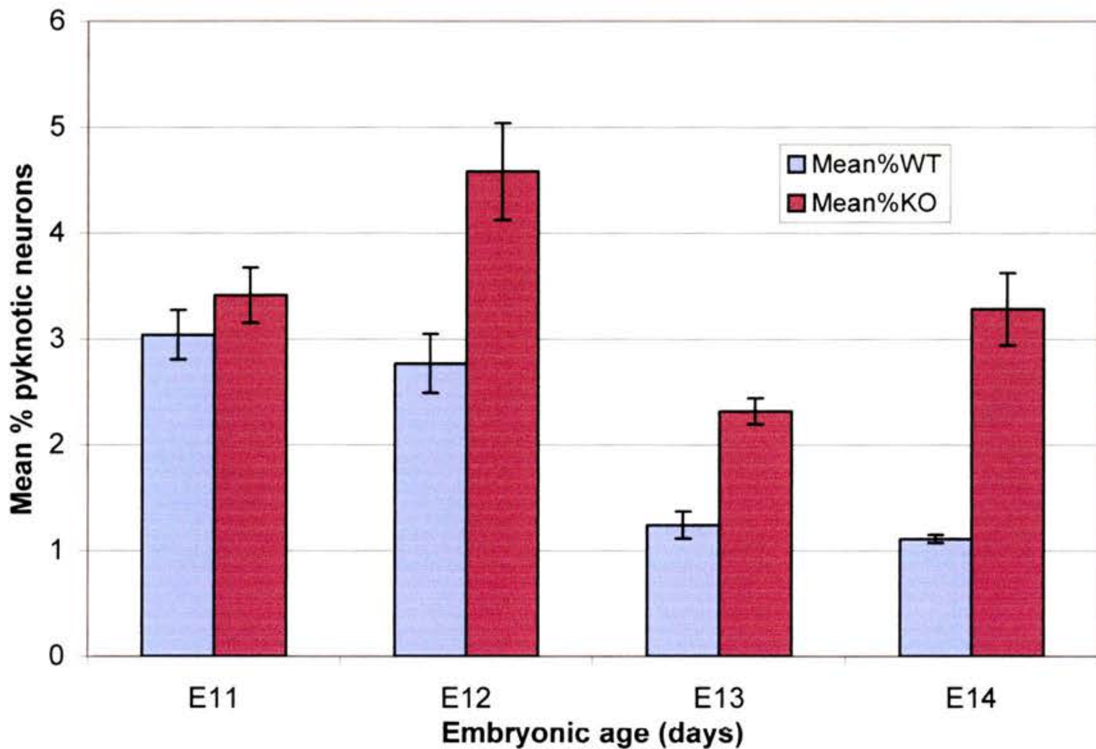


Mean neuronal density in trigeminal ganglia of E11 – E14 embryos with no mutations (blue) or a homozygous null mutation for the ErbB3 receptor (red). Bars represent the mean ± standard error (n=6-8 for each data point).

The number of pyknotic neurons in the trigeminal ganglion as a proportion of the total number can be seen in figure 6.12. Whereas the proportional levels of apoptosis seemed equal at the early stages of trigeminal ganglion development (Mann-Whitney; E11: $p>0.2$), the number of pyknotic neurons in the trigeminal ganglion were subsequently higher than in wildtype embryos, being 66% higher at E12 (Mann-Whitney; $p<0.05$), 87% higher at E13 (Mann-Whitney; $p<0.005$) and 195% higher at E14 (Mann-Whitney; $p<0.005$). These observations suggest that some of the reduction in the neuronal complement of the trigeminal ganglia of *ErbB3-/-* mice is related to a higher proportion of neurons undergoing apoptosis. As there are no significant differences between the neuronal numbers in knockout animals at the ages

studied (apart from the significant increase in numbers between E12 and E13), increased cell death must be compensated by neurogenesis in the ganglion to some extent.

Figure 6.12: Mean percentage of pyknotic neurons as a proportion of total neuronal number in the trigeminal ganglion of wildtype and *ErbB3-/-* embryos during development

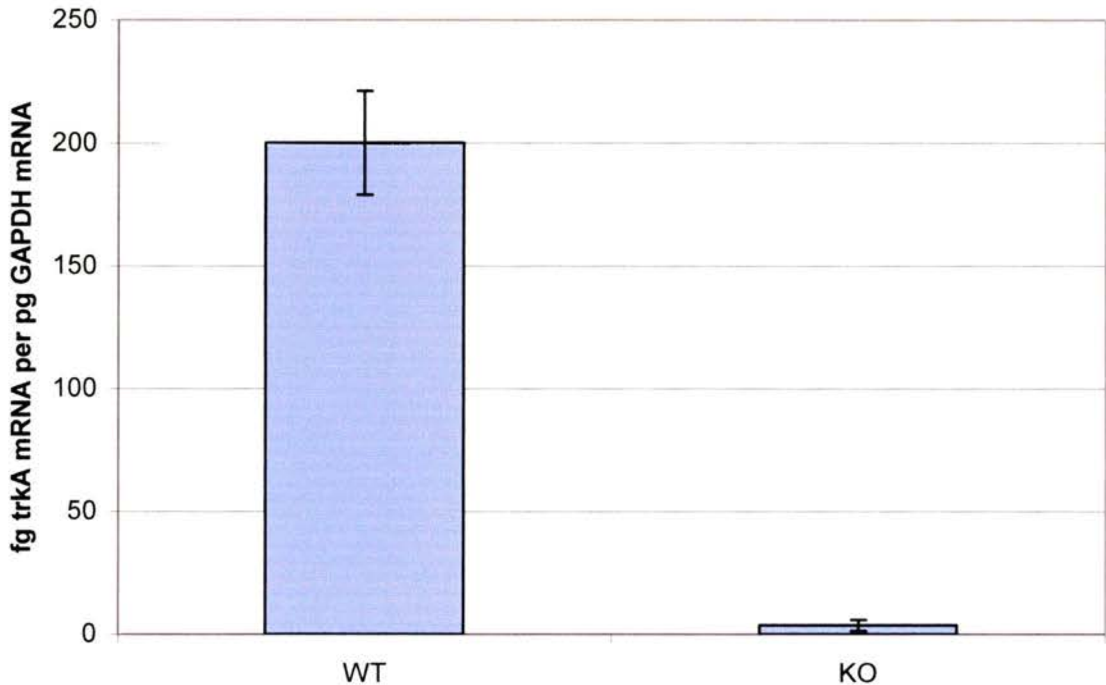


Mean percentage of pyknotic neurons in trigeminal ganglia of E11-E14 embryos with no mutations (blue) or a homozygous null mutation for the ErbB3 receptor (red). Bars represent the mean ± standard error (n=6-8 for each data point).

Relative levels or trk expression in the nodose and trigeminal ganglia were assessed using quantitative RT-PCR as outlined in chapter 2. Figure 6.13 compares the trkA mRNA levels relative to GAPDH mRNA in trigeminal ganglia of E12 *ErbB3-/-* embryos and wildtype littermates. *ErbB3* mutants displayed a 98% reduction in trkA mRNA levels compared with wildtype littermates (t-test; $p<0.00001$). An attempt

was made to quantify the relative trkB mRNA levels in E12 ErbB3 null mutants, however, the levels were below the limits of detection.

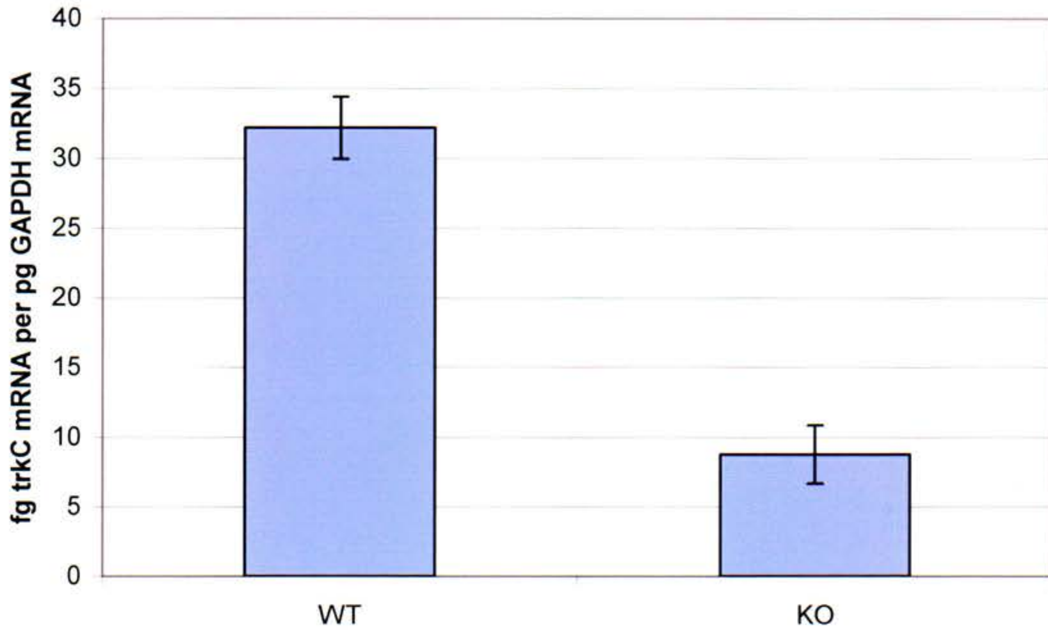
Figure 6.13: Relative levels of trkA mRNA in the trigeminal ganglion of wildtype and *ErbB3-/-* embryos at E12



Mean relative levels of trkA mRNA relative to GAPDH mRNA in the trigeminal ganglion of wildtype (WT) and *ErbB3-/-* embryos (KO) at E12. Bars represent the mean ± standard error (n=6 per data point).

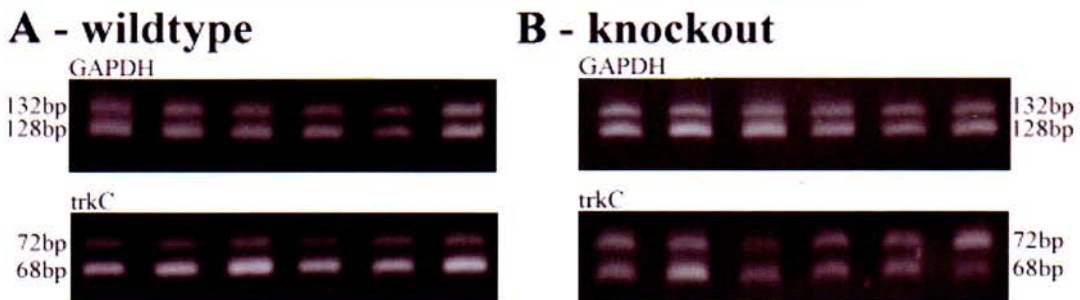
Figure 6.14 shows the trkC mRNA level relative to GAPDH mRNA in the trigeminal ganglia of E12 *ErbB3-/-* embryos and wildtype littermates. ErbB3 mutants displayed 73% lower levels of trkC mRNA expression than their wildtype littermates (t-test; $p<0.00001$). RT-PCR-gels showing differences in trkC mRNA in wildtype and knockout animals are shown in Figure 6.15.

Figure 6.14: Relative levels of trkC mRNA in the trigeminal ganglion of wildtype and *ErbB3-/-* embryos at E12



Mean relative levels of trkC mRNA relative to GAPDH mRNA in the trigeminal ganglion of wildtype (WT) and *ErbB3-/-* embryos (KO) at E12. Bars represent the mean ± standard error (n=6 per data point).

Figure 6.15: Gels showing the relative levels of trkC mRNA in the trigeminal ganglion of wildtype and *ErbB3-/-* embryos at E12

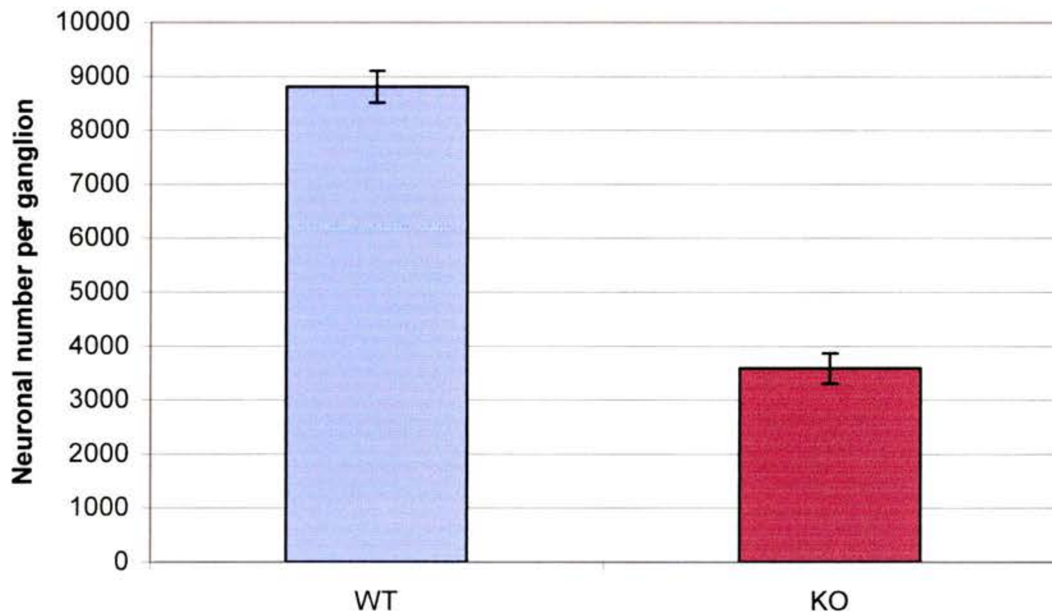


SYBR®Gold (Molecular Probes) stained gels showing the products of RT-PCR reactions amplified with either GAPDH-specific or trkC-specific primers. (A) Reactions containing 500fg of the GAPDH competitor RNA (132bp) and 6.7fg of the trkC competitor (72bp) plus total RNA from wildtype E12 trigeminal ganglia (GAPDH: 128bp; trkC: 68bp). (B) Reactions containing 83fg of the GAPDH competitor RNA (132bp) and 0.83fg of the trkC competitor (72bp) plus total RNA from *ErbB3-/-* E12 trigeminal ganglia (GAPDH: 128bp; trkC: 68bp).

6.2.4 The superior cervical ganglion

Although the main focus of this section is the survival of sensory neurons, the superior cervical ganglion was also assessed at E14 to determine whether any changes could be observed in sympathetic neurons. The phenotype of the superior cervical ganglion in ErbB3 knockout embryos at E14 is shown in Figure 6.1. Analysis of neuronal numbers revealed a major difference between *ErbB3-/-* and wildtype embryos (Figure 6.16). In null mutants, there were 59% fewer neurons than in the wildtype littermates (t-test; $p<0.00001$). Pyknotic neurons were not counted in the SCG due to the small size and high density of the neurons, making adequate identification of pyknotic nuclei impossible using CFV-staining (see chapter 2).

Figure 6.16: Mean neuronal numbers in the E14 superior cervical ganglion of wildtype and *ErbB3-/-* embryos

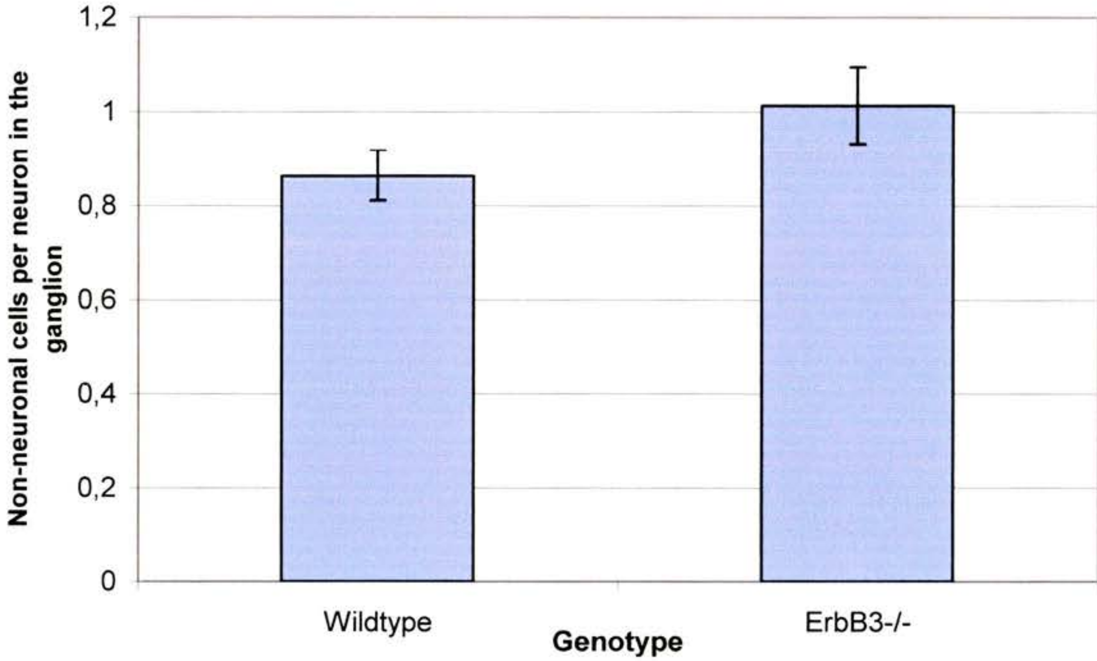


Mean neuronal numbers in the superior cervical ganglion at E14. Wildtype embryos (blue) and homozygous null mutants (red) are represented. Bars represent the mean ± standard error (n=6 per data point).

6.2.5 Non-neuronal cells in the nodose, DRG and trigeminal ganglia

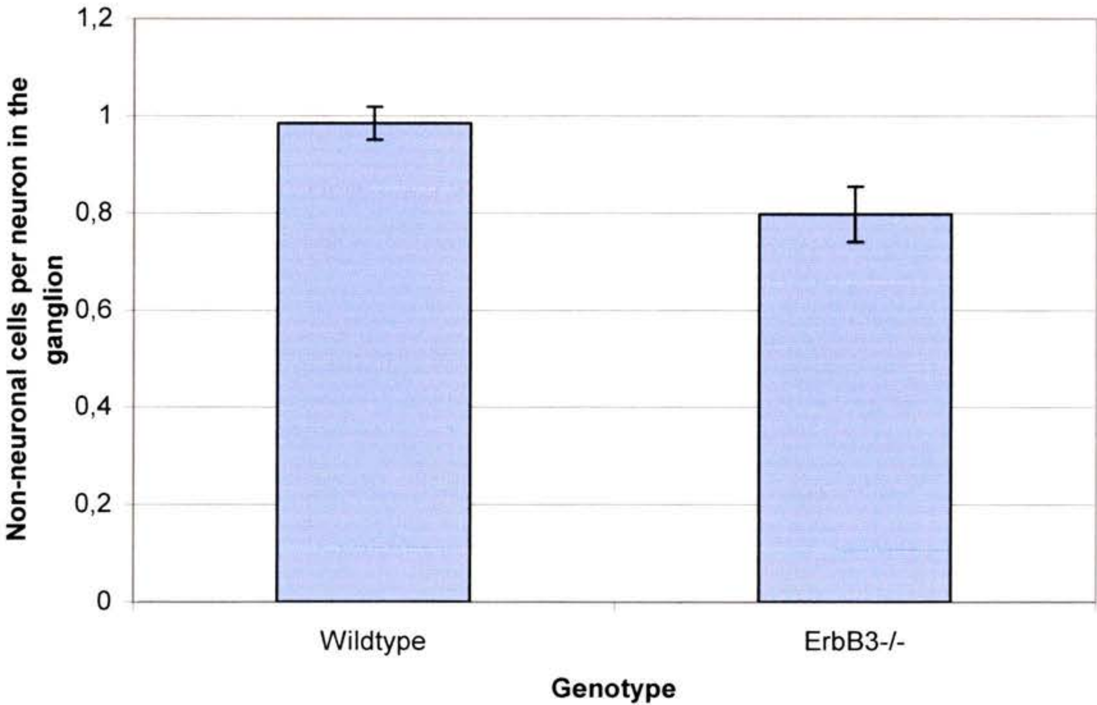
To assess the effect of the *ErbB3* null mutation on non-neuronal cells in the ganglia studied, the total number of visible cells in CFV-stained sections at E14 (neuronal cells, identified by their large pale nucleus surrounded by stained cytoplasm, and non-neuronal cells, identified as all cells not displaying neuronal criteria) were quantified and corrected as described for neuronal cells (chapter 2). The number of non-neuronal cells was then expressed as a proportion of the number of neuronal cells. In all the sensory ganglia studied the proportion of non-neuronal to neuronal cells in wildtype mice was approximately 1:1 (Figures 6.17 – 6.19). In the trigeminal ganglion, this proportion remained unchanged in *ErbB3-/-* embryos (Figure 6.17; Mann-Whitney; $p>0.1$). In the DRG and the nodose ganglia there were significantly less non-neuronal cells per neuronal cell than in the wildtype embryos. In the DRG, the ratio was reduced by approximately 20% to 0.8 non-neuronal cells per neuronal cell (Figure 6.18; Mann-Whitney; $p<0.05$), whereas in the nodose ganglion the ratio was reduced by over 60% to 0.4 non-neuronal cells per neuronal cell (Figure 6.19; Mann-Whitney; $p<0.005$).

Figure 6.17: Mean proportion of non-neuronal to neuronal cells in the trigeminal ganglion of E14 wildtype and *ErbB3-/-* embryos



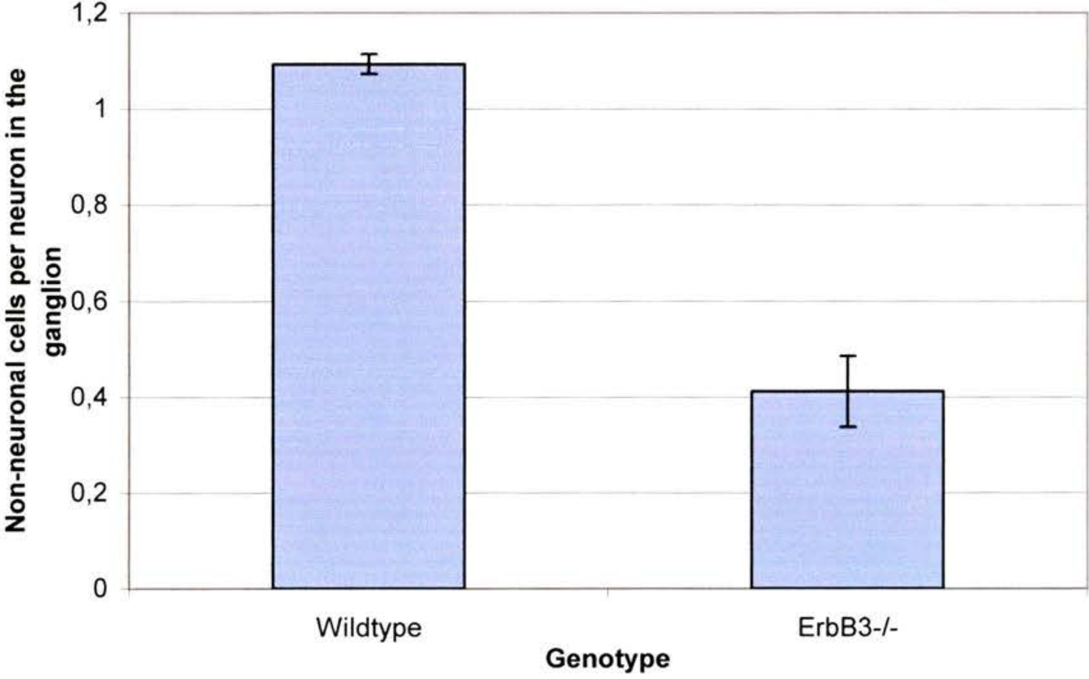
Bars represent the mean ± standard error (n=6 per data point).

Figure 6.18: Mean proportion of non-neuronal to neuronal cells in the dorsal root ganglion of wildtype and *ErbB3-/-* embryos



Bars represent the mean ± standard error (n=12 per data point).

Figure 6.19: Mean proportion of non-neuronal to neuronal cells in the nodose ganglion of wildtype and *ErbB3-/-* embryos



Bars represent the mean ± standard error (n=6 per data point).

6.3 Discussion

The aim of this study was to establish the importance of Schwann cell precursors for the survival of populations of embryonic sensory neurons using *ErbB3-/-* mice which lack these cells. The number of neurons in the nodose ganglia of ErbB3-deficient embryos remained normal until E13 and E14, where only a slight reduction in number was observed. No differences in the number of pyknotic neurons were found at any of the ages studied, and the relative levels of trkA, trkB and trkC mRNA in the ganglion were unchanged in E12 *ErbB3-/-* mice. Dorsal root ganglia of *ErbB3-/-* mice displayed little loss of neurons at E12, followed by a large loss in the neuronal complement by E13 and E14. This was reflected by increased numbers of pyknotic neurons at these ages. Neuronal numbers in trigeminal ganglia of *ErbB3* knockouts were greatly reduced from the earliest age studied (E11), remaining low throughout the normal period of neurogenesis of this population of neurons. Reduced neuronal numbers appeared to be most prevalent in the dorsomedial part of the ganglion. The number of pyknotic neurons expressed as a proportion of total neuron number was normal in the trigeminal ganglion at E11, but at subsequent ages there were higher proportional amounts in *ErbB3-/-* compared to wildtype embryos. The relative levels of both trkA and trkC mRNA were greatly reduced in E12 *ErbB3*-deficient trigeminal ganglia compared to wildtype ganglia. Neuron numbers in the superior cervical ganglia were also reduced in *ErbB3*-deficient mice at E14 compared to wildtype mice. The number of non-neuronal cells approximately matched the number of neurons in the trigeminal and DRG ganglia of wildtype and *ErbB3-/-* mice. In wildtype nodose ganglia the number of non-neuronal cells also matched the number

of neurons, however, in *ErbB3-/-* nodose ganglia the ratio of non-neuronal to neuronal cells was greatly reduced.

These results indicate that nodose neurons are less dependent on intermediate trophic support from Schwann cell precursors while growing to their target field than trigeminal, DRG and SCG neurons. However, non-neuronal cells in the nodose ganglion appear to be more sensitive to the *ErbB3* mutation than non-neuronal cells in the other ganglia, indicating different survival requirements. The nature of the non-neuronal cells lost preferentially in the nodose ganglion is unknown at present.

Nodose neurons have previously been found to survive for long periods of time in culture independently of trophic factors at the stage when their axons are growing to their target fields (Vogel and Davies, 1991). This prolonged period of neurotrophin independence appears to be related to the long distance the peripheral and central axons of these neurons are required to grow to their target fields, further than other sensory neuron population. This period of neurotrophin independence appears to be intrinsically programmed in nodose and other placode-derived neurons (Vogel and Davies, 1991). Nodose neurons may thus not depend on intermediate neurotrophic support from cells and tissues lying en route to their targets before these targets are reached. The unchanged proportion of pyknotic neurons, together with the unaltered expression of trk receptors further suggests that nodose neurons are independent of intermediate support from Schwann cell precursors. The slight decrease in neuronal numbers in *ErbB3* null mutants suggests that some intermediate support by Schwann cell precursors is required by a minority of nodose neurons while their axons are growing towards their target fields. In summary, most nodose neurons appear to be

independent of intermediate support by Schwann cell precursors during the period of growth to their target fields, consistent with their independence on trophic factors during this period.

Findings in the DRG confirmed that neurogenesis in *ErbB3-/-* mice initially occurs normally in this neuronal population (Riethmacher et al., 1997), with little loss of neurons at E12. The massive subsequent loss of neurons in the ganglion, however, coincides with a significant increase in pyknotic neurons at E13 and E14. This period of enhanced neuronal loss during which neuronal loss in the DRG of *ErbB3-/-* embryos coincides with the stage of development when many axons are still growing towards their targets, suggesting that this neuronal population is dependent on intermediate trophic support from factors released by Schwann cell precursors. The increased neuronal death coincides with the period of Schwann cell precursor generation between E12 and E13 in the mouse embryo (Mirsky et al., 2002). However, the extent of neuronal loss observed in the DRG of *ErbB3-/-* animals studied here (~42%) is less than previously reported (~70%) (Riethmacher et al., 1997). There are two possible explanations for this. First, there may be differences in the background strain of the mice used. The previous study was carried out in a C57/BL6 background, whereas mice used in the current study had been bred into a CD1 background. This raises the interesting possibility that neurons in different strains of mouse could be affected differently by deficits in Schwann cell precursors. Differences in background strains have previously been reported to affect the number of DRG neurons in *BDNF*-deficient mice (Fariñas, 1999; (Liebl et al., 1997). Second, the effect observed may be due to the rostral-caudal position of the DRG counted. In

the present study, the C2 and C3 DRG were counted, whereas the previous study assessed L3 and L4 DRG (Riethmacher et al., 1997). It is possible that neurons in the DRG follow the same rostral-caudal phenotype seen with the sympathetic chain, with neuronal loss being more severe in caudal areas (see below).

The neuronal deficit observed in the trigeminal ganglion of *ErbB3-/-* mice early in development confirms non-quantified deficits observed earlier in the development of *neuregulin-/-* and *ErbB2-/-* mice (Meyer and Birchmeier, 1995; Lee et al., 1995). These deficits were attributed to neuregulin signalling through ErbB2/ErbB3 being essential for the neurogenic lineage of the cranial neural crest.

In birds, neural crest-derived neurons occupy a dorsomedial position in the developing trigeminal ganglion, whereas those of placodal origin lie in the peripheral rim of the ganglion, forming a ventrolateral 'cap' (Noden, 1980; Davies and Lumsden, 1990). Also, avian trigeminal ganglion neurons of different embryonic origins tend to make contact with different target tissues (Davies, 1988). Therefore, an interesting observation in the *ErbB3-/-* mice studied was that the remaining neurons occupied a ventrolateral position in the ganglion (see Figure 6.6). This may suggest that the remaining cells in the trigeminal ganglion of *ErbB3-/-* embryos were of placodal origin, and that the neural crest-derived complement was reduced or missing, in accordance with previous observations in *neuregulin-/-* and *ErbB2-/-* embryos (Meyer and Birchmeier, 1995; Lee et al., 1995).

The strongly decreased levels of trkA mRNA observed in *ErbB3-/-* trigeminal ganglia would support this hypothesis. In the chicken, neurons expressing trkA transcripts are confined to the dorsomedial portion of the early trigeminal ganglion,

representing the neural crest-derived portion of the ganglion. Neurons expressing trkB and trkC are located ventrolaterally, representing the placode-derived portion of the ganglion (Williams et al., 1995). This would explain the massively reduced levels of trkA mRNA observed in the trigeminal ganglion of *ErbB3-/-* embryos at E12, whereas reductions in trkC mRNA levels were less drastic. However, if one assumes that trkA-expressing neural crest-derived neurons are lost in the trigeminal ganglion at this stage with little effect on the placode-derived neuronal complement, one would expect a proportional increase of trkB and trkC relative to GAPDH mRNA in the *ErbB3-/-* embryo. This would be a consequence of the relative reduction in absolute GAPDH mRNA levels in the ganglion of *ErbB3-/-* mice following the loss of neurons of neural crest origin. TrkB mRNA, although detectable in wildtype ganglia, was below detectable levels in *ErbB3-/-* mice, with trkC mRNA greatly reduced too. Schwann cells, but not Schwann cell precursors have been shown to express trkC. This makes it unlikely that the reductions in trkC receptor expression were related to the absence of Schwann cell precursors. This suggests that, even if the cells seen in the ganglion were of placodal origin, they were still affected by the *ErbB3* null mutation.

In early *ErbB2* null mutants, the maxillary and ophthalmic nerves of the trigeminal ganglion appear normal, which appears to be the case for the *ErbB3* mutants as well, at least at E11 (see figure 6.10). This would suggest that early trigeminal, presumably placode-derived, neurons extend ophthalmic and maxillary axons normally. At later ages in *ErbB3-/-* embryos, few projections were observed from the trigeminal ganglion (Figures 6.10 and 6.6), suggesting that the initially normal

outgrowth is not maintained later in development. This correlates with the increased proportion of pyknotic neurons observed from E12 to E14. The increased neuronal death correlates with the onset of Schwann cell precursor generation in the mouse between E12 and E13 (Mirsky et al., 2002). This would rule out any direct implication of Schwann cell precursors in the initial deficit observed in the trigeminal ganglion at E11, but suggests that they mediate trophic effects on the remaining population of neurons later in development. It is thus possible that many placode-derived neurons of the trigeminal ganglion develop normally initially, but subsequently die due to a lack of Schwann cell precursor-mediated trophic support. Neurons extending axons to the maxillary process are known to require intermediate trophic support by BDNF and NT-3 during axonal growth (Buchman and Davies, 1993; Paul and Davies, 1995). The results suggest that at least some placode-derived neurons in the trigeminal ganglion initially extend axons to the maxillary process independently of intermediate trophic support, but subsequently become dependent on such trophic support from Schwann cell precursors.

Neuronal numbers in the SCG at E14 were reduced in ErbB3 null mutants in comparison with wildtype embryos. Severe hypoplasia of the primary sympathetic chain has previously been reported in *ErbB3-/-* mice, as well as in *ErbB2* and *neuregulin-1* null mutants at E10.5 (Britsch et al., 1998). This was attributed to an impaired migration of neural crest cells to the anlage of the primary sympathetic chain. Normal migration of neural crest cells was observed to the anlage of DRG, followed by a severe reduction in the cells migrating beyond this point towards the sympathetic anlage. This previous study found only small changes in the size of the

SCG at E12.5, with effects being more severe in the caudal portion of the sympathetic chain, illustrating a rostral-caudal phenotype. This would suggest a migration pattern closer to wildtype in the rostral part of the embryo. There are indeed indications that the migration of neural crest cells to the SCG occurs differently from other ganglia in the sympathetic chain, and may thus not require the neuregulin signalling system required by trunk sympathetic neural crest cells for migration. The SCG is predominantly generated from sympathogenic neural crest cells from the hindbrain rather than trunk crest cells (Durbec et al., 1996).

Reduction in the number of SCG neurons observed at E14 would likely be related to neuronal death due to the lack of Schwann cell-derived support rather than to deficits in migration. By E17.5 a 50% reduction of the SCG was observed in previous studies, whereas the remainder of the sympathetic chain was almost completely absent (Britsch et al., 1998). Interestingly, in the CD1 background mutants of this study, a reduction in neuronal numbers in the SCG of *ErbB3-/-* mice of approximately 60% was found at E14 already, suggesting potential differences in the support mediated by Schwann cells depending on the mouse strain examined.

The results of non-neuronal cell counts at E14 are interesting, because of the apparent deficiency in non-neuronal cells in relation to neurons in the nodose ganglia of *ErbB3-/-* mice. In the other ganglia studied, the trigeminal and the DRG, the number of non-neuronal cells appears to match the number of neurons (or vice-versa). It will be important to establish the type of cell excessively lost in the nodose ganglia of *ErbB3-/-* mice, since these appear to be more vulnerable to this mutation than the neurons in the ganglion and non-neuronal cells in other sensory ganglia. Of

the glial cells within the ganglion, satellite cells have been detected in the trigeminal, DRG, nodose and SCG of *ErbB3-/-* mice (Britsch et al., 2001). These measurements were made at the earlier developmental stage of E12.5, however, allowing for the possibility of an excessive loss of this cell type in the nodose ganglion after this period of development. It will be necessary to establish whether the number of satellite cells within the nodose and other ganglia studied remains unaffected in *ErbB3-/-* mice by E14, using antibodies such as B-FABP (Brain-specific fatty acid binding protein; Kurtz et al., 1994). Should it be found that there is a deficiency in satellite cells in the nodose ganglion, this would further illustrate the ability of this population of neurons to survive in the absence of intermediate trophic support, since other sensory neuron populations undergo more severe losses in mice lacking both Schwann cell precursors and satellite cells (Britsch et al., 2001).

In summary, normal neuronal numbers were observed in the dorsal root and nodose ganglia of *ErbB3-/-* mice at E12. Both the losses observed in the trigeminal ganglion and the SCG can be partially explained by deficits in migration of the neurogenic neural crest lineage. The DRG and nodose ganglia subsequently lost a proportion of neurons between E12 and E13. However, neuronal deficiency in the DRG was double that seen in the nodose ganglion. Additionally, increases in the proportion of apoptosis were observed in the DRG, whereas none were seen in the nodose ganglion at later stages. Together these data suggest that neural crest-derived neurons in the DRG are more reliant on intermediate trophic support by Schwann cell precursors than placode-derived neurons in the nodose ganglion. This correlates with the requirement of the majority of neural crest-derived sensory on intermediate trophic

support, whereas most placode-derived sensory neurons at similar stages survive for varying lengths of time without neurotrophins in vitro that are correlated with target distance. Interestingly, the remaining neurons in the trigeminal ganglion, lying in a placodal location, appear to be dependent on Schwann cells. The apparently normal ophthalmic and maxillary projections in *ErbB3-/-* embryos at E11 suggest that these neurons extend axons to these target fields. However, these projections subsequently appear to be reduced, which together with the increased proportion of pyknotic neurons and the decreases in relative trkB and trkC mRNA levels suggests a loss of neurons extending axons towards these targets during the period of Schwann cell precursor generation. Neurons extending axons to the maxillary process are known to require intermediate trophic support from surrounding tissue, which is likely to be derived at least in part from Schwann cell precursors.

Analysis of pyknotic neuron counts revealed that the proportion of dying neurons in the trigeminal and dorsal root ganglia of *ErbB3-/-* embryos started increasing at E12/E13, compared with wildtype embryos, coinciding with the generation of Schwann cell precursors. This suggests that prior to this period the neurons were independent of factors released by Schwann cell precursors, consistent with the differentiation of sensory neurons preceding Schwann cell precursor generation in the DRG (Lawson and Biscoe, 1979). Since there can be no signal from the Schwann cell precursors to trigger this dependency in *ErbB3-/-* embryos, it is possible that this increased neuronal death is due either to an intrinsic programme in the neurons or to signals from other cells which govern the onset of Schwann cells precursor dependence.

Some aspects of this study will require further investigation. Lee et al. (1995) determined that it was the neural crest derived portion of the trigeminal ganglion missing in *ErbB2* knockout mice (see Figure 6.1 for an indication of this in ErbB3 null mutants). This was done on the assumption that the dorsomedial region of the ganglion was missing and the ventrolateral region of the ganglion still present, a line of thought adopted in the analysis of this study. However, even though the indications suggest that the neurons remaining in older ganglia are of placodal origin, this will have to be determined with certainty. It could be that the neurons are neural crest derived but misplaced in relation to their normal position. Neuregulin signalling has been shown to be important for the positioning of neurons into distinct ganglia. For example, *ErbB4* null mutations result in near-fusion of the trigeminal and the geniculate ganglia (Gassman et al., 1995). No such effect has been reported in *ErbB3-/-* mice, however, which signifies that this receptor is not involved in such processes. The degree of absence of neural crest-derived neurons and the presence of placode-derived neurons will need to be assessed using specific markers, which are currently only available for the chick (Covell and Noden, 1989; Stark et al., 1997). It would be interesting to examine whether placode-derived sensory neuron populations that have closer targets than nodose neurons, such as the geniculate ganglion, become dependent on Schwann cell support earlier in development than nodose ganglion neurons.

To study more fully the influence of Schwann cells and their precursors on neural crest-derived neurons, it will be necessary to look at alternative methods, since all of the null mutants studied thus far have deficits in neurogenic neural crest migration

and differentiation. Conditional mutations, eliminating *ErbB3* purely in sensory ganglia and thus allowing normal migration of neural crest cells, may provide an alternative approach to solving this problem.

Regulation of the onset of Schwann cell precursor dependence will need to be addressed, possibly using co-cultures of neurons with Schwann cell precursors, a strategy that would be suitable to study the effects of Schwann cell precursors on neural crest-derived neurons as well. Methods for culturing Schwann cell precursors have recently been identified (Dong et al, 1999; Lobsiger et al., 2000).

The importance of other glial cells in providing neurotrophic support in the peripheral nervous system will also need to be examined. Satellite cells, for example, are still present in the ErbB3 null mutant at early stages of development (although there may be deficiencies later in development, as suggested by non-neuronal cell counts in the nodose ganglion) (Britsch et al., 2001). The recently generated *Sox10* null mutant mouse (a transcription factor for, among others, ErbB3), was found to lack both satellite and Schwann cell precursors. This mutant was found to have more severe sensory and motor deficits than the ones observed in the *ErbB3* knockout (Britsch et al., 2001). It would thus be of interest to study this null mutant to determine whether the variations between neuronal populations requiring intermediate support and those who do not may be even more accentuated, due to variations in the requirement of trophic support from within the ganglion, by satellite cells.

Conclusions

The results presented in this section support the idea that early trigeminal and dorsal root ganglion neurons are dependent on trophic support from Schwann cells and their precursors when their axons are growing to their targets whereas nodose neurons are mostly independent of such support which is consistent with previous in vitro data suggestive of a prolonged period of neurotrophic independent survival in nodose neurons when the axons of these neurons are growing to their targets. This difference in dependence is summarised in Figure 6.20.

The molecular nature of trophic signals from Schwann cell precursors in vivo is currently unclear. Members of the GDNF family of trophic factors have been suggested to play a part in the survival effects on motoneurons. GDNF mRNA is found in Schwann cell precursors and immature Schwann cells, which together with the loss of motoneurons in *GDNF-/-* mice by birth suggesting that this is one of the factors released by Schwann cells and their precursors to promote motoneuron survival. GDNF is unlikely to promote the survival of DRG neurons, however, since its receptor Ret is expressed at very low levels at the stages these neurons undergo cell death in *ErbB3-/-* embryos. NT-3 is produced by early Schwann cells, and may thus be a factor important for the survival of sensory neurons during axonal growth, since both the dorsal root and trigeminal neurons are believed to require this factor early in development. However, NT-3 secretion by Schwann cell precursors still remains to be demonstrated (reviewed by Jessen and Mirsky, 1999).

Figure 6.20: Differences in intermediate trophic dependence on Schwann cells and their precursors between the trigeminal/dorsal root ganglia and the nodose ganglion

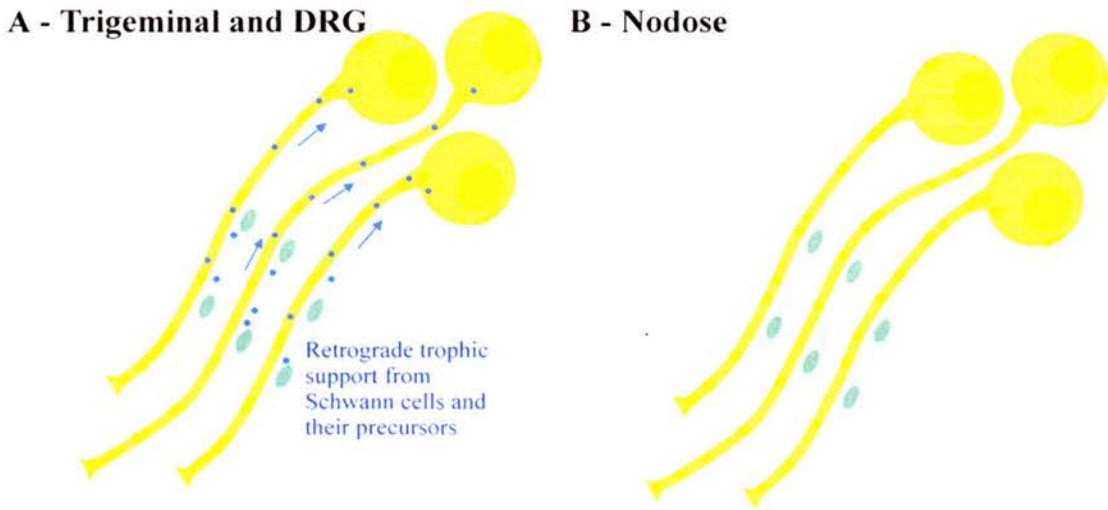


Figure illustrating the differences in intermediate survival requirements of trigeminal and dorsal root ganglia neurons (A) and of nodose neurons (B) as they extend axons to their target fields. While trigeminal and dorsal root neurons require intermediate trophic support from Schwann cells and their precursors en route to their targets (blue), nodose neurons appear to be less dependent on such intermediate support (adapted from Davies, 2003).

CHAPTER VII

Final Discussion

The key findings of the thesis may be summarised as follows:

1. Contrary to previous assumptions, trkA mRNA levels in the trigeminal ganglion do not appear to be solely regulated by intrinsic mechanisms. The sharp intrinsic increase in trkA expression taking place between E11 and E13 (coinciding with the onset of NGF-dependence) is dampened down by substance(s) released from the target fields *in vitro*. This period covers the time between innervation and programmed cell death *in vivo*. trkA expression levels then decrease between E14 and E15, with the target fields having no effect on trkA expression in the ganglion at these later ages.

2. Further to the effect of the target on ganglion trkA expression, there appears to be a reciprocal interaction controlling target NGF expression in early development. *In vitro* results suggest that initial early innervation promotes NGF expression in the target field. *In vivo*, a small early decrease in NGF mRNA was detected in target fields of mice with an early deficiency of neurons.

3. The pattern of neuronal death in the trigeminal ganglion of *trkA*, *trkB* and *trkA/trkB* knockouts suggests that during certain stages in development there are subsets of neurons, which can survive with either one or the other receptor, whereas at other stages both receptors are required.

p75 appears to be required for survival in the trigeminal ganglion from E14 onwards, with a truncated receptor isoform promoting survival earlier in

development than the full-length receptor. This demonstrates a role for p75 in regulating embryonic sensory neuron survival *in vivo*.

4. Early trigeminal and dorsal root neurons appear to be dependent on trophic support from Schwann cell precursors earlier in development, when their axons are growing to their targets, whereas nodose neurons are more independent of such support, possibly reflecting the greater distance these neurons axons cover prior to innervation.

Placing these results in context with each other, they are all concerned with factors mediating the survival of embryonic sensory neurons. Both aspects of their regulation (Chapters III and IV) and the effect of their absence (Chapters V and VI) have been studied. Figure 7.1 attempts to bring these different factors together using the example of the trigeminal ganglion.

One common aspect to the chapters is that each observation in early development can be interpreted as a means to optimize survival prior to competition and programmed cell death (PCD) (Figure 7.1, A and B).
Reduction of trkA expression in early development (Chapter 3) could serve to delay the onset of NGF-dependence in some neurons, enabling more to survive before PCD and thereby maximizing competition, enabling the target field to 'select' the most appropriately connected neurons. It could be speculated that downregulation by the target field and an associated trkA-signalling independence is the explanation for apparent discrepancies between neuronal losses in the trigeminal ganglion of trkA

knockouts being observed from E13 onwards (Piñon et al., 1996), whereas in *in vitro* dissociated cultures (i.e. with no target field present), the onset of trkA-survival response has been determined as E11 to E12 (Buchman and Davies, 1993).

The positive effect of innervation on target NGF production (Chapter 4) further helps optimize selection. Increased expression of NGF would enable neurons that are dependent on NGF prior to competition to be maintained with sufficient NGF supply. Further mechanisms to aid survival prior to competition include the early survival-dependence of trigeminal neurons on BDNF and NT-3, signalling through trkB, and the subsequent phase where neurons appear to have the potential to survive through either BDNF and NT-3 or NGF, signalling through trkB or trkA (Chapter 5).

Finally, the early dependence of neuronal populations on Schwann cells and their precursors (Chapter 6) may be an additional mode of support before neurons are required to compete for survival factors. In this instance, however, there is no information on the neuronal support requirement on Schwann cells and their precursors later in development, during neurotrophin competition and thereafter. It may thus be that Schwann cells and their precursors support neurons at later stages as well. This could be tested using conditional knockouts or cultures in Schwann cell conditioned medium.

Figure 7.1: Interaction between neurons, glia and target field cells in regulating the survival of cranial sensory neurons

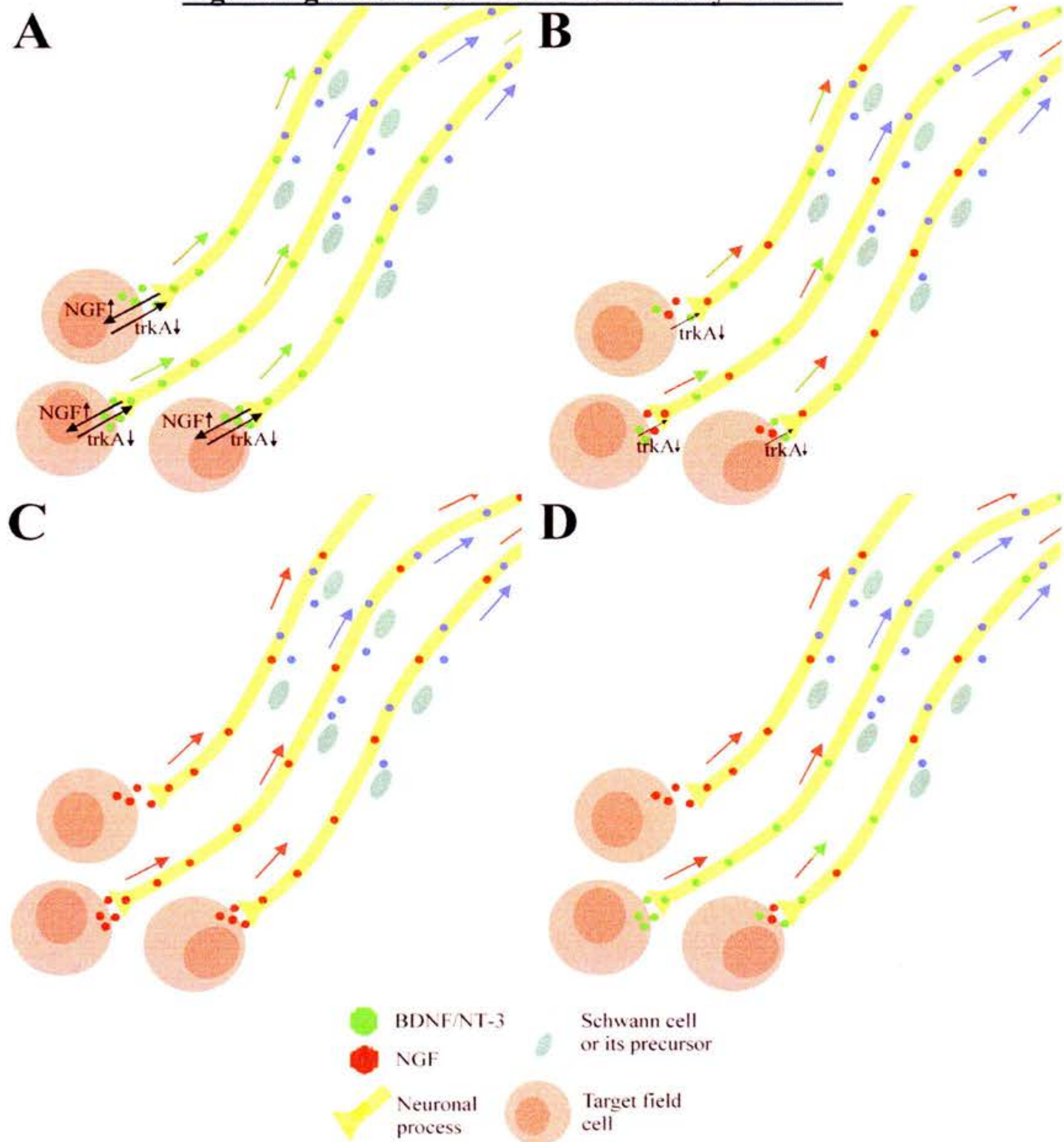


Figure summarizing the survival factors for mouse trigeminal sensory neurons and their regulation early in development. (A) Trigeminal ganglion neurons and their target field just before and upon innervation. After neurotrophin independence, trigeminal neurons become mainly dependent on BDNF and NT-3 released from intermediate tissue and from their target field (Chapter 5). They are also dependent on Schwann cell precursors for their survival at this stage (Chapter 6). The arrival of neurons at the target field appears to promote the expression of NGF (Chapter 4), while trkA expression on neurons is downregulated in the stages immediately prior to, and after, target field contact (Chapter 3). (B) Between innervation and programmed cell death (PCD), neurons can be maintained by either NGF or BDNF (Chapter 5), with inhibition of trkA expression being less pronounced than at earlier stages (Chapter 3). (C) Time of trophic factor competition, where the majority of neurons compete for NGF (Chapter 5). (D) After programmed cell death, there appears to be a diversification of survival-dependency, with some neurons dependent on NGF or BDNF alone and some neurons dependent on both simultaneously for survival (Chapter 5). Schwann cells and their precursors may have important functions for neuronal survival at later stages of development and are hence shown at all ages (adapted from Davies, 2003).

In addition, it was found that different neuronal populations have different ways to maximize survival prior to trophic factor competition, with trigeminal ganglion neurons relying on a variety of factors (Chapters 3,4,5 and 6), whereas nodose ganglion neurons stay independent of trophic support for a longer period (Davies and Vogel, 1991, Chapter 6).

The finding that the survival-dependency of neurons diversifies after the period of neurotrophin competition (Chapter 5) is interesting. Assuming that the most appropriately connected neurons have been selected, these can now be differentiated from other neurons by making them dependent on different survival factors or a combination of them. Although this hypothesis was not directly tested, studies in other sensory neurons indicate that neurotrophins are involved in the determination of neuronal phenotype (Lewin, 1996). It could be speculated that the single or joint trophic factor dependence of different neuronal subpopulations at these late stages of programmed cell death enables the selection and phenotype determination of neurons innervating specific targets or specific cells within targets. Neurotrophins may thus not only be a means to match neuron and target cell populations, but may also serve to select and differentiate neurons with different functions.

Neurotrophins have also been demonstrated to regulate dendritic growth in the peripheral nervous system (reviewed in McAllister, 2000). Single or joint trophic factor dependence may thus also play a role in shaping the dendritic length and arborisation of different subgroups of neurons, depending on their function.

More information is needed to fully elucidate the points raised in this thesis. There are various avenues of research that could be pursued to give more insight about some of the observations made in chapters III to VI. Firstly, it may be useful to identify and isolate the substance responsible for the downregulation of trkA mRNA, in order to determine whether it plays important roles at other stages of development or in disease, and to identify the mechanism of trkA-downregulation. Secondly, the extent to which early arriving sensory neurons can regulate NGF mRNA levels in the maxillary target field will need to be determined using younger ages, adapting the tissue culture methodology to enable the trigeminal ganglion to stay intact in culture at these stages. Identifying mouse mutants with reduced or no innervation of the maxillary target field *in vivo* may further improve the understanding of events up-regulating NGF mRNA early in development. The extra- and intracellular mechanisms mediating this NGF upregulation also require investigation. Thirdly, it will be interesting to determine the future phenotype of neurons that respond to different neurotrophins signalling through their trk or p75 receptors at different stages of development. Such a study could include other trophic factors, such as cytokines. This may give insights into the way neuronal phenotype is determined by the individual action or combination of survival factors acting on a neuron during development. Finally, the study of conditional mutants, with deficits in the survival of Schwann cells and their precursors but not in the migration of neural crest cells, should give a better insight on the timing and magnitude of neuronal cell loss in some sensory ganglia at different stages of development. New tissue culture methodology, enabling the culture of Schwann cells and their precursors (Lobsiger et

al., 2000), will be useful in determining the sensitivity of different neuronal populations to Schwann cell trophic support.

The findings in this thesis do not contradict the neurotrophic hypothesis, but the data would support a modified theory, at least prior to programmed cell death, in which a dynamic interplay regulating supply and reception of survival signals, rather than passive receipt/competition for that signal takes place. There may thus be more aspects to survival in early embryonic development than previously thought, with regulatory mechanisms acting in conjunction with other factors in order to maximize choice during the period of cell death. In summary, it can thus be said that trophic factors do not only play an important role during the period of naturally occurring cell death; they are also needed at other stages of development, taking place in the period before or late in programmed cell death, and can be released from tissues other than the target fields, such as surrounding intermediate tissues or Schwann cells and their precursors. Further study of these complex interactions should give more insight into the importance of the findings in regulating the development of the peripheral nervous system.

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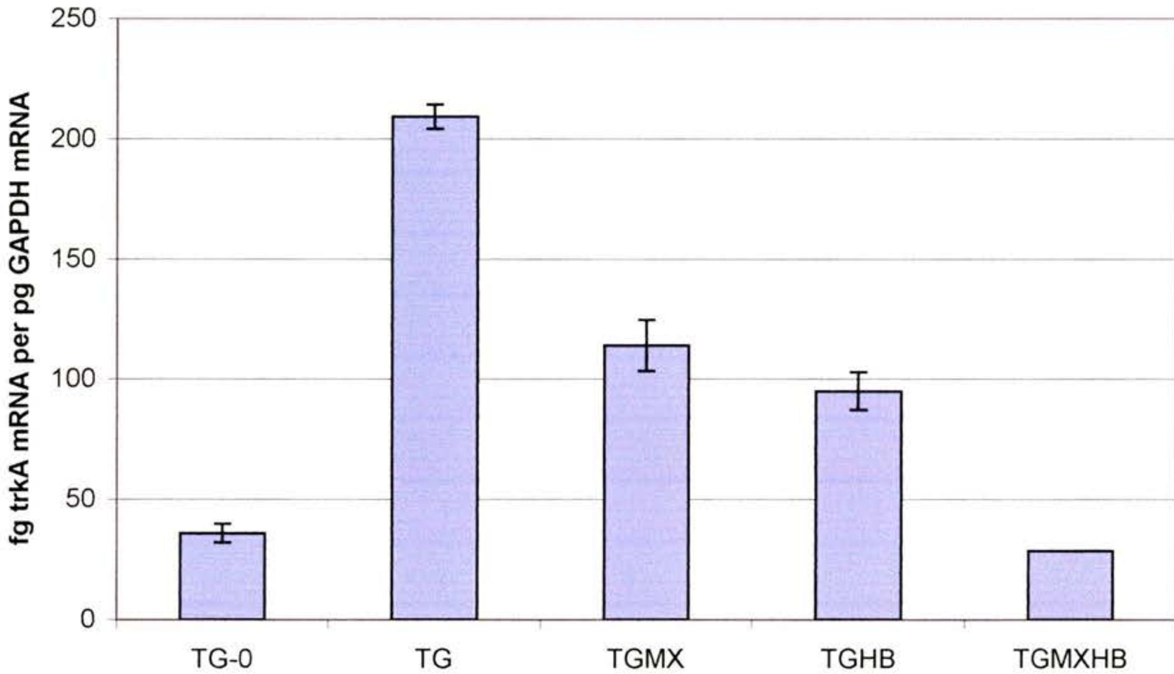
APPENDIX

Preliminary findings

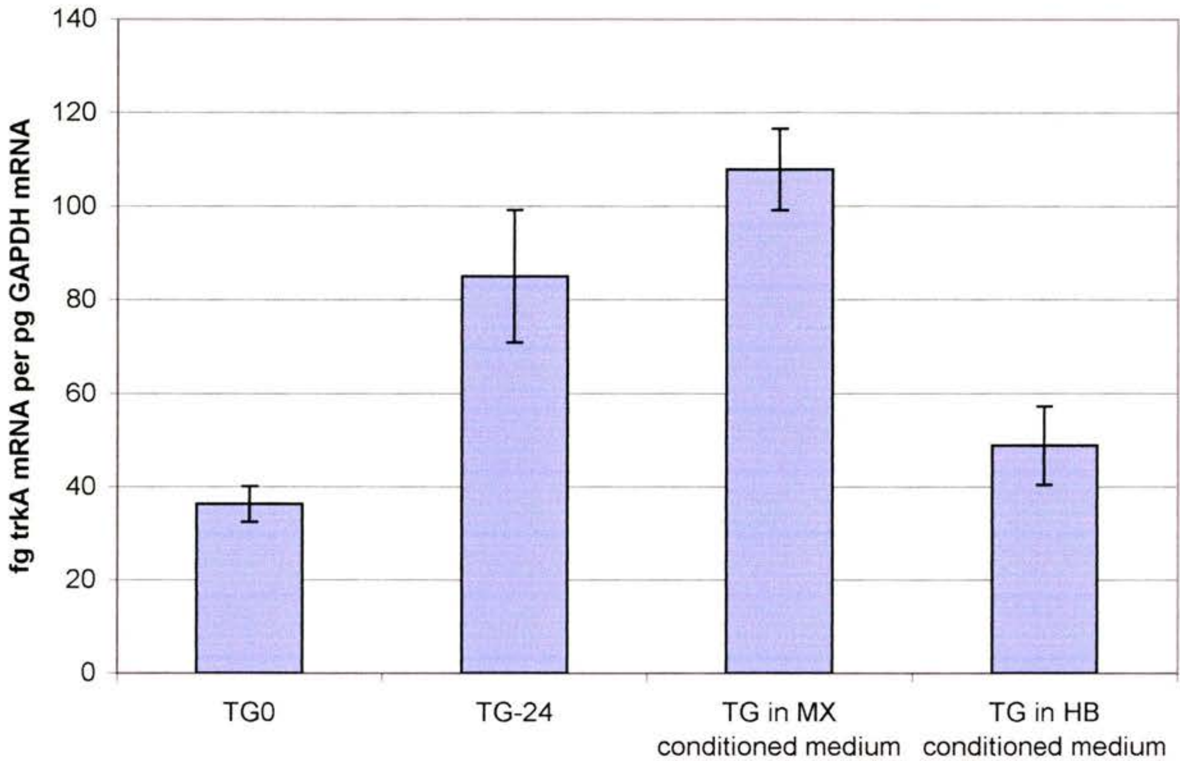
This section shows some preliminary findings that have been mentioned in the results section of chapter 3.

Figure A.1: trkA expression in the E11 trigeminal ganglion after 24 hrs in culture without or with its peripheral and central targets attached (cultured without growth factors in medium)



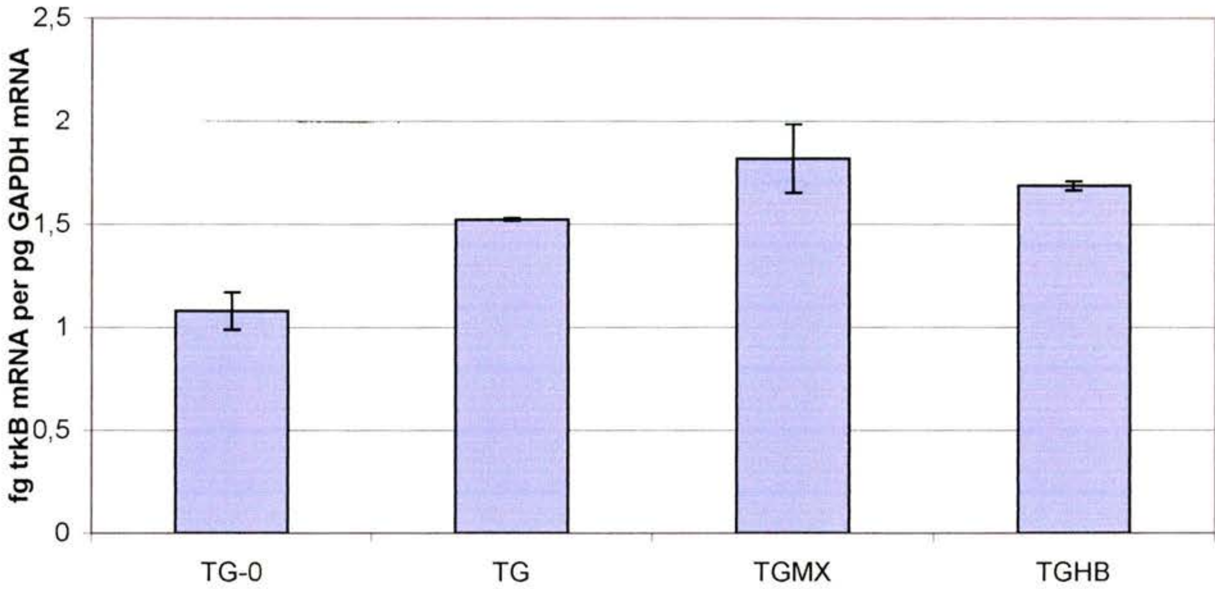
Expression of trkA in E11 trigeminal ganglia after 24hrs in culture without growth factors added to the medium. TG-0: Expression in ganglia before culture. TG: Expression in the ganglion grown on its own. TGMX: Expression in the ganglion grown with the maxillary process. TGHB: Expression in the ganglion grown with the hindbrain. TGMXHB: Expression in the ganglion grown with both target fields. As can be seen, trkA expression still increases, and the target fields still effect a reduction in trkA expression when ganglia are grown without survival factors. Bars represent the mean ± standard error (n=1-5 per data point).

Figure A.2: **trkA expression in E11 trigeminal ganglia after 24 hrs in culture with conditioned medium from its peripheral and central targets**



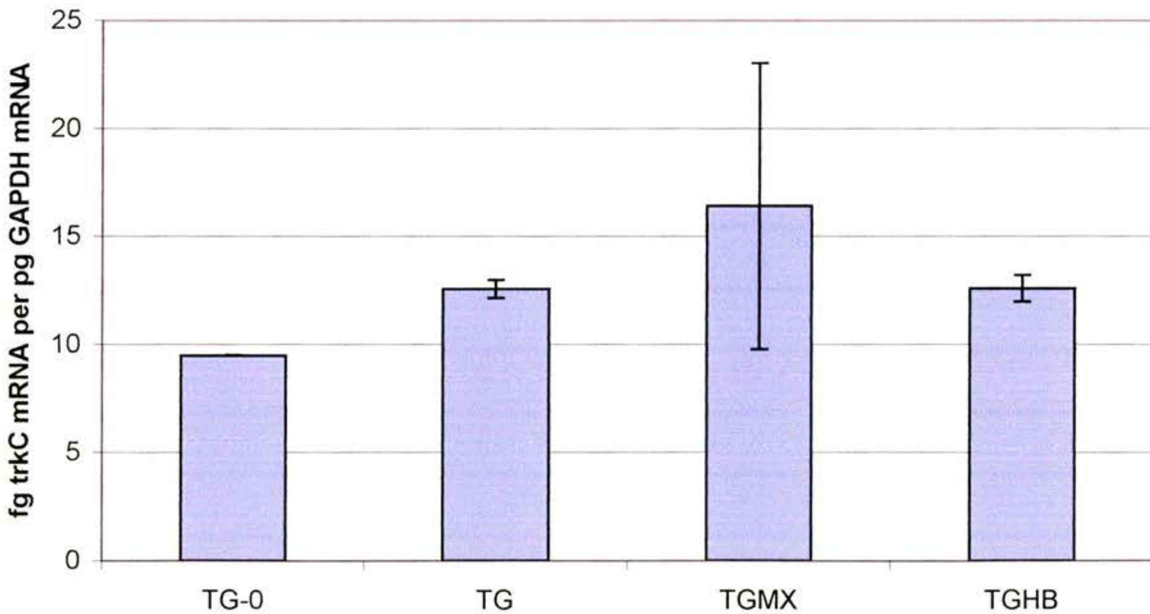
Expression of trkA in E11 trigeminal ganglia after 24hrs in culture with NGF, BDNF and NT-3 in maxillary and hindbrain conditioned medium obtained by growing these target fields in 50μl of medium for 24hrs. TG-0: Expression in ganglia before culture. TG: Expression in the ganglion grown alone in control (incubated for 24hrs without targets) medium. TG in MX conditioned medium: Expression in the ganglion grown alone in maxillary process conditioned medium. TG in HB conditioned medium: Expression in the ganglion grown alone in hindbrain conditioned medium. Whereas the maxillary process conditioned medium had no effect on trkA expression, the hindbrain conditioned medium resulted in reduced trkA expression in the trigeminal ganglion. Hindbrain conditioned medium thus replicates the observations made with hindbrain homogenate. Bars represent the mean ± standard error (n=4 per data point).

Figure A.3: trkB expression in the E11 trigeminal ganglion after 24 hrs in culture without or with its peripheral and central targets attached



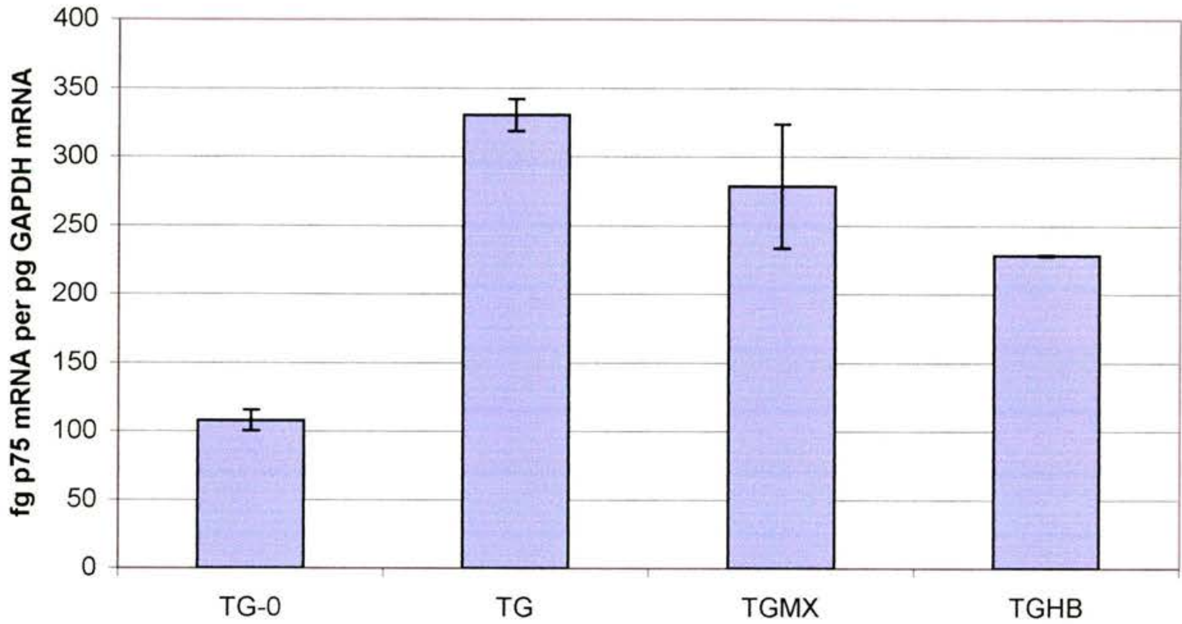
Expression of trkB in E11 trigeminal ganglia after 24hrs in culture with NGF, BDNF and NT-3. TG-0: Expression in ganglia before culture. TG: Expression in the ganglion grown on its own. TGMX: Expression in the ganglion grown with the maxillary process. TGHB: Expression in the ganglion grown with the hindbrain. TGMXHB: Expression in the ganglion grown with both target fields. As can be seen, trkB expression increases less than trkA over 24hrs in culture, and the target fields appear to have little effect on trkB expression. Bars represent the mean ± standard error (n=2 per data point).

Figure A.4: trkC expression in the E11 trigeminal ganglion after 24 hrs in culture without or with its peripheral and central targets attached



Expression of trkC in E11 trigeminal ganglia after 24hrs in culture with NGF, BDNF and NT-3. TG-0: Expression in ganglia before culture. TG: Expression in the ganglion grown on its own. TGMX: Expression in the ganglion grown with the maxillary process. TGHB: Expression in the ganglion grown with the hindbrain. TGMXHB: Expression in the ganglion grown with both target fields. As can be seen, trkC expression increases less than trkA over 24hrs in culture, and the target fields appear to have little effect on trkC expression. Bars represent the mean ± standard error (n=2 per data point).

Figure A.5: p75 expression in the E11 trigeminal ganglion after 24 hrs in culture without or with its peripheral and central targets attached



Expression of p75 in E11 trigeminal ganglia after 24hrs in culture with NGF, BDNF and NT-3. TG-0: Expression in ganglia before culture. TG: Expression in the ganglion grown on its own. TGMX: Expression in the ganglion grown with the maxillary process. TGHB: Expression in the ganglion grown with the hindbrain. TGMXHB: Expression in the ganglion grown with both target fields. As can be seen, p75 expression increases strongly over 24hrs in culture, and co-culture with the hindbrain appears to reduce p75 expression in the ganglion. The maxillary process has little effect on p75 expression in this experiment. Bars represent the mean ± standard error (n=2 per data point).