

# **The Role of Tumour Necrosis Factor $\alpha$ and NF- $\kappa$ B Signalling in Regulating Neuronal Survival During Development**

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## Abstract

Neurons are generated in excess in the developing vertebrate peripheral nervous system, and the superfluous neurons are lost during a phase of programmed cell death that occurs shortly after they innervate their targets. Using a variety of *in vitro* and *in vivo* experimental approaches in wild type and transgenic mice, I have demonstrated that tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) has opposite effects on neuronal survival at different stages in development. Early during the period of naturally occurring neuronal death it enhances the survival of sensory and sympathetic neurons, whereas later in development it promotes, by an autocrine mechanism, the death of neurons that fail to obtain sufficient target-derived neurotrophic factor to sustain their survival. The early survival-enhancing effect of TNF $\alpha$  and the survival-enhancing effect of nerve growth factor (NGF) are mediated by activation of the transcription factor NF- $\kappa$ B in the peripheral nervous system. However, in the case of retinal ganglion cells and motoneurons of the developing central nervous system, NF- $\kappa$ B appears to play a role promoting cell death during development. Finally, I demonstrated that Bad, a member of the Bcl-2 family of proteins, is required for programmed cell death of many sensory and sympathetic neurons during development. These findings show that the same extracellular and intracellular signalling proteins can exert opposite effects on neuronal survival in different locations in the nervous system and at different stages of development.



## **Declaration**

I Victoria Maedhbh Barker, candidate for a PhD in neuroscience, declare that this thesis has been composed solely by myself, that the work here-in described is my own, except where indicated, and that this work has not been submitted for any other degree or professional qualification.

Victoria M. Barker BSc.

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## Abbreviations

**AD** - Alzheimer's disease  
**Aif** - Apoptosis inducing factor  
**ALS** - Amyotrophic lateral sclerosis  
**AP-1** - Adaptor protein 1  
**Apaf-1** - Apoptotic protease activating factor 1  
**aPKC** - Atypical protein kinase C  
**Bcl-2** - B-cell lymphoma/leukaemia-2  
**BDNF** - Brain derived neurotrophic factor  
**BH** - Bcl-2 homology domain  
**bp** - Base pair  
**cAMP** - Cyclic adenosine monophosphate  
**Ca<sup>2+</sup>** - Calcium  
**Caspase** - Cysteine aspartate kinase  
**cDNA** - Cellular deoxyribonucleic acid  
**cIAP** - Cellular inhibitor of apoptosis  
**CFV** - Cresyl fast violet  
**CNS** - Central nervous system  
**°C** - Degrees Celsius  
**DAB** - Diaminobenzide tetrahydrochloride  
**ddH<sub>2</sub>O** - Double distilled water  
**DED** - Death effector domain  
**DFF** - DNA fragmentation factor  
**DNA** - Deoxyribonucleic acid  
**DRG** - Dorsal root ganglia  
**ERK** - Extracellular signal regulated kinase  
**E** - Embryonic day (e.g., E12)  
**F12/14** - Ham nutrient mixture F-12/14  
**FADD** - Fas associated death domain protein  
**G protein** - GTP-binding regulatory protein  
**g** - Gram  
**HBSS** - Hank's balanced salt solution  
**HIHS** - Heat inactivated horse serum  
**HRP** - Horseradish peroxidase  
**IGF1** - Insulin-like growth factor 1  
**IFN $\alpha$**  - Interferon alpha  
**I $\kappa$ B** - Inhibitory kappa B  
**IKK** - Inhibitory kappa B kinase  
**IL-1** - Interleukin 1  
**JNK** - c-Jun NH<sub>2</sub> terminal kinase  
**kDa** - Kilodalton  
**L15** - Leibovitz's L15 nutrient mixture  
**M** - Molar  
**mg** - Milligram



**µg** - Microgram  
**ml** - Millilitre  
**mM** - Millimolar  
**MAPKKK** - Mitogen activated protein kinase kinase kinase  
**MEKK1** - Mitogen activated protein/Erk kinase kinase 1  
**mRNA** - messenger ribonucleic acid  
**NADE** - p75<sup>NTR</sup> associated cell death executor  
**NGF** - Nerve growth factor  
**NIK** - NF-κB inducing kinase  
**NF-κB** - Nuclear factor kappa B  
**NMR** - Nuclear magnetic resonance  
**NRAGE** - Neurotrophin receptor interacting MAGE homologue  
**NRIF** - Neurotrophin receptor interacting factor  
**NSAIDs** - Non-steroidal anti-inflammatory drugs  
**NT-3** - Neurotrophin 3  
**NT-4/5** - Neurotrophin 4/5  
**NT-6** - Neurotrophin 6  
**NTS** - Nuclear translocation signal  
**P** - Postnatal day  
**PARP** - Poly-ADP ribose polymerase  
**PBS** - Phosphate buffered saline  
**PCNA** - Proliferating cell nuclear antigen  
**PCR** - Polymerase chain reaction  
**PD** - Parkinson's disease  
**PDK1** - Phosphoinositide dependent kinase 1  
**PEST** - Proline (P), glutamic acid (E), serine (S) and threonine (T) rich protein region  
**PGE<sub>2</sub>** - Prostaglandin E<sub>2</sub>  
**PI3'K** - Phosphatidyl inositide 3'-OH kinase  
**PLCγ** - Phospholipase C gamma  
**RIP1** - Receptor interacting protein 1  
**RHD** - Rel homology domain  
**RNA** - Ribonucleic acid  
**rpm** - Revolutions per minute  
**SCG** - Superior cervical ganglion  
**SOSS** - Silencing of survival signals  
**SODD** - Silencer of death domains  
**TACA** - TNF alpha converting enzyme  
**T-BP-1** - TNF binding protein 1  
**TBS** - Tris-buffered saline  
**TdT** - Deoxynucleotidyl transferase  
**TGFβ** - Transforming growth factor beta  
**sTNFR:Fc** - Fitc conjugate anti-TNFR  
**TMN** - Trigeminal mesencephalic nucleus  
**TNF** - Tumour necrosis factor  
**TRADD** - TNF receptor 1-associated death domain protein

**TRAF** - TNF receptor associated factor

**TrkA** - Tropomyosin related kinase A

**TUNEL** - Transferase mediated dUTP biotin nick end labelling

**UV** - Ultra violet

**V** - Volts

**VDAC** - Voltage-dependant anion channel

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# CHAPTER 1: INTRODUCTION

In this thesis the extracellular signals and intracellular molecular mechanisms that regulate neuronal survival have been studied in several populations of neurons in the developing mouse. The sensory neurons of the trigeminal and nodose ganglia, the sympathetic neurons of the superior cervical ganglia (SCG), the motoneurons of the spinal cord and retinal neurons were selected for these studies for the following reasons, 1) though they have been studied previously and developmental cell death is known to occur within these populations, the molecules that stimulate and the receptors that mediate this cell death remain to be fully elucidated 2) they are easily accessible to both *in vivo* and *in vitro* study, 3) these neurons are known to be dependent upon neurotrophins for survival during the stage of developmental cell death and thus their survival or demise can easily be manipulated so that the pathways involved can be studied.

Initially the dual cytotoxic and cytoprotective pathways mediated by transcription factor NF- $\kappa$ B in the spinal cord, retina and SCG were investigated. The findings of these experiments led to an examination of the role of TNF $\alpha$  as an inducer of cell survival in neurotrophin responsive neurons of the SCG, trigeminal and nodose ganglia. This was followed by the discovery of its role as a cytotoxic stimulus in these ganglia later in development. The research described in this study led to an examination of the role of

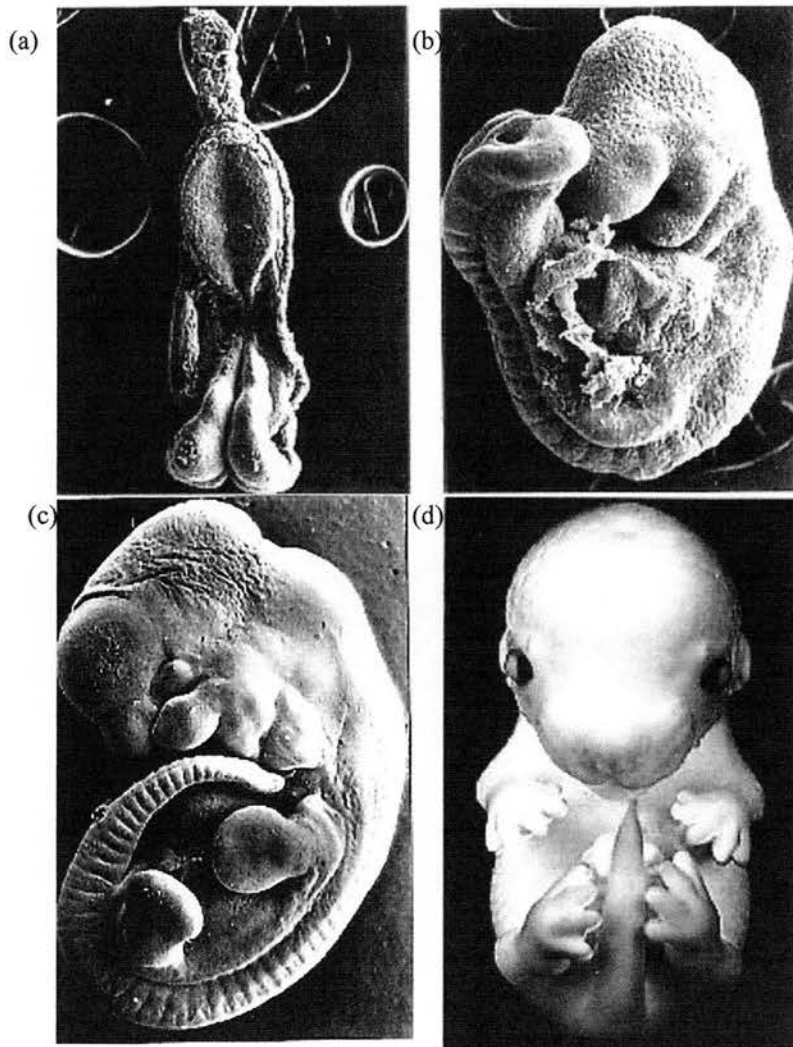
Bcl-2 family member Bad in the developmental cell death cascade, in order to ascertain whether it was acting downstream from  $\text{TNF}\alpha$  in inducing cytotoxicity.

As an introduction to this work I will briefly outline the development of the nervous system, focusing on the above populations of neurons, and describe what is known about the roles of the neurotrophic factors in regulating the survival of these neurons during development and the signalling pathways involved.

# 1.1 Neurodevelopment of Vertebrates

## 1.1.1 The Central Nervous System

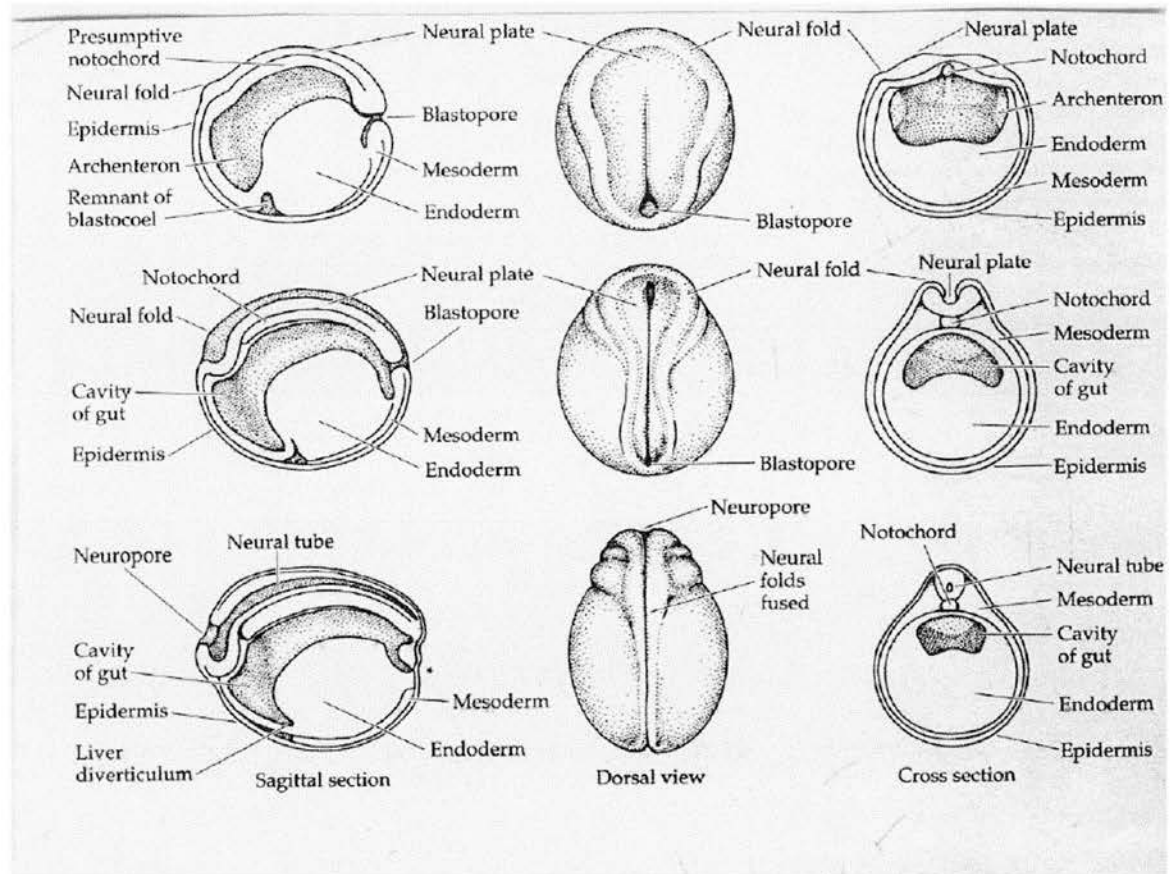
A full understanding of the development of the nervous system requires that the developmental origins and relationships between cells and structures be known. The vertebrate nervous system develops in a multi-stage process (see figure 1.1).



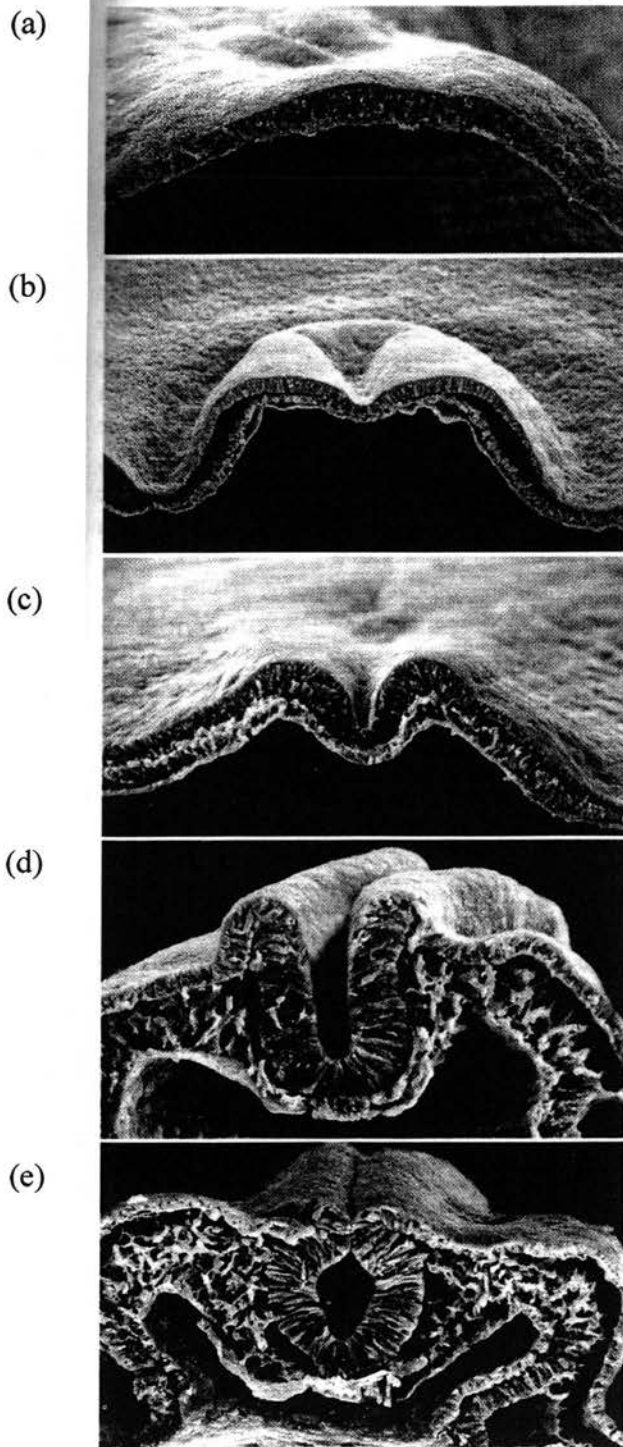
**Figure 1.1: Scanning electron micrograph of representative mouse embryos over the period E8-E14.5. (a) E8: early evidence of neural-fold apposition. (b) E9.5: The rostral neuropore has closed, but the caudal neuropore has yet to do so and the forelimb bud is starting to differentiate. (c) E11.5: The distal part of the limb buds is paddle-shaped and the tail has elongated and narrowed. (d) E14.5: The primitive features are recognisable as those seen in the adult. (from Kaufman, 1992)**

The initial stage of this process is known as neurulation. This process begins when the neural plate is formed from the thickening dorsal ectoderm of the gastrula in the future occipital (cervical) region (reviewed in Bard, 1994). This process is induced by the underlying notocord and precaudal mesodermal cells, commonly known as the primitive node (Hensen's node in the developing chicken). Primary induction involves the induction of neural properties at Hensen's node at the gastrulation stage of development. Hensen's node acquires its 'neuralising' capacity as it passes the dorsal lip of the blastopore during gastrulation (Brown, Hopkins and Keynes, 1992). Subsequent to induction three major morphogenetic events occur. These are 1) the shaping of the neural plate, 2) the bending of the neural plate and 3) neural tube closure (see figure 1.2 and 1.3). The neural plate narrows and elongates due to oriented mitosis and the active rearrangement of cells. As shaping of the neural plate progresses it begins to bend and the formation of the neural groove ensues. During neural groove formation the lateral edges of the neural plate rise up to form ridges along a midline groove. These ridges are the neural folds, which subsequently close over the cephalic region and fuse, demarcating the most rostral section of the intact neural tube. The neural tube is the precursor from which the entire adult central nervous system develops (for further details, see Jacobson and Tam, 1982; Kaufman, 1992). Neural folds form as a result of localised interactions occurring at the interface between their two components: lateral neural plate and medial skin ectoderm. Cell behaviours within the neural plate and skin ectoderm, such as mitosis, rearrangement and shape changes, produce the forces needed

for the uplifting of the neural folds and neural groove formation (Schoenwolf, 1997). The intact neural tube submerges beneath the surface and the ectoderm is repaired. The rostral section of the primitive neural tube ultimately gives rise to the cerebral hemispheres, cerebellum and pons, whereas the caudal section gives rise to the spinal cord.



**Figure 1.2: Neurulation I.** diagram showing neurulation. As a result of induction, the neurectoderm forms a neural plate, bounded by neural folds. The folds arch up and fuse along the embryo's dorsal midline to create the neural tube (Schoenwolf, 1997).



**Figure 1.3: Neurulation II.**  
 Transverse sections of neurulation in the chick embryo (x140). (a) The neural plate on the embryo's dorsum can be seen. (b) the lateral edges of the neural plate rise up to form the neural groove. (c) The midline groove becomes more prominent. (d) the ridges on either side of the neural groove are neural folds. (e) the neural folds close over and fuse to form the neural tube which subsequently submerges beneath the surface ectoderm. (Crossman and Neary, 2000).



As development proceeds the neural tube becomes subdivided into three primary vesicles, called the prosencephalon, the mesencephalon and the rhombencephalon. The prosencephalon then further divides into the telencephalon and diencephalon. The former develops into structures including the adult cerebrum. The latter forms the adult thalamus, hypothalamus, pineal and posterior pituitary glands. The mesencephalon forms the corpora quadrigemina and the cerebral peduncles. The rhombencephalon subdivides into the metencephalon and myelencephalon, which form the cerebellum and pons and the medulla oblongata respectively. Within these five secondary vesicles and the spinal cord, there are further subdivisions, called neuromeres (Kaufman and Bard, 1999).

The primitive neural tube is initially a straight tube in which the vesicles are arrayed like beads on a string. Regions of the adult nervous system have specific spatial relationships to each other which are attained through the processes termed “torsion” and “flexion”. During torsion the embryonic axis, including the neural tube, twists rostrocaudally. In flexion the neural tube bends back on itself at four levels to give rise to four flexures termed the cephalic, cervical, pontine and tail flexures (Gilbert and Raunio, 1997). Thus, considerable growth of the central nervous system can occur during development despite neural tube elongation being restricted by its encasement in the developing skull and vertebral column.

### 1.1.1a The Spinal Cord

The development of the spinal cord begins as the primitive brain region, (or rostral extremity of the neural axis) becomes demarcated from the future spinal cord, (the derivative of the caudal part of the neural axis at the cervical flexure). This occurs when the brain region folds, as described above, whereas the spinal cord region remains straight and the cells within the neural epithelium divide and produce different cell types (Walbot and Holder, 1987). The segmented spinal nerves and trunks of the peripheral nervous system appear. The neural tube becomes concentrically layered into the ependymal (ventricular), mantle and marginal layers (see figure 1.3). The ependymal layer forms the lining of the lumen of the spinal cord and ventricles of the brain. This layer is primarily responsible for the production and resorption of cerebrospinal fluid. In the hindbrain and spinal cord the marginal and mantle layers give rise to the anuclear (outer) white and nuclear (intermediate) grey matter, respectively. Support cells derived from the neural precursor cells, for example astrocytes and oligodendrocytes are located in the mantle layer. The neural tube shows ventral and dorsal thickenings. The ventral thickening (basal plates), which contain ventral motor horn cells form the motor areas of the spinal cord. The dorsal thickening (alar plates) form the sensory areas. Motor nerve fibres appear at week four, arising from the nerve cells in the basal plates. These fibres collect into bundles called ventral nerve roots. The axons from basal plate neurons penetrate the marginal zone and appear on the ventral aspect of the spinal cord. They are known collectively as the ventral motor root of the spinal nerve and they conduct

motor impulses from the spinal cord to the muscle (Sadler, 2000). At this stage the floor- and roof - plate form as a result of neural tissue thinning. From this point onwards the neural tube caudal to the cervical flexure is, by convention, termed the spinal cord (Kaufman and Bard, 1999).

### **1.1.1b The Eye**

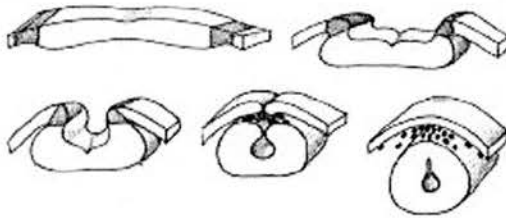
Sensory organs develop in close association with the neural tube. In the mouse, approximately at the stage when the neural folds first become evident (embryonic day 7.5-8.5) a slightly flattened area of neural ectoderm in the central part of the primitive forebrain region represents the earliest sign of the optic placode (epithelial thickening). A slight indentation forms in the centre of this placode, in corresponding regions in the two prospective hemispheres, forming the optic pits. The optic pits increase in depth and are connected by a pair of shallow grooves. These are the optic sulci and are in the area of the future optic chiasma, where the neurons from opposite visual fields cross the midline of the brain to the visual cortex of the contralateral hemispheres. Meanwhile on the lateral (outer) aspects of the cephalic (head) regions, protrusions termed the optic eminences, develop overlying the optic pits. The eventual closure of the neural folds results in the formation of the optic vesicles and the optic stalks by which they are connected to the forebrain vesicle. The outer section of the optic vesicles collapses inwards giving rise to the optic cups. The inner layer of optic cup cells form the neural layer of the retina and the outer layer gives rise to the pigmented layer (Kaufman, 1992).

The optic vesicles interact with the overlying surface ectoderm that covers the neural tube resulting in the formation of the cornea and the lenses of the eyes, which subsequently become nestled in the optic cups (Schoenwolf, 1997). The iris of the eye develops from the rim of the optic cup (Oppenheimer and Lefevre, 1984). The overall volume of the globe of the eye increases in subsequent stages of embryonic development. Unmyelinated nerve fibres appear, originating from the primitive retinal ganglia, grow towards the optic pit and from there into the optic stalk and the region of the future optic chiasma. The neural retina becomes increasingly differentiated. It consists of a number of cell layers including the outer nuclear or neuroblastic layer, which will eventually form the horizontal cells and the nuclei of the photoreceptor cells, a transient intermediate layer (layer of Chievitz), and an inner nuclear layer which is destined to form the ganglion cells (Kaufmann, 1992).

### **1.1.2 The Peripheral Nervous System.**

The peripheral nervous system consists of the sensory and autonomic ganglia and their associated peripheral nerves that lie external to the brain and spinal cord. At the time of neural tube fusion and submersion a number of cells, termed the neural crest cells detach from the periphery of the neural tube having developed as a transient structure along the boundary between the surface and neural ectoderm (see figure 1.4). The neural crest cells detach initially in the mesencephalic region and subsequently in a cranio-caudal (head to tail) direction along the neural axis. They then migrate along a

number of defined routes through the mesoderm to target regions in the developing embryo where they form a variety of derivatives, including the majority of the ganglia and support cells of the peripheral nervous system. Adrenal medullary chromaffin cells and craniofacial connective tissue elements (bone, cartilage and dentine) and the melanocytes of the skin are also formed from the neural crest cells (Le Douarin, 1986).



**Figure 1.4: Neural crest formation.**

The neural crest forms from cells at the juncture between the neuroectoderm and the epidermis and migrate through the mesoderm to target regions including the peripheral nervous system. (Balinsky, 1981).

The particular fate of each individual cell has been shown by cell lineage studies to be generally undetermined at the time of migration (Bronner- Fraser and Fraser, 1988). Their final lineages are decided by the migratory route taken and their destination. The exception to this is the cephalic neural crest cells, the fates of which are determined prior to migration from the hindbrain neural tube (reviewed by Gilbert 1997). The migration of neural crest cells along specific pathways in the periphery is probably controlled by adhesive interactions between the crest cell surface receptors (integrins) and local extracellular matrix molecules (e.g., laminin, fibronectin)(Bronner- Fraser, 1984). If either the receptor (Bronner-Fraser and Lallier, 1988) or its ligand (Bronner-Fraser, 1985) is blocked, gross perturbations of migration occur.

In the trunk region, neural crest cells take either a ventral pathway through the anterior part of the sclerotome region<sup>1</sup> of the somite<sup>2</sup> giving rise to the cells of the peripheral nervous system. Alternatively it can take a dorsal route between the epidermis and the dermomyotome region<sup>3</sup> of the somite leading to the formation of melanocytes (reviewed by Gilbert, 1997). The progenitor cells of the peripheral nervous system may also originate in the neurogenic (nervous tissue) placodes of the embryonic cephalic ectoderm. These are thickenings associated with the branchial arches in the developing craniofacial region, also called epibranchial placodes, they give rise to cells that migrate away and join other migratory cells derived from the neural crest. (Noden, 1978; D'Amico-Martel, 1982, D'Amico-Martel and Noden, 1983; Le Douarin, 1986). Particular ganglia and nerve plexuses in the peripheral nervous system arise from distinct levels of the crest. Each level of the neural crest gives rise to the specific phenotypes of cell of its associated ganglia or plexus (Brown, Hopkins and Keynes, 1992).

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<sup>1</sup> Gives rise to skeletal tissue.

<sup>2</sup> A paired, segmented division of mesoderm.

<sup>3</sup> Forms the dermis and associated tissue

### 1.1.2a The Sensory Ganglia

The sensory ganglia of the peripheral nervous system are classified into the cranial and dorsal root ganglia (DRG). The cranial sensory ganglia are located on five of the twelve pairs of cranial nerves. The trigeminal nerve is the fifth cranial nerve and supplies sensory innervation to the face and motor innervation to the muscles of mastication. The two populations of primary sensory neurons of this system are those of the trigeminal ganglion and the trigeminal mesencephalic nucleus (TMN). Three major nerves arise from the trigeminal ganglion: the ophthalmic nerve (cranial nerve V<sub>1</sub>), the maxillary nerve (cranial nerve V<sub>2</sub>), both of which are wholly sensory, and the sensory component of the mandibular nerve (cranial nerve V<sub>3</sub>) (Moore and Dalley, 1999). The trigeminal ganglion innervates mainly mechanoreceptors, thermoreceptors and nociceptors in the face and the oral and nasal cavities. The TMN innervates muscle spindles and tendon organs of the muscles of mastication. Its central axons terminate on several groups of second order sensory neurons in the brainstem, including the trigeminal motoneurons (Davies, 1997). Trigeminal neurons are derived from either the neurogenic trigeminal placode or the neural crest population, depending on their position.

The nodose ganglion (superior vagal ganglion) is a group of placode derived neurons, that provides sensory innervation to the thoracic and abdominal viscera. It is located in the jugular foramen on the vagus nerve (cranial nerve X). Other sensory ganglia include the geniculate ganglion (VII), the inferior vagal ganglion (X), the superior

glossopharyngeal ganglion (IX), the petrosal ganglion (IX) and the vestibulocochlear ganglion (VIII). Neural crest and placode derived cell transplantation experiments have allowed the mapping of origins of avian cranial sensory neurons. These demonstrate that the neurons of the ventrolateral region of trigeminal ganglia and the nodose ganglia, vestibulocochlear, geniculate and petrosal ganglia of the developing chick are derived from the neurogenic trigeminal, epibrachial and otic placodes. The neurons of dorsomedial trigeminal ganglia, DRG, jugular ganglion and trigeminal mesencephalic nucleus stem from the neural crest (Weston, 1962; Le Douarin, 1973; D'Amico-Martel, 1982). The DRG are located on the dorsal roots of the spinal nerves adjacent to the spinal cord. The sensory cells within the DRG transmit impulses received from the periphery to the spinal cord.

### **1.1.2b The Autonomic Ganglia**

The autonomic system, classically described as 'the visceral motor system' consists of fibres that innervate smooth muscle, modified cardiac muscle and glands. The efferent nerve fibres and ganglia of the autonomic nervous system are organised into the sympathetic (thoracolumbar) division, the parasympathetic (craniosacral) division and the enteric sub-division.

Preganglionic sympathetic neurons are located in the intermediolateral grey columns of the thoracic spinal segments. Postganglionic sympathetic grey neurons are located in



the paravertebral sympathetic ganglia of the sympathetic trunks and the prevertebral sympathetic ganglia. The largest and most cranial ganglion of the sympathetic trunks is the superior cervical ganglion (SCG) whose postganglionic fibres form a plexus on the carotid artery and several of its branches allowing it to reach much of the smooth muscle of the head (Poritsky, 1984). Other paravertebral ganglia include the middle cervical ganglion, stellate ganglion and sympathetic chain ganglia. The prevertebral ganglia are in the plexuses that surround the origins of the main branches of the abdominal aorta. These prevertebral ganglia are the coeliac, superior mesenteric, inferior mesenteric and aorticorenal ganglia. The chromaffin cells of the adrenal medulla receive an innervation from preganglionic sympathetic neurons directly (Poritsky, 1984).

Parasympathetic neuron cell bodies are located in two sites within the central nervous system. Those located in the grey matter of the brain stem, contribute preganglionic parasympathetic fibres to cranial nerves III, VII, IX and X, and constitute the cranial parasympathetic outflow. Those located in the grey matter of the sacral segments of the spinal cord, S2, S3 and S4 contribute preganglionic parasympathetic fibres to corresponding sacral spinal nerves and constitute the sacral parasympathetic outflow. The cranial outflow provides parasympathetic innervation to the head, thoracic and abdominal viscera. The sacral outflow provides the innervation of the pelvic viscera (Moore and Dalley, 1999). Parasympathetic ganglia include the ciliary, otic, and submandibular ganglia and the terminal ganglia of the vagus nerve and pelvic plexus. Neuronal and glial cells of both the sympathetic and parasympathic ganglia arise from

neural crest cells (Le Douarin, 1980; D'Amico-Martel and Noden, 1983; Bronner and Fraser, 1988).

### **1.1.2c The enteric Ganglia**

The enteric nervous system consists primarily of Meissner's (submucosal) plexus and Auerbach's (myenteric) plexus. It provides innervation to the smooth muscle, vessels and secretory glands of the gastrointestinal tract. The enteric nervous system's innervation derives from two distinct neural crest cell populations. The majority of which, combined with that of the SCG, are derived from post-otic hindbrain or neural crest cells (vagal and sacral). A second lineage of cells from trunk neural crest forms the more posterior sympathetic ganglia and contributes to the foregut enteric nervous system (Le Douarin and Teillet, 1973, 1974; Bronner and Fraser, 1988; Serbedzija et al., 1991; Durbec et al., 1996).

It is the role of developmental cell death in these regions that is under examination. Cell death is a process that is vital for normal neurodevelopment and thus it is important that it be fully understood.

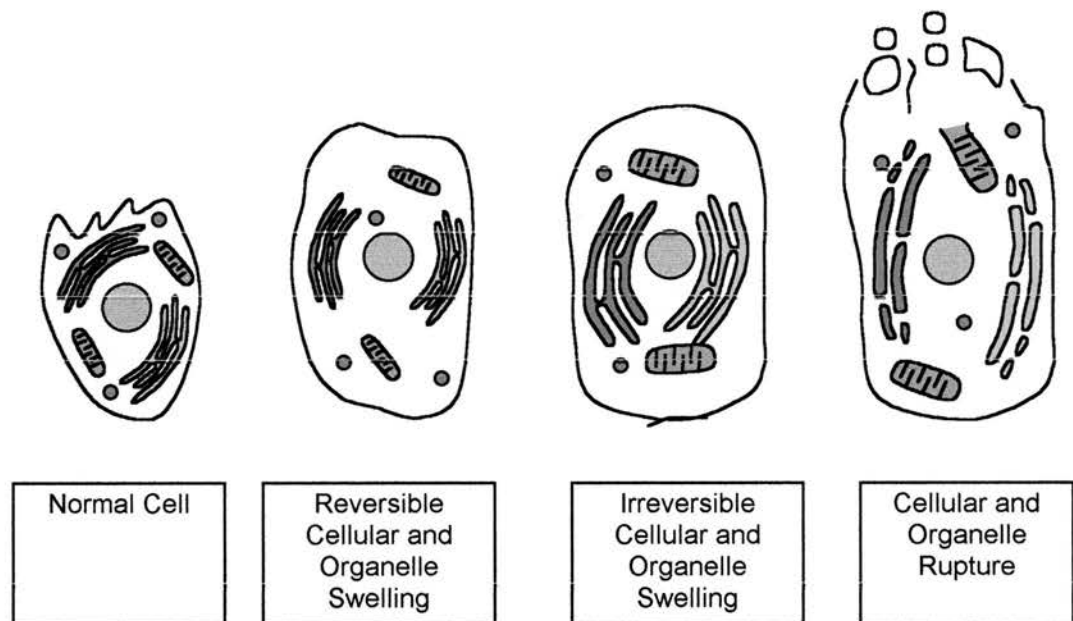
## 1.2 Apoptosis

### 1.2.1 Types of Cell Death

‘Normal cell death’ was first discovered in amphibian metamorphosis (Vogt, 1842). It has since been observed in many developing tissues of both vertebrates and invertebrates (reviewed by Glucksmann, 1951; Clarke and Clarke, 1996). Cell death can take two distinct forms. The first, necrotic cell death is the cellular response to toxic and pathological stimuli (Clarke, 1990; Ellis and Horvitz, 1991). The second, physiological or programmed cell death, is the process of intrinsically regulated cell death (Pommerville and Kochert, 1982).

The process of necrosis involves chromatin clumping, organelle swelling, membrane degradation and mitochondrial flocculence. The cells and organelles rupture expelling their contents and initiating an inflammatory response (Kerr et al., 1972) (Figure 1.5). These are passive responses to the pathological stimulus and do not require *de novo* transcription or translation (Martin et al., 1988; Scott and Davies, 1990; Ellis and Horvitz, 1991; Saunders and Wride, 1995). The characteristics of necrotic cell death are consistent with a failure in osmotic regulation that may be stimulated by a loss of cellular energy supplies (Oppenheim, 1991). In contrast, physiological cell death is an active process, involved in both vertebrate and invertebrate development and is essential for tissue homeostasis. It may require *de novo* gene transcription and protein synthesis.

Physiological cell death can be further sub-divided into three categories, all of which are known to occur during development (Schweichel and Merker, 1973). The essential difference between these three types of physiological cell death is the role played by lysosomes.



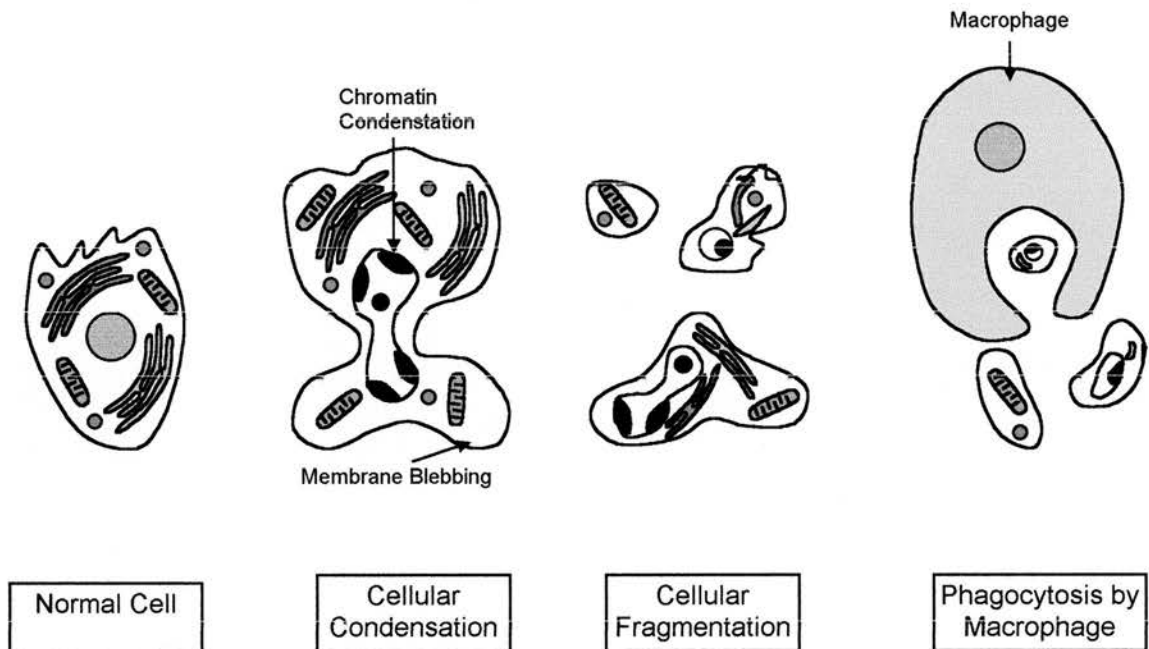
**Figure 1.5 Morphological features of necrosis.** Cytotoxic stimuli cause the cells and organelles to swell and then rupture expelling their contents and initiating an inflammatory response. (Adapted from figure donated by Oliver Schmidt).

Autophagic degeneration involves the cellular presence of autophagic vacuoles into which the hydrolytic enzymic contents of primary lysosomes are emptied. The endoplasmic reticulum and mitochondria of the cell dilate, and enlargement of the golgi apparatus occurs. The cytoplasmic components of the cell are packaged into double membrane structures known as autophagosomes and are delivered to lysosomes or

vacuoles for destruction. The cell is digested and the remnants removed by heterophagy (Schweichel and Merker, 1973; Seglen and Bohley, 1992; Dunn, 1994; reviewed by Bursch et al., 2000). This type of cell death occurs to remove cells, tissues and organs that have had a transient function at one stage but are no longer required once a more mature developmental stage is attained, for example, during metamorphosis, when tadpoles' tails regress (reviewed by Oppenheim, 1991). Autophagy is proposed to be involved in various physiological processes including cellular remodelling (Bolender and Weibel, 1973; Masaki et al., 1987), differentiation (Tsukada and Ohsumi, 1993) and non-apoptotic cell death during embryogenesis (Clarke, 1990). In sympathetic neurons NGF-deprivation and cytosine arabinoside treatment were both found to result in a thirty fold increase in the levels of autophagic particles. The anti-autophagic drug 3-methyladenine also delayed cell death (Xue et al., 1999). This suggests a role for autophagy in normal neuronal responses to cytotoxic stimuli.

The process of non-lysosomal degradation, as the name infers, takes place without the aid of lysosomal degradative activity. Phagocytosis by the surrounding cells is also uninvolved. Instead the intracellular organelles swell and organelle-free vacuoles appear in the cytoplasm. These fuse with each other and with the extracellular space. Cellular structure is gradually degraded, destroying the cell (Schweichel and Merker, 1973). This type of cell death is primarily seen during the mineralisation of vacuolated cartilage, prior to resorption of calcified intercellular substance (Schweichel and Merker, 1973).

The final type of physiological cell death is apoptosis. It is this type of physiological cell death that will be focused on in this thesis. Apoptosis was first defined as an active, inherently programmed process that is initiated or inhibited by a number of environmental stimuli. It involves membrane blebbing (zeiosis), cytoplasmic condensation (Kerr et al., 1972) and the activation of endogenous endonucleases, which cleave double stranded nuclear DNA in the internucleosomal linker regions, producing mono- and oligo-nucleosomes. The mono- and oligo-nucleosomes accumulate in the apoptotic cell as DNA degradation occurs prior to nuclear membrane breakdown (Wyllie, 1980). This gives apoptotic neuronal nuclei their characteristic pyknotic appearance of dark, spherical structures surrounded by pale staining cytoplasm contained within the membrane, when stained with cresyl fast violet (CFV) dye. In contrast to nuclear DNA, the mitochondrial DNA remains intact. Nuclear and cellular membranes become convoluted as zeiosis occurs, cytoplasmic organelles degenerate, the nucleus and cytoplasm become compartmentalised into membrane bound apoptotic bodies which are phagocytosed by surrounding cells (Kerr et al., 1972; reviewed by Oppenheim, 1991) (see figure 1.6). Thus there is no leakage of cellular contents and no inflammatory response is induced suggesting that this process is well suited to a role in tissue homeostasis.



**Figure 1.6 Morphological features of apoptosis.** Membrane blebbing and cytoplasmic condensation occur. Nucleosomes accumulate, cytoplasmic organelles degenerate and the nucleus and cytoplasm become compartmentalised into membrane bound apoptotic bodies which are phagocytosed. (Adapted from figure donated by Oliver Schmidt).

Apoptosis is an intrinsic part of many regulatory processes. For example, it occurs during embryogenesis with the destruction of autoreactive T-cells during maturation (Raff, 1992; Dwyer, 1993). Apoptosis can be stimulated by the removal of cell type specific growth factors, such as IL-2 from haematopoietic cells or the addition of certain substances, e.g., glucocorticoids or tumour necrosis factor (TNF) to those cells during development (Zheng et al., 1995). The dysregulation of apoptosis may have significant detrimental consequences, including tumorigenesis, autoimmunity, neurodegenerative diseases, haematopoietic deficiencies and infertility (Vaux et al., 1999; Pellegrini and Strasser, 1999).

## 1.2.2 The Roles of Apoptosis in Development

The role of apoptosis *in vivo* is to establish and maintain the functional properties of tissues and organs (Saunders, 1966; Kerr et al., 1972). Cell death has a number of roles during development. It is involved in morphogenetic events, giving rise to species-typical patterns of tissue, organ and body structure. An example of this is the removal of interdigital webbing in the majority of higher vertebrates (Saunders and Fallon, 1966). If apoptosis is inhibited by treatment with peptide caspase inhibitor, interdigital webbing fails to degenerate (Milligan et al., 1995; Jacobson et al., 1996). Another instance in which this type of morphogenetic cell death can be seen is the creation of space to allow for axonal growth, for example the growth of the optic nerve (reviewed by Oppenheim, 1991). Cells that have fulfilled their function are removed by programmed cell death. For example, the tail-spike cells of *Caenorhabditis elegans* (Sulston et al., 1983), which die having generated the filaments that shape the tail. Apoptosis is involved in the removal of vestigial structures, e.g., pronephric tubules which form the kidneys in fish but are not used in mammals are eliminated by apoptosis. Alternatively structures that are required in one sex but not the other are removed by apoptosis, for example the removal of the Mullerian duct that forms the uterus and oviducts from males (reviewed by Jacobson et al., 1997).



### **1.2.2a Apoptosis in the Development of the Spinal Cord**

During the development of the spinal cord, 50-60% of motoneurons degenerate by apoptosis (Lance-Jones, 1982; Oppenheim et al., 1986; Oppenheim 1986). This is believed to generate space for the axonal growth of both sensory and motoneurons within the spinal cord. Another role for apoptosis is thought to be the elimination of unwanted cellular phenotypes (Frade et al., 1996; Homma et al., 1994). Also specific neurogenic progenitors may be selectively eliminated by apoptosis in the dorsomedial regions of the CNS as a means of attaining a balance in the numbers of neuronal and non-neuronal cells required in different regions (Homma et al., 1994).

### **1.2.2b Apoptosis in the Development of the Eye**

During the development of the eye, the loss of significant numbers of cells can allow for the expansion or displacement of cells in neighbouring areas. The distribution of degenerating cells in the development of the mammalian eye was related to sites which subsequently alter their shape (Glücksmann, 1951). From this, it was concluded that cell death allows for the displacement of cells during morphogenic movements, for example invagination, seam formation and rudiment separation. Apoptotic cells are seen to be located close to the optic disc. This indicates that apoptosis may create space for the axons of the optic nerve to form (Frade and Barde, 1999). This is supported by the fact that all mammals show apoptosis in the region of the ventrally located optic

fissure, which subsequently provides passage of the optic nerve and intraocular blood vessels. The pattern of degeneration sites during development of the eye is now believed to reflect a number of roles for cell death, including the inhibition of invagination within the death centres, integration of invaginations which shape the dorsal optic cup and optic fissure, assistance of formation of the pigment epithelium monolayer and orientation of the lens vesicle within the eye cup (Silver and Hughes, 1973). Cell death is also thought to have a role in the elimination of neurons that have formed inappropriate connections in the developing visual system (Cowan et al., 1984). During early isthmo-optic nucleus development, a number of neurons extend axonal projections to the ipsilateral, as opposed to contralateral eye. These neurons are removed by physiological cell death (Frade and Barde, 1999).

### **1.2.2c Apoptosis in the Development of the Peripheral Nervous System**

Neuronal apoptosis occurs among virtually all major taxonomic groups and involves many different types of neurons in the peripheral nervous systems. This includes neurons of the sympathetic, nodose and trigeminal ganglia (reviewed by Oppenheim, 1991). During neurodevelopment both progenitor cells and post-mitotic neurons are produced in excess. The nervous system then eliminates cells that have made inappropriate connections or that have not contacted their target field. This occurs during major periods of apoptosis, one of which takes place in the ventricular and sub-ventricular regions of the developing nervous system, where neural stem and progenitor

cells differentiate to produce the neurons and glial cells that migrate and populate the brain and spinal cord. This period is thought to have two functions; to eliminate progenitors that do not differentiate appropriately and to ensure the production of appropriate cell numbers in rapidly growing tissues (Miller et al., 2000). A further period of apoptosis in the nervous system takes place when newly generated neurons have migrated to their final destination, extended their axons and attempted to establish appropriate connections. This period of neuronal apoptosis eliminates about half the neurons in any given population (Oppenheim, 1991). The survival of neurons during this period depends upon trophic support provided on the formation of appropriate connections at the target field (reviewed by Miller and Kaplan, 2001). An example of this is the neurons of the developing DRG (Levi-Montalcini, 1987) and the trigeminal ganglia (Harper and Davies, 1990) the survival of which is matched to the size of the target field by the magnitude of production of essential neurotrophic factors by that target-field. This process is thought to ensure that there is an adequate balance between the size of a neuronal population and that of its target. It is the basis for the neurotrophic hypothesis which proposes that substances are present in the target field of developing neurons upon which these neurons are dependant for survival. Neurotrophic factors are present in limiting concentrations so that those neurons that do not get an adequate supply, die by apoptosis (Cowan et al., 1984; Davies 1988a and b; Purves, 1988; Barde, 1989). This will be discussed in greater detail in section 1.6. It has also been suggested that the large reductions in neuronal populations, such as sensory neurons, creates an environment in which precursor cell differentiation is enhanced.

This was suggested as it was seen that the premature differentiation of sensory neuron progenitor cells is a consequence of enhanced neuronal death and that DRG precursor cells of NGF deficient mice are seen to differentiate prematurely together with increases in the level of apoptosis of NGF receptor, TrkA, expressing neurons (Farinas et al., 1998, 1999-review). Apoptosis is also thought to be involved in the developmental refinements of synaptic circuitry. This synapse elimination can be seen as a mechanism for creating large numbers of specific circuits from initially diffuse and redundant connections (reviewed by Lichtman and Colman, 2000).

For many types of neurons that exhibit apoptosis the control of the timing and extent of cell death is, at least partially influenced by stimuli derived from or associated with their synaptic targets.

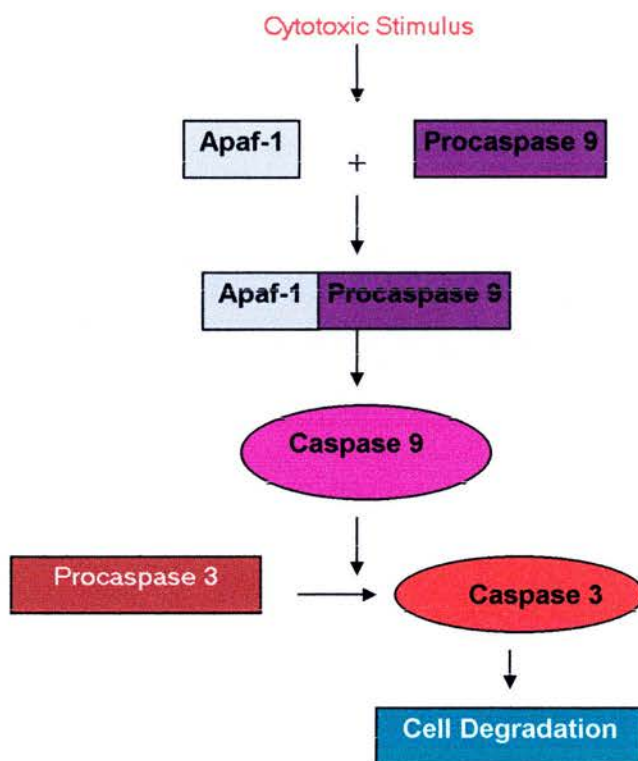
Apoptosis is a complex, all or nothing process, thus, the pathway that leads to it contains a number of checks and controls which ensure that it is highly regulated. The final checkpoint for apoptosis is the Bcl-2 family. This family of proteins controls the activity of the caspases which are responsible for cellular destruction. Prior to this are a number of cellular events that occur depending upon the pathway that is activated. NF- $\kappa$ B is a transcription factor that has been found to initiate transcription of the genes involved in both cell survival and cell death. NF- $\kappa$ B can be activated by a number of extracellular signals including the binding of both NGF and TNF $\alpha$  to their respective cellular receptors. For the sake of clarity events will be discussed in this reverse order.

### 1.3 The Role of the Bcl-2 Family and the Caspases in Apoptosis

The importance of the apoptotic process is emphasised by the level of evolutionary conservation. Genetic pathways of cell death have been defined in the nematode *C. elegans* and the fruit fly *D. melanogaster* (Abrams, 1999; Ellis et al., 1991). The basic components of the vertebrate apoptotic mechanism are similar to those of the invertebrates, although they are more varied and complex.

The Bcl-2 family regulate a highly conserved pathway that results in cell death. The pathway leads to the cleavage of vital target proteins by proteases of the caspase (cysteine aspartate protease) group. In vertebrates the Bcl-2 family of proteins act upstream of the caspases to regulate their activity (Chinnaiyan et al., 1996). Caspases are essential effectors in pro-apoptotic pathways induced in response to cytotoxic stimuli, including TNF $\alpha$  (reviewed in Budihardjo et al., 1999). In order to reach the stage of cell death a highly regulated cascade must be initiated in which the caspases are synthesised as low activity precursors that require processing at the sites of caspase cleavage for the generation of the active enzyme. This is done by effector caspases which are processed by upstream caspases (Stennicke and Salvensen, 2000; Adams and Cory, 2001). These upstream caspases are termed the regulatory caspases and initiate the caspase cascade following autoactivation (Li, P. et al., 1997). This autoactivation requires multimerisation, which is assisted by an adaptor or scaffold protein, such as apoptotic protease-activating factor-1 (Apaf-1). Apaf-1 primes autoactivation of

procaspase-9 (Hengartner, 2000; Wei et al., 2000; Budihardjo et al., 1999) and plays a very important part in the apoptotic pathway. Caspase 9 acts upstream from effector caspase 3 (Kuida et al., 1998). The effector caspases, once activated go on to cleave a number of substrates, resulting in the destabilisation of cellular integrity, leading to nuclear and cytoskeletal disassembly, metabolic stress and inhibition of DNA repair and replication (see figure 1.7). Substrates of the effector caspases include the lamins (Orth et al., 1996; Takahashi et al., 1996), the cleavage of which leads to the loss of nuclear integrity. The degradation of Poly-ADP ribose polymerase (PARP) by caspases prevents normal DNA repair and turnover thereby crippling the cell (Marks and Berg, 1999 - review). Caspases also activate DNA fragmentation factor (DFF) (Liu, X. et al., 1997).



**Figure 1.7: Summary diagram of the caspase cascade in response to cell death stimuli.** A cell death stimulus leads to the association of procaspase 9 with apoptotic protease-activating factor 1 (Apaf-1) and its conversion to caspase 9. Caspase 9 activates caspase 3, which is responsible for cleavage a number of substrates, resulting in the destabilisation of cellular integrity, leading to nuclear and cytoskeletal disassembly, metabolic stress and inhibition of DNA repair and replication.



Caspase activity has been found to be highly involved in developmental cell death following neurotrophin deprivation. Cysteine proteases have been found to act endogenously in the programmed death of cells following growth factor deprivation (Ray et al., 1992; Gagliardini et al., 1994; Kuida et al., 1995). Caspase inhibitors can prevent neuronal apoptosis induced by growth factor withdrawal *in vitro* (Armstrong et al., 1997; Keane et al., 1997) and the treatment of SCG neurons with caspase inhibitors prevents their death following NGF withdrawal (Werth et al., 2000). This indicates that neurotrophin withdrawal initiates a pathway that ultimately leads to caspase-mediated apoptosis.

As mentioned before, the Bcl-2 family represent the final checkpoint before caspase activation. Proteins of the Bcl-2 family fall into three distinct groups. Firstly there are those that repress apoptosis such as Bcl-2 (Vaux, 1993; Steller, 1995; Thompson, 1995), Bcl-x<sub>L</sub> (Boise et al., 1993) and Mcl-1 (Kozopas et al., 1993). Secondly there are members such as Bax (Oltvai et al., 1993), Bak (Kiefer et al., 1995; Chittenden et al., 1995) Bcl-x<sub>S</sub> (Fang et al., 1994), and Bik (Kelekar and Thompson, 1998) which promote apoptosis. The Bcl-2 and Bax subfamilies share three of four conserved BH (Bcl-2 homology) sequence motifs and can take on similar conformations (Suzuki et al., 2000; Sattler et al., 1997; Muchmore et al., 1996). A further group of pro-apoptotic members constitutes the third group. These are pro-apoptotic members that share only the BH3 domain and are otherwise unrelated to one another and members of the other groups (Kauffman and Hengartner, 2001). This group includes Bad (Yang, E. et al., 1995), Bid

and Bim (Kelekar and Thompson, 1998) and is referred to as the ‘BH3-only’ group (See table 1.1).

**Table 1.1: Different classes of the Bcl-2 family of proteins.**

<b>Anti-Apoptotic Members</b>	<b>Pro-Apoptotic Members</b>	<b>BH3-Only Members</b>
Bcl-2	Bax	Bad
Bcl-x <sub>L</sub>	Bak	Bid
Mcl-1	Bcl-x <sub>S</sub>	Bim
A1	Bik	
	Bok	

### **1.3.1 Anti-Apoptotic Bcl-2 Family Members**

Bcl-2, protects cells against programmed cell death. It has been demonstrated to protect cells from a number of apoptosis-inducing stimuli, including growth factor deprivation, glucocorticoids, ionising radiation and oxidant stressors (reviewed by Williams and Smith, 1993). It was found that sympathetic neurons deprived of NGF are rescued by Bcl-2 (Garcia et al., 1992; Muira et al., 1993). Bcl-2 also protects chick sensory neurons deprived of NGF (Allsopp et al., 1993). Other anti-apoptotic members of the family have similar effects including Bcl-x<sub>L</sub> (Gonzalez-Garcia et al., 1995; Middleton et al., 1996) and Bcl-w (Middleton et al., 2001)

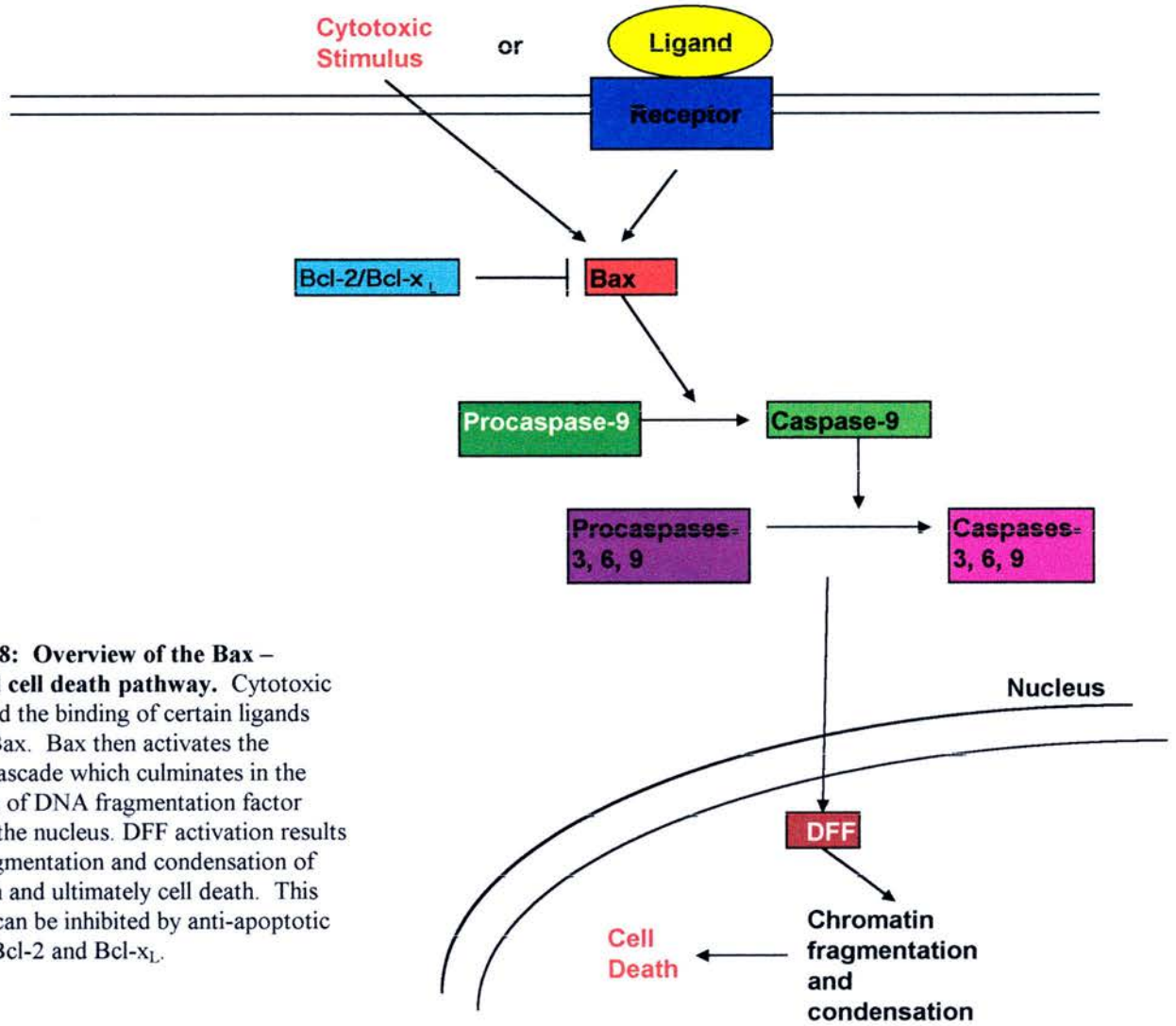


The examination of Bcl-2 knockout mice showed that endogenous Bcl-2 is required for the sustained survival response of trigeminal and nodose neurons to neurotrophins during development. Its absence results in decreased numbers of neurons *in vivo* (Pinon et al., 1997; Michaelidis et al., 1996) and *in vitro* (Pinon et al., 1997). It was shown that neonatal sympathetic neurons from Bcl-2 knockout mice died more rapidly following NGF withdrawal than those from normal mice (Greenlund et al., 1995). The over-expression of Bcl-2 in mice induces the production of excess neurons in the facial nucleus, retinal ganglion cell layer (Martinou et al., 1994) and DRG sensory neurons (Farlie et al., 1995). This Bcl-2 over-expression also rescues retinal ganglion cells from developmental cell death (Martinou et al., 1994). The Bcl-2 protein is normally expressed in cells of the retinal ganglion at low levels but after intraorbital crush of the optic nerve there is a reduction in expression combined with a significant increase in the moderate baseline expression of Bax protein (Isenmann et al., 1997). This suggests that there is a down-regulation of pro-survival proteins and an up-regulation of cell death enhancing proteins when apoptosis is imminent.

Functionally opposite members of the family readily heterodimerise. The amphipathic BH3 region of the pro-apoptotic proteins can bind to the hydrophobic groove on the anti-apoptotic proteins created by the  $\alpha$  helices of the BH1-3 domains (Sattler et al., 1997; Muchmore et al., 1996). It is through this interaction that the anti-apoptotic proteins exert their activity

### 1.3.2 Pro-Apoptotic Bcl-2 Family Members

The pro-apoptotic members of the Bcl-2 family, such as Bax, can assume a conformation that permits dimerisation through the BH3 region and leads to cell death. They also appear to be able to kill cells through an alternative mechanism, which may work without activating caspases (Gross et al., 1999). Bax, has been shown by immunohistochemical analysis to be widely expressed in both the central and peripheral nervous systems (Merry and Korsmeyer, 1997), indicating its involvement in cell death in the nervous system. It demonstrates 21% sequence homology to Bcl-2 and was first identified by co-immunoprecipitation with Bcl-2 (Merry and Korsmeyer, 1997). According to the “Rheostat model” (Oltvai et al., 1993), the primary determinant of the death decision is the concentration of Bax homodimers. The theory suggests that pro-survival proteins such as Bcl-x<sub>L</sub> and Bcl-2 inactivate Bax by heterodimerisation. The ability of Bax to dimerise is thought to be dependant upon the BH3 domain (Zha et al., 1996) (see figure 1.8). The mechanistic basis of this commonly accepted model is, as of yet, unclear.



**Figure 1.8: Overview of the Bax – mediated cell death pathway.** Cytotoxic stimuli and the binding of certain ligands activate Bax. Bax then activates the caspase cascade which culminates in the activation of DNA fragmentation factor (DFF) in the nucleus. DFF activation results in the fragmentation and condensation of chromatin and ultimately cell death. This pathway can be inhibited by anti-apoptotic proteins Bcl-2 and Bcl-x<sub>L</sub>.

During neurodevelopment levels of Bax expression are high throughout the period of physiological cell death, after which there is a decrease. Furthermore, micro-injection of a Bax expression vector into cultured sympathetic neurons induces apoptosis in the presence of NGF and increases the rate of apoptosis on NGF withdrawal (Vekrellis et al., 1997). This reflects the involvement of Bax during the period of developmental cell death in which only neurons that obtain sufficient growth factor support survive.

A study of *bax* gene knockout mice (Deckwerth et al., 1996) further indicates an important role for Bax in regulating developmental and neurotrophin deprivation-induced neuronal apoptosis. *bax*-deficient developing SCG and facial nuclei showed an increased number of sympathetic and motoneurons respectively at birth, combined with increased resistance to death induced by neonatal axotomy (Deckwerth et al., 1996). Neonatal sympathetic neurons cultured from *bax*-deficient mice survived NGF deprivation for longer periods than their wildtype counterparts (Deckwerth et al., 1996). This indicates that Bax is required for neuronal death during both naturally occurring developmental processes and after deprivation of neurotrophic factors in these regions. In both the trigeminal and nodose neurons from *bax*-deficient mice apoptosis was seen to be reduced but not fully abolished (Middleton et al., 2000b). *bax* knockout mice were seen to have a virtual elimination of apoptosis in the sympathetic neurons of the SCG (Deckerth et al., 1996), whilst the over-expression of Bax *in vitro* is able to accelerate apoptosis following neurotrophin withdrawal (Martinou et al., 1998; Vekrellis et al.,

1997). This suggests that while Bax is the primary inducer of cell death in the SCG, other pro-apoptotic proteins are involved in mediating apoptosis in trigeminal and nodose neurons.

It was proposed that the increase in sympathetic and motoneurons during the development of Bax deficient mice was as a result of their inability to complete the usual apoptotic pathway in the absence of Bax (Deckwerth et al., 1996). It was shown that the increases in number of neurons coincide with decreases in naturally occurring apoptosis. Further support for the role of Bax in developmental cell death comes from the findings that showed that naturally occurring apoptosis in the DRG is prevented in Bax deficient mice (White et al., 1998) and also, by the *in vivo* demonstration that apoptosis induced by NGF deprivation during development is dependent on the presence of Bax (Patel et al., 2000). Deletion of the *bax* gene has also been demonstrated to prevent apoptosis of SCG neurons deprived of NGF (Werth et al., 2000).

Cell factors that determine the subcellular localisation of Bax may be involved in controlling its function. In healthy cells Bax is free to diffuse in the cytosol, whereas after the apoptotic stimulus, it is targeted to the mitochondria (Wolter et al., 1997). Bax translocation to the mitochondria requires a conformational change. This may be triggered by a transient rise in pH (Khaled et al., 1999) or by Bid (Eskes et al., 2000). Altered confirmation allows Bax to homodimerise on the mitochondrial membrane and

associate with Bcl-2, an interaction that is implicated in the cytotoxic action of Bax (Gross et al., 1999).

It has been suggested that Bax can be post-translationally regulated by Bcl-2, indicating the existence of a feedback mechanism that may help to maintain the ratio of Bcl-2 to Bax protein in physiologically appropriate ranges (Miyashita et al., 1995). The Bax protein acts upstream of the caspases and has been shown to be responsible for their activation in sympathetic neurons, it is the activity of the caspases that mediate the pro-apoptotic effects of Bax (Martinou et al., 1998). It is unclear whether members of the Bax group initiate the apoptotic process or alternatively ensure cellular demise, through mitochondrial damage, after the anti-apoptotic Bcl-2 proteins are inactivated (Adams and Cory, 2001). Mitochondria are thought to play an important role in apoptosis (This will be discussed in section 1.3.4).

Thus Bax has clearly been demonstrated to be involved in programmed cell death in the developing nervous system and a significant amount is known about the mechanism by which it induces apoptosis. What is less clear is the involvement of other pro-apoptotic Bcl-2 members and particularly those of the BH3-only group.

### 1.3.3 The 'BH3-Only' Members of the Bcl-2 Family

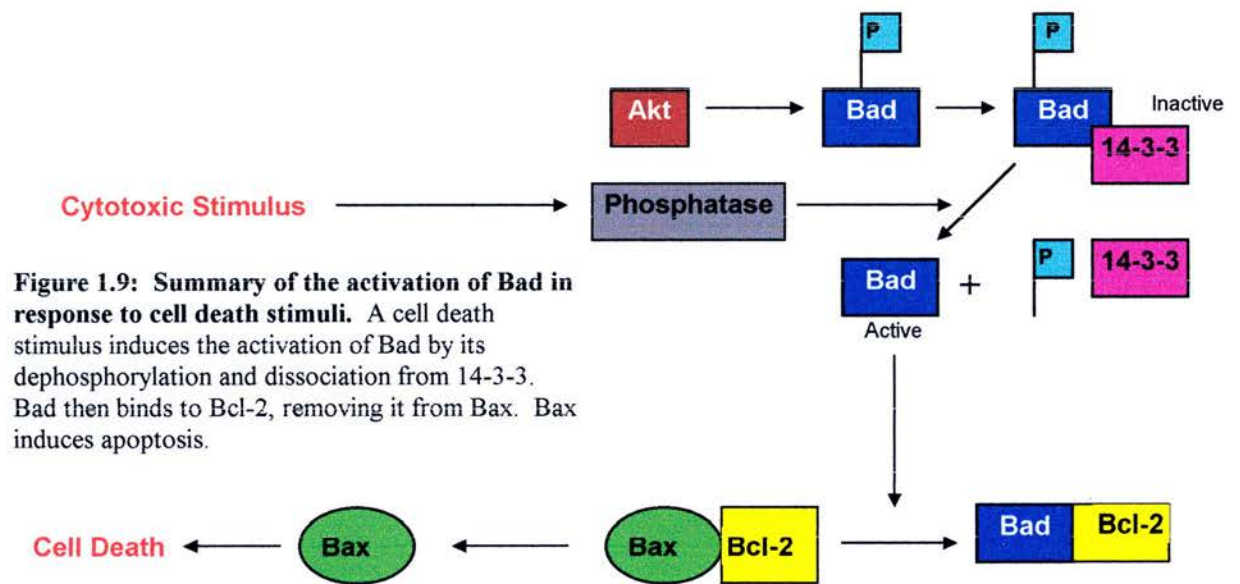
In the other pro-apoptotic group of the Bcl-2 family, the 'BH3-only' group it is the short 9-16 residue BH3 region that is essential and probably sufficient for its death promoting activity (Kelekar and Thompson, 1998). All of the members of this group identified so far are pro-apoptotic and have been shown to interact with one or more of the death suppresser family members. Thus, it seems that a BH3-only protein can serve as a ligand that locks pro-survival proteins into an inactive or possibly apoptogenic conformation. In this way the BH3-only proteins apparently kill entirely through binding other family members (Adams and Cory, 2001). BH3-only proteins may induce death by binding to a Bcl-2-related death suppressor such as Bcl-2 or Bcl-x<sub>L</sub> and disrupting the complex of the death suppressor with a caspase activator, such as Apaf-1, which in turn leads to the activation of caspases (e.g., caspase-9) and the death of the cell (Lutz, 2000). The BH3-only proteins appear to be sentinels for cellular damage and essential triggers of apoptosis. This however does not take into account for the presence of pro-apoptotic Bcl-2 family members such as Bax and Bak, which may be activated by the BH3-only proteins (Shimizu et al., 1999; Li et al., 1997), or for the involvement of the Bcl-2 proteins in regulating the release of cytochrome c from the mitochondria (discussed in section 1.3.4).

Bad is a BH3-only protein that is 24kDa in size and consists of two hundred and four amino acids. Several PEST (proline, glutamic acid, serine and threonine rich protein region) motifs are contained within this (Yang et al., 1995), and these motifs are usually found in proteins that undergo rapid degradation, indicating that Bad has a relatively short half life (Rogers et al., 1986). *Bad* mRNA has been detected in both the central and peripheral nervous systems (Yang et al., 1995; Merry and Korsmeyer, 1997), suggesting a role for Bad in cell death in the nervous system. Also, the over-expression of Bad in NGF supported sympathetic neurons induced apoptosis indicating that Bad may be involved in the regulation of cell death in these neurons (Hamner et al., 2001). As with all BH3-only proteins, Bad shows only limited homology to the other Bcl-2 family members. It dimerises with Bcl-2 and Bcl-x<sub>L</sub> but not with Bax, Bcl-x<sub>S</sub> or itself. BH3-only proteins can bind directly to anti-apoptotic members such as Bcl-x<sub>L</sub> (Sattler et al., 1997) and inhibit their interaction with death effector members, e.g., Bax and Bak *in vitro* (Ottillie et al., 1997; Diaz et al., 1997), thus inducing apoptosis. The targeting of anti-apoptotic proteins such as Bcl-x<sub>L</sub> and Bcl-2 by Bad, is essential to its pro-apoptotic activity (Lutz, 2000). Bad requires the BH3 domain for its interaction with Bcl-x<sub>L</sub> and its death-inducing activity (Zha et al., 1997; Ottillie et al., 1997; Kelekar et al., 1997). When Bad is phosphorylated by the serine-threonine kinase, Akt, it associates with 14-3-3, a signal transducer protein that interacts with several signalling enzymes (Aitken et al., 1995), including Raf-1 (Muslin et al., 1996; Datta et al., 1997; del Peso et al., 1997). This maintains Bad in its inactive state, preventing its binding to Bcl-x<sub>L</sub>. There are two potential phosphorylation sites in murine Bad but only one (serine 136) is



phosphorylated by Akt (Franke and Cantley, 1997). Akt is a downstream target of the phosphatidylinositol-3'-OH kinase (PI3'K) pathway and binds directly to phosphoinositide products of PI3'K by an N-terminal pleckstrin homology domain (Franke et al., 1997). This binding results in partial activation of Akt. There is then further phosphorylation by other serine/threonine kinases leading to full Akt activation. One such kinase is phosphoinositide-dependent kinase 1 (PDK1), which phosphorylates and activates Akt *in vitro* and is activated itself by products of PI3'K (Alessi et al., 1997a, b). Withdrawal of IL-3 from haematopoietic cell lines that are dependant on it for survival induces the dephosphorylation of Bad resulting in its disassociation from 14-3-3 and binding to Bcl-x<sub>L</sub>, thus preventing the survival promoting effects of Bcl-x<sub>L</sub> (Zha, I. et al., 1996). When Bad is phosphorylated its interaction with 14-3-3 ends its binding to Bcl-x<sub>L</sub> (Franke and Cantley, 1997). This suggests that phosphorylation may inhibit the pro-apoptotic activity of Bad, by blocking its interaction with death suppresser proteins and indeed, Bad mutants that cannot be phosphorylated show increased cytotoxicity as compared to the wildtype (Lutz, 2000). It has been shown that the phosphorylation of Bad is induced by certain cell survival factors, such as NGF (Datta et al., 1997). Akt is activated in response to these survival factors in a PI3'K-dependent manner. The phosphorylation of Bad induced by these factors can be blocked by inhibitors of PI3'K (Datta et al., 1997; del Peso et al., 1997). Activation of Akt is sufficient to promote the phosphorylation of Bad *in vivo* and *in vitro* (Datta et al., 1997; del Peso et al., 1997). In keeping with this, the dephosphorylation of Bad would be expected to lead to increased cell death. In fact, it has been found that the calcium activated protein phosphatase,

calcineurin, dephosphorylates Bad and induces apoptosis (Wang, et al., 1999). Calcium-mobilising agents induced the dephosphorylation of Bad, resulting in its dissociation from 14-3-3 and translocation from the cytosol to the mitochondria, where it is associated with Bcl-x<sub>L</sub> (Wang, et al., 1999). All this supports an activation mechanism for Bad that involves its phosphorylation by Akt and a deactivation mechanism reliant upon its dephosphorylation (see figure 1.9).



### 1.3.4 Regulation of Cell Death and Survival by the Bcl-2 Family

Most members of the Bcl-2 family contain a hydrophobic C-terminal segment that facilitates their interaction with the endoplasmic reticulum, nuclear envelope and mitochondrial membrane, where pro-survival members usually reside and most other members congregate during apoptosis (Adam and Cory, 2001). The mitochondrial

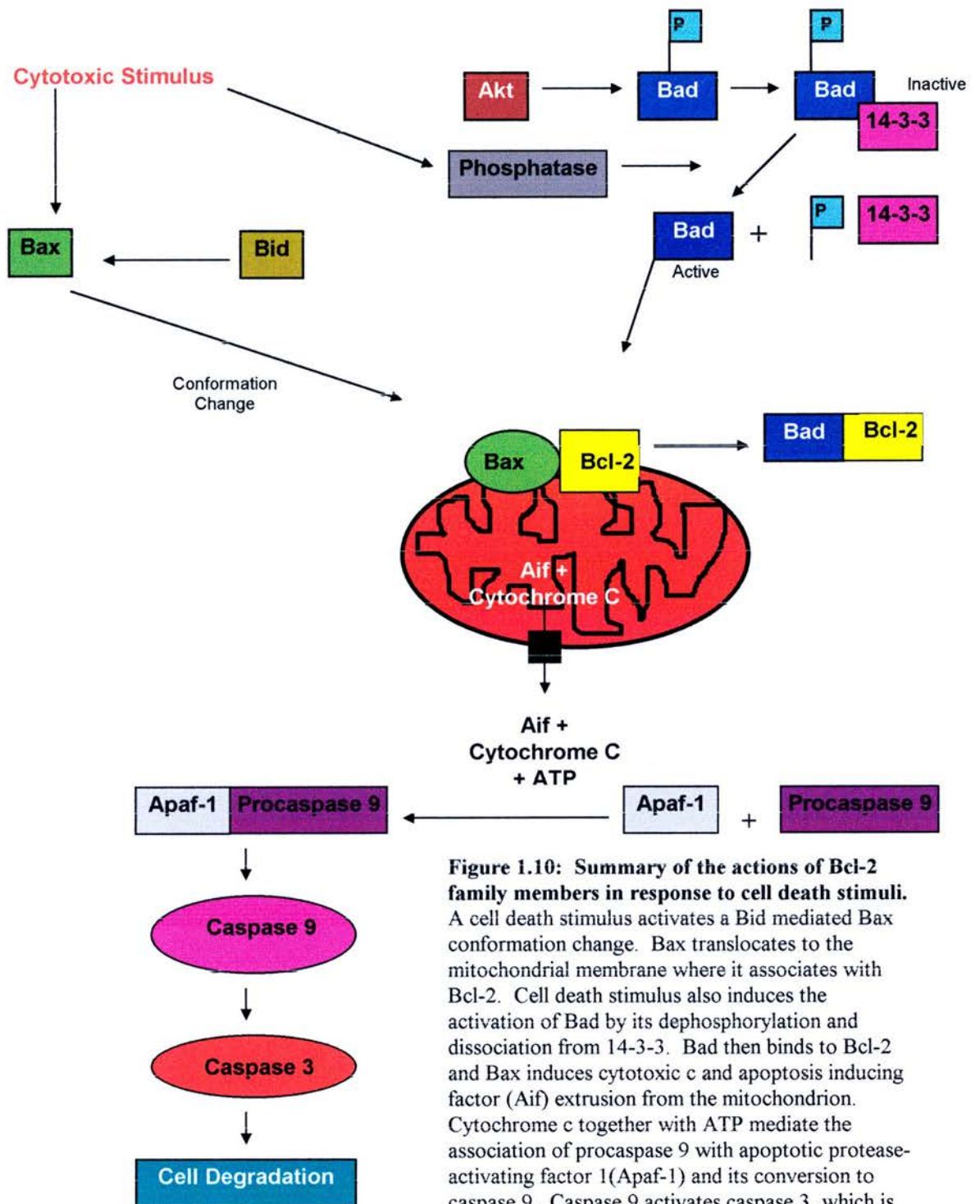
intermembrane space contains several pro-apoptotic proteins and their release on mitochondrial disturbance is probably involved in the process of cell death. Cytochrome c is one such protein (reviewed in Budihardjo et al., 1999; Green and Reed, 1998). The release of cytochrome c leads to the activation of Apaf-1 downstream of the Bcl-2 block. With several cytotoxic stimuli, cytochrome c release precedes caspase activation (Yoshida et al., 1998) (see figure 1.9). Apaf-1-deficient mice show reduced levels of apoptosis in the central nervous system (CNS), their cells are resistant to a variety of apoptotic stimuli and caspase-2, -3 and -8 processing and activation is impaired in these mice, implying that Apaf-1 plays a central role in apoptosis (Cecconi et al., 1998; Yoshida et al., 1998). Mice deficient in Apaf-1 and caspase -3 and -9 all show late embryonic to perinatal lethality due to CNS deficits. The neonates show large scale neuronal hyperplasia and structural disorganisation indicating a failure of apoptosis during neurodevelopment (Cecconi et al., 1998; Hakem et al., 1998; Kuida et al., 1998, 1996; Woo et al., 1998; Yoshida et al., 1998). This, together with the fact that cytochrome c deficiency results in early embryonic lethality (Li, K. et al., 2000), indicates that the apoptotic cascade directed by cytochrome c is important in neurogenesis.

Further support comes from the demonstration that mitochondria release cytochrome c (Bossy-Wetzel et al., 1998; Kluck et al., 1997; Yang, J., et al., 1997; Rosse et al., 1998; Eskes, et al., 1998; Jurgensmeier, et al., 1998) together with apoptosis inducing factor (Aif) (Susin et al., 1996). Also, elimination of cytochrome c from the cytosol was

shown to reduce apoptotic activity. This activity was restored by subsequent addition of cytochrome c (Liu, X. et al., 1996a). Cells undergoing apoptosis *in vivo* were found to have increased levels of cytochrome c in their cytosol (Liu, X. et al., 1996a). This combined with the fact that cytochrome c has been found to be released from mitochondria in cells undergoing apoptosis stimulated by death receptor activation, DNA damaging agents and kinase inhibitors (Yang, J. et al., 1997; Scaffidi et al., 1998) indicates that cytochrome c is highly involved in the apoptotic cascade. In the presence of cytochrome c and ATP, Apaf-1 binds to procaspase-9 via their respective NH<sub>2</sub>-terminal domains, thus activating it. Caspase-9 in turn cleaves and activates procaspase-3, which then cleaves a number of other substrates, inducing cellular disassembly (Zou et al., 1997; Li et al., 1997, Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997; Villa et al., 1997; Wang et al, 1996, Cohen, 1997, Friedlander and Yuan, 1998). Apaf-1 is required for the apoptotic effect of cytochrome c (Zou et al., 1997).

Members of the Bcl-2 family are important in the regulation of cytochrome c release and thus in regulating the apoptotic cascade (see figure 1.10). Bcl-2 and Bcl-x<sub>L</sub> have been discovered to inhibit cytochrome c release (Bossy-Wetzel et al., 1998; Kluck et al., 1997; Yang, J. et al., 1997), whereas Bax stimulates it both *in vitro* in isolated mitochondria and in intact cells (Bossy-Wetzel et al., 1998; Eskes, et al., 1998; Jurgensmeier, et al., 1998). Also, Bcl-2 over-expression inhibits cytochrome c release from the mitochondria and in doing so prevents apoptosis (Yang and Korsmeyer, 1996). Early in the apoptotic process Bax has been seen to undergo a conformational change in

cerebellar granule cells deprived of serum and potassium (Desagher et al., 1999) and it has also been found to translocate to the mitochondria (Hsu et al., 1997; Wolter et al., 1997). This conformational change in Bax was observed to be triggered by the BH3-only protein, Bid (Eskes et al., 2000). Following this Bax is oligomerised and inserted into the outer mitochondrial membrane (Goping et al., 1998; Gross et al., 1998) where it triggers cytochrome c release. Thus Bax exerts at least part of its activity by inducing mitochondrial release of cytochrome c. Bcl-2 and Bcl-x<sub>L</sub> prevent Bid induced Bax conformation change by binding directly with Bax (Eskes et al., 2000) and thus prevent cytochrome c release and apoptosis.



**Figure 1.10: Summary of the actions of Bcl-2 family members in response to cell death stimuli.** A cell death stimulus activates a Bid mediated Bax conformation change. Bax translocates to the mitochondrial membrane where it associates with Bcl-2. Cell death stimulus also induces the activation of Bad by its dephosphorylation and dissociation from 14-3-3. Bad then binds to Bcl-2 and Bax induces cytochrome c and apoptosis inducing factor (Aif) extrusion from the mitochondrion. Cytochrome c together with ATP mediate the association of procaspase 9 with apoptotic protease-activating factor 1 (Apaf-1) and its conversion to caspase 9. Caspase 9 activates caspase 3, which is responsible for cellular degradation.



The processes by which members of the Bcl-2 family regulate cytochrome c release are uncertain although a number of theories have been proposed. It was found that the 3D structure of Bcl-x<sub>L</sub> was similar to that found in membrane penetrating bacterial toxins, such as diphtheria. This prompted the idea that Bcl-2 members containing the hydrophobic BH1/2 domain might form pores in organelle membranes (Muchmore, et al., 1996). Under certain conditions Bcl-x<sub>L</sub>, Bcl-2 and Bax can create pores in liposomes (reviewed in Vander-Heiden and Thompson, 1999) and mitochondrial membranes (Antonsson et al., 1997). The presence of these pores could influence the flow of ions and therefore water into and out of the mitochondria (Shimzu et al., 1998). It is proposed that osmotic and electrical gradient disturbances result in mitochondrial swelling and release of calcium and intermembrane proteins such as cytochrome c and Aif (Kroemer et al., 1997). Aif was thought to be a protease but it was then discovered that it is a flavoprotein homologous to many bacterial oxidoreductases (Susin et al., 1999b). It can translocate to the nucleus where it induces chromatin condensation and DNA fragmentation (Susin et al., 1999b). This implicates it as being induced along with cytochrome c in the apoptotic process mediated by the Bcl-2 family.

Another proposed theory is that Bcl-2 members either stabilise or destabilise a pre-existing channel, for example the permeability transition pore which is believed to form across sites where the inner and outer membranes are in contact. This channel is a voltage-dependent anion channel (VDAC) and it is gated at both inner and outer

membranes (Vander-Heiden et al., 2000; Vander-Heiden et al., 1999). Some evidence suggests that Bcl-x<sub>L</sub> associates with VDAC and closes it in liposomes, whereas Bax and Bak keep it open (Vairo et al., 2000; Shimizu et al., 1999) allowing the passage of intermembrane space molecules such as cytochrome c. In contrast, it is also possible that Bcl-x<sub>L</sub> may keep the outer membrane channel open (Vander-Heiden et al., 2000) and that a death signal then recruits BH3-only proteins to the pro-survival proteins. This interaction might then shut the VDAC. The ensuing ionic and pH changes could initiate the translocation and oligimerisation of pro-apoptotic members, such as Bax. Finally, the multimerised family members may create pores which allow the escape of intermembrane space proteins, triggering the caspase cascade.



Bid has been demonstrated to mediate cytochrome c release from the mitochondria after being cleaved by caspase 8 or caspase 3 (Luo et al., 1998; Li et al., 1998). This is thought to represent an amplification of apoptosis when levels of caspase 8 are low and can be inhibited by Bcl-2 and Bcl-x<sub>L</sub> (Vander-Heiden, 1997; Medema et al., 1997; Srinivasula et al., 1998). It has been found that high levels of BH3 proteins can induce cytochrome c release (Cosulich et al., 1997). Indicating cytochrome c release as a potential mechanism of BH3-only protein induced cell death. Very little is known about the role played by BH3-only proteins in the apoptotic cascade. Although considering the significant involvement of Bax in developmental cell death it is possible that BH3-only proteins also play a role in this process. In fact they may be responsible for the residual cell death found when the *bax* gene is knocked out as seen in the trigeminal, nodose (Middleton et al., 2000b) and to a small extent in the SCG (Deckwerth et al., 1996).

The Bcl-2 proteins are produced in response to the action of a transcription factor, which initiates their synthesis as they are required by the cell. Thus the relative levels of pro- and anti-apoptotic members of the Bcl-2 family can be regulated at the level of their synthesis. One such transcription factor induced in the apoptotic pathway in response to cytotoxic stimuli is NF- $\kappa$ B.

## 1.4 The Role of NF- $\kappa$ B Activation in Cell Survival and Apoptosis

The NF- $\kappa$ B/Rel family of transcription factors is thought to be highly involved in apoptotic responses to cytotoxic stimuli, together with infection and inflammation, as they are key regulators of the expression of various cellular and viral genes (Ghosh et al., 1998; Baldwin, 1996). NF- $\kappa$ B is as a transcription factor and was first identified as a lymphoid-specific protein that binds to a decameric oligonucleotide present in the  $\kappa$ -light chain gene intronic enhancer (Sen and Baltimore, 1986). It is a sequence-specific DNA-binding protein complex composed of homo- or hetero-dimers of members of the Rel family. Mammals express five Rel proteins that belong to two classes. The first class includes RelA, RelB and c-Rel, which are synthesised as mature products. The second class is encoded by the genes *Nfkb1* and *Nfkb2*, whose products are initially synthesised as precursor proteins p105 and p100. These are then proteolytically processed to give mature p50 and p52 NF- $\kappa$ B proteins (Karin and Lin, 2002 - review). Members of the Rel family of proteins share a 300 amino acid conserved DNA-binding and dimerisation domain called the Rel-homology domain (RHD) and are concerned with transcriptionally regulated processes (Baeuerle and Baltimore, 1996). The Rel-homology domain containing proteins are regulated by a mechanism of nuclear-cytoplasmic shuttling (Baldwin, 1996). The highly conserved RHD is responsible for dimerisation and DNA binding (Chen et al., 1998). The two Rel proteins of the dimer each contact half the DNA binding site. Slight variations in the ten base pair DNA consensus sequence ( $5'$ GGGGYNNCCY $3'$ ) confer a preference for selected Rel

combinations (Kunsch et al., 1992). The RHD contains the nuclear translocation signal (NTS), and it is this that is the site for binding of inhibitors of NF- $\kappa$ B (reviewed in Baldwin, 1996).

The Rel proteins p65 (Rel A) and p50 are known to heterodimerise to form NF- $\kappa$ B and p65/p50 heterodimers are the most abundant form of NF- $\kappa$ B in cells, although p50 homodimers that lack transactivation domains are found in the resting cell nuclei (Kang et al., 1992; Ten et al., 1992). The inducible NF- $\kappa$ B complex was originally purified from human cell lines, rabbit lung tissues, and human placenta (reviewed by Miyamoto and Verma, 1995). The genes encoding p50 and p65 subunits of NF- $\kappa$ B are related to each other. p50 is synthesised as a precursor protein of 105kDa to 110kDa (p105) and is cleaved by a ubiquitin-dependent proteasome to form the p50 protein. Agents which activate NF- $\kappa$ B also enhance the proteolysis of p105, thus the processing of p105 can regulate the level of p50 production (reviewed by Miyamoto and Verma, 1995). If p50 is disrupted, a number of focal defects in immune responses occur (Sha et al., 1995), supporting role in the regulation of the immune system. Only four amino acid substitutions in p65 to the corresponding residues in p50 in the N terminal region altered p65 DNA-binding specificity to that of p50 (Coleman et al., 1993). In spite of this p65 is functionally very different from p50. Unlike p50, p65 is not produced as a precursor. It can form homodimers and bind directly to  $\kappa$ B sites on DNA. The presence of a potent C-terminal transactivation domain allows  $\kappa$ B-dependent transactivation and as there is significant transcriptional activity with p50/p65 dimers but not p50 homodimers, it

seems that the transactivation potential of NF- $\kappa$ B is provided by the p65 subunit (reviewed by Miyamoto and Verma, 1995). This is supported by the fact that p65/p50 heterodimers have greater affinity for the classical  $\kappa$ B site than p50 homodimers (Phelps et al., 2000). A number of studies support the proposal that it is p65 phosphorylation that is responsible for the transcriptional activity of NF- $\kappa$ B (Bird et al., 1997; Madrid et al., 2000, 2001; Wang et al., 2000). In contrast to this role of p65, p50 (and p52) homodimers have been seen to repress the transcription of their target genes (Zhong et al., 2002). In keeping with this role for p50, dimers containing p50 have been found to bind to the  $\kappa$ B site within certain target gene promoters and inhibit their activity. When the cell is stimulated, activated p65/p50 heterodimers, following the phosphorylation of p65, translocate to the nucleus and displace the p50 containing inhibitory protein (Zhong et al., 2002).

PCR-based selection of  $\kappa$ B binding sites using bacterially expressed p50 and p65 showed that three guanines in the 5' half site are essential for p50 binding whereas two cytosines at the 3' end are necessary for p65 binding. Also it was found that sites recognised by neither p50 or p65 alone can be recognised by the p50/p65 heterodimer (Kunsch et al., 1992). *p65* knockout mice show embryonic lethality between E14.5 and E15 as a result of extensive liver apoptosis (Beg et al., 1995). Increased sensitivity to pro-apoptotic stimuli is also demonstrated by *p65*<sup>-/-</sup> fibroblasts when treated with tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) (Beg and Baltimore, 1996; Van Antwerp et al., 1996). The dependence of the foetal liver on NF- $\kappa$ B for its survival is due to the production of

large amounts of  $\text{TNF}\alpha$  by the haematopoietic progenitors that populate the foetal liver (Rosenfield et al., 2000).

#### 1.4.1 Inhibition of NF- $\kappa$ B

In most cell types NF- $\kappa$ B is maintained in its inactive form in the cytosol due to its interaction with one of a group of inhibitory proteins. These include  $\text{I}\kappa\text{B}\alpha$ ,  $\text{I}\kappa\text{B}\beta$ ,  $\text{I}\kappa\text{B}\gamma$ ,  $\text{I}\kappa\text{B}\epsilon$ , p105 and p100. Each member of this inhibitory family has regions of protein-protein interaction called ankyrin-repeats. The proteins are usually composed of a core of six or more ankyrin repeats, with an N-terminal regulatory domain and a C-terminal domain that contains a PEST motif (reviewed in Baldwin, 1996). The number of ankyrin repeats varies between the inhibitory proteins and it is this that influences the specificity with which the protein binds to NF- $\kappa$ B dimers, inhibiting them. It is thought that the first two ankyrin repeats prevent the binding of importins to the NTS of NF- $\kappa$ B (Huxford et al., 1998; Jacobs and Harrison, 1998). It is thought that the existence of different forms of  $\text{I}\kappa\text{B}$  protein is to selectively inhibit the various NF- $\kappa$ B complexes composed of different Rel proteins. The p50/p65 NF- $\kappa$ B complex can be inhibited by  $\text{I}\kappa\text{B}\alpha$ ,  $\text{I}\kappa\text{B}\gamma$ , p105 and p100 (reviewed by Miyamoto and Verma, 1995). By binding to NF- $\kappa$ B the inhibitors conceal the NTS and retain it in the cytoplasm. In response to various stimuli, including  $\text{TNF}\alpha$ , IL-1 and bacterial endotoxin, the  $\text{I}\kappa\text{B}$  proteins are completely proteolytically degraded in a ubiquitin-mediated manner and p105 and p100 precursors are partially degraded. This mechanism has been best studied for the inhibitor  $\text{I}\kappa\text{B}\alpha$ .

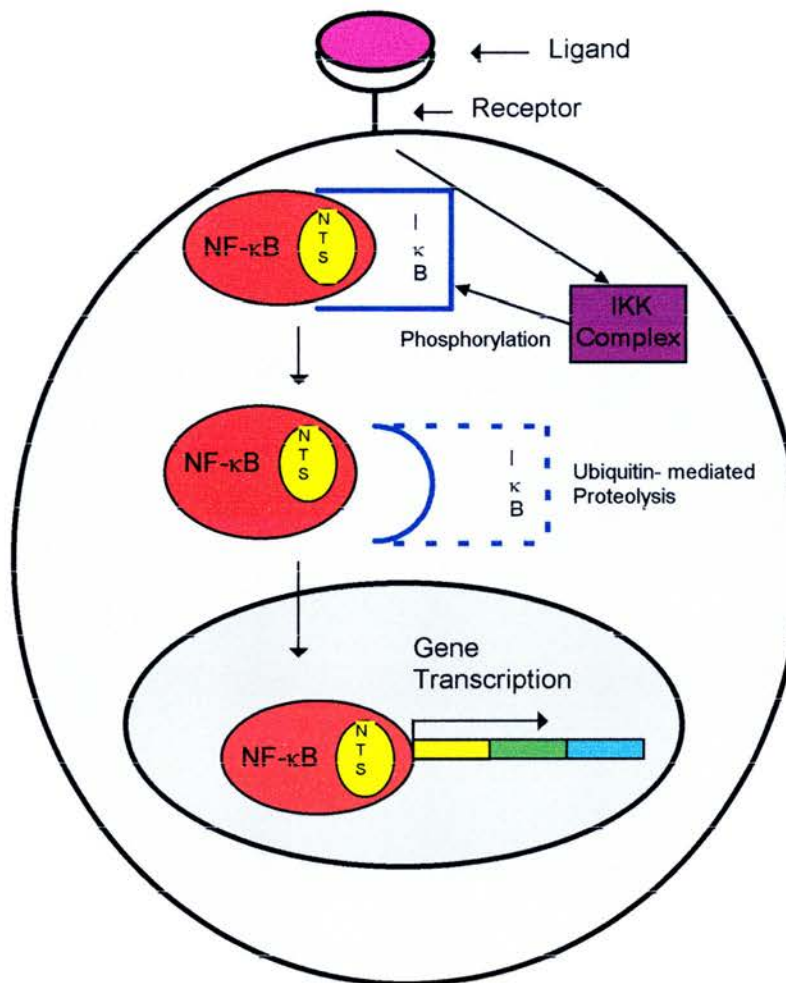


Biochemical purification and immunoprecipitation studies indicate that I $\kappa$ B $\alpha$  inhibits the vast majority of p50/p65 heterodimers (Thompson et al., 1995, Whiteside et al., 1997). On activation NF- $\kappa$ B translocates to the nucleus where it induces the transcription of target genes (see figure 1.11) (Israel, 2000), including I $\kappa$ B genes. This leads to rapid re-synthesis of the I $\kappa$ B proteins, (Arenzana-Seisdedos et al., 1995). Some of these I $\kappa$ B proteins, such as I $\kappa$ B $\alpha$ , contain a nuclear export sequence. In the nucleus they bind to NF- $\kappa$ B dimers resulting in their exportin-mediated transport to the cytoplasm (Arenzana-Seisdedos et al., 1997). This binding and export of NF- $\kappa$ B by I $\kappa$ B $\alpha$  has been found to be controlled by the acetylation of p65. Acetylated p65 will not bind to I $\kappa$ B $\alpha$  and following its activation of gene transcription p65 is deacetylated, associates with I $\kappa$ B $\alpha$  and is exported to the cytoplasm (Chen et al., 2001). Thus NF- $\kappa$ B activity is inhibited by an auto-regulatory feedback loop mechanism.

I $\kappa$ B is removed by the ubiquitin-proteasome pathway. As a result of two phosphorylation events on serine 32 and 36 of the N-terminus, I $\kappa$ B is targeted for ubiquitin-mediated proteolysis (Di Donato, 1996; Brown et al., 1995; Chen et al., 1995), indicating their importance in this step. Directly following phosphorylation poly-ubiquitination occurs. The primary acceptor sites for ubiquitin in I $\kappa$ B $\alpha$  are arginines 21 and 22. The substitution of these with lysines significantly inhibits I $\kappa$ B $\alpha$  degradation (Di Donato et al., 1995; Scherer et al., 1995). The only regulated step in the degradation process is the phosphorylation reaction. The ubiquitinating activity that specifically



recognises phosphorylated I $\kappa$ B $\alpha$  is constitutive and the addition of ubiquitin acts as a destruction signal. The process of degradation is mediated by proteosomes which are hollow cylinders with four seven-member rings that degrade I $\kappa$ B into short peptides (Arenzana-Seisdeos et al., 1995). The destruction of I $\kappa$ B is mediated by the 26S proteosome (Whiteside and Israel, 1997; Verma et al., 1995; Beg and Baldwin, 1993) and it has been found that in the presence of proteosome inhibitors, N-terminally phosphorylated I $\kappa$ B $\alpha$  accumulates very rapidly, indicating that phosphorylation precedes I $\kappa$ B $\alpha$  degradation as opposed to its dissociation from NF- $\kappa$ B (Alkalay et al., 1995; Di Donato et al., 1995).



**Figure 1.11: NF- $\kappa$ B activation.** Ligand binding to the transmembrane receptor stimulates the I $\kappa$ B kinase (IKK) complex to initiate the degradation of inhibitory  $\kappa$ B (I $\kappa$ B) by the ubiquitin-proteosome pathway. This uncovers the nuclear translocation signal (NTS) on nuclear factor  $\kappa$ B (NF- $\kappa$ B), which then moves into the nucleus, where it initiates gene transcription.

There are exceptions to this universal pathway of NF- $\kappa$ B activation. Firstly, there is the activation of NF- $\kappa$ B in response to UV radiation, which is dependent on I $\kappa$ B degradation but not N-terminal phosphorylation of I $\kappa$ B (Bender et al., 1998; Li and Karin, 1998). Secondly, anoxia, which stimulates phosphorylation of I $\kappa$ B $\alpha$  at tyrosine 42 (Imbert et al., 1996). This then binds to the SH2 domain of PI3'K, which pulls it away from NF- $\kappa$ B (Beraud et al., 1999). Treatment with NGF has also been demonstrated to activate NF- $\kappa$ B following tyrosine 42 phosphorylation in PC12, human neuroblastoma and rat hippocampal cells. This activation was found to occur without significant I $\kappa$ B $\alpha$  degradation as determined by western blotting and time lapse imaging of neurons expressing fluorescent protein tagged I $\kappa$ B $\alpha$  (Bui et al., 2001). The over-expression of a Y42F mutant of I $\kappa$ B $\alpha$  potently suppressed NGF-induced NF- $\kappa$ B activation (Bui et al., 2001), supporting this mechanism of NF- $\kappa$ B activation. However, tyrosine 42 is not conserved in other I $\kappa$ Bs and perhaps, depending on which I $\kappa$ B proteins are present in a given cell type, different NF- $\kappa$ B/I $\kappa$ B complexes will be activated by various stimuli. Thus, cell-specific regulation and synergistic effects of numerous different stimuli may be accommodated.

Thirdly, at least one NF- $\kappa$ B dimer, RelB/p52, is subject to an alternative form of regulation. In the cytoplasm of non-stimulated cells, RelB is associated with p100, the C-terminal domain of which is degraded after stimulation, allowing the RelB/p52 dimers to move to the nucleus. The activation of this process has been found to be dependent



on IKK $\alpha$  and IKK $\alpha$ -/- B lymphocytes exhibit a specific deficiency in RelB/p52 processing (Senftleben et al., 2001). This processing is highly regulated and can be induced in response to ectopic expression of NIK (Xiao et al., 2001). NIK-induced RelB/p52 processing is not affected if IKK $\beta$  or  $\gamma$  are deficient (Senftleben et al., 2001).

#### **1.4.2 The IKK complex**

The regulation of I $\kappa$ B phosphorylation in response to all other NF- $\kappa$ B activating stimuli may be dependent on a complex of protein kinases called the I $\kappa$ B kinase (IKK) complex. This is a serine specific complex that phosphorylates the N-terminal regulatory serines of I $\kappa$ Bs. It is responsive to a number of NF- $\kappa$ B stimuli, including TNF $\alpha$  and IL-1, which induce its activity with kinetics matching those of I $\kappa$ B $\alpha$  degradation (Di Donato et al., 1997; Mercurio et al., 1997). In addition to this, the extent to which the IKK complex is activated appears to determine the kinetics of I $\kappa$ B degradation and IKK shows high degree of specificity for serines 32 and 36 of the I $\kappa$ Bs. The over-expression of catalytically inactive IKK $\alpha$  or IKK $\beta$  inhibits NF- $\kappa$ B activation in response to TNF $\alpha$ , as measured by the translocation of p65 to the nucleus (Zandi et al., 1997). Thus implicating the IKK complex as an activator of NF- $\kappa$ B via I $\kappa$ B $\alpha$  phosphorylation and degeneration.

Gel filtration, protein purification, micro-sequencing and molecular cloning lead to the identification of three protein kinase subunits that make up the IKK complex (Karin, 1999 - review). These were I $\kappa$ B kinase - $\alpha$  (IKK $\alpha$ ) and - $\beta$  (IKK $\beta$ ), which are catalytically active subunits that have protein kinase domains at their N terminus, and form hetero- and homo-dimers (Zandi et al., 1998; Di Donato et al., 1997; Mercurio et al., 1997; Zandi et al., 1997). These dimers together with the regulatory subunit, IKK $\gamma$  (NEMO) form the IKK tripartite complex (Rothwarf et al., 1998; Yamaoka et al., 1998). IKK $\gamma$  does not contain a recognisable catalytic domain but is composed of three large  $\alpha$ -helical regions (Karin, 1999 - review). The IKK subunits are highly homologous proteins with IKK $\alpha$  and IKK $\beta$  showing 52% sequence identity (Israel, 2000). Although cross-linking experiments indicate that IKK $\alpha$  and IKK $\beta$  can form both homo- and hetero-dimers *in vitro* (Zandi et al., 1998), biochemical analysis has shown that the predominant type of IKK is an IKK $\alpha$ :IKK $\beta$  heterodimer that is associated with either a dimer or trimer of IKK $\gamma$  (Rothwarf et al., 1998).

Treatment of purified, activated IKK complex with protein phosphatase 2A resulted in its inactivation, indicating a role for phosphorylation in IKK activation and this was verified when the treatment of cells with protein phosphatase 2A inhibitor was found to lead to the activation of IKK and NF- $\kappa$ B (Di Donato et al., 1997). The incubation of HeLa cells with TNF was discovered to stimulate the phosphorylation of IKK  $\alpha$  and IKK $\beta$  subunits (Delhase et al., 1999). The activation of IKK depends on the

phosphorylation of its IKK $\beta$  subunit (Delhase et al., 1999). The phosphorylation sites of IKK $\alpha$  and IKK $\beta$  are exclusively serines, some of which are located in the activation loop of the kinase domain (Delhase et al., 1999; Zheng and Guan, 1994). Serine phosphorylation of the activation loop of IKK $\beta$ , but not IKK $\alpha$ , is required for NF- $\kappa$ B activation by cytokines, indicating a more important role for IKK $\beta$  (Mercurio et al., 1997) in this process. Phosphorylation moves the activation loop away from the catalytic pocket, thus allowing it to interact with substrates (Johnson et al., 1996). The replacement of the two phosphoacceptor serines, Ser-177 and Ser 181, of IKK $\beta$  with alanines prevents activation (Delhase et al., 1999; Mercurio et al, 1997) and the replacement of the two equivalent serines in IKK $\alpha$ , Ser-176 and Ser-180, abolishes autophosphorylation of this subunit but has no effect on the stimulation of IKK complex activation by TNF, IL-1, or the upstream kinases NIK or MEKK1 (Delhase et al., 1999). This indicates that IKK is activated in response to IKK $\beta$  phosphorylation and that although IKK $\alpha$  phosphorylation is concurrent with that of IKK $\beta$ , it is not vital for the stimulation of IKK complex activity. The negative regulation of IKK activity also involves phosphorylation. IKK $\beta$  is extensively phosphorylated at its C-terminal serines. This depends on autokinase activity and mutagenesis experiments indicate that these sites are involved in the termination of the kinase activity that induces IKK activation (Delhase et al., 1999). This is supported by the fact that the replacement of nine or ten of the C-terminal serines with alanines leads to the inactivation time IKK being prolonged to four times that of the wildtype enzyme (Delhase et al., 1999).

### 1.4.2a IKK $\alpha$

When mice with null mutation in IKK $\alpha$  were examined they showed a very specific phenotype. Complete IKK $\alpha$  deficiency leads to perinatal lethality. *IKK $\alpha$  -/-* mice are born alive but die within 30 minutes of birth (Hu et al., 1999). These mice have severe craniofacial deformities, immature limbs and tails and taut, shiny, unwrinkled skin. The most dramatic alteration in these mice is that the epidermis is up to ten times thicker than in the wildtype. This is due to hyper-proliferation of basal layer cells, which is normally one cell thick. Keratinocyte differentiation is also inhibited, giving the epidermis a uniform rather than stratified constitution (Hu et al., 1999).

Histopathological examination has shown that the *IKK $\alpha$  -/-* mice have limb bones of almost normal size, but that they are restricted under the thickened skin (Li, Q., et al., 1999; Hu et al., 1999; Takeda et al., 1999). Other skeletal abnormalities include a partially split sternum, fused vertebrae and severe skull truncation (Hu et al., 1999).

When *IKK $\alpha$  -/-* embryonic fibroblasts, primary keratinocytes and liver cells were stimulated with TNF, IL-1 and endotoxin normal activation of IKK and degradation of I $\kappa$ B occurred, although despite this *IKK $\alpha$  -/-* fibroblasts showed a 50% reduction in total NF- $\kappa$ B binding activity (Hu et al., 1999). Thus although IKK $\alpha$  is not involved in IKK activation it may have a role in the stimulation of NF- $\kappa$ B nuclear translocation or DNA binding activity. The IKK complex found in *IKK $\alpha$  -/-* cells shows normal size and regulation, supporting the fact that IKK $\alpha$  is not involved in IKK complex activity. None

of the available knockout mouse mutants deficient in known NF- $\kappa$ B subunits or components of the TNF and IL-1 pathways demonstrate a similar phenotype, suggesting that the defects found in *IKK $\alpha$* <sup>-/-</sup> mice may not be due to alterations in NF- $\kappa$ B activity (Karin, 1999 - review).

#### **1.4.2b IKK $\beta$**

Inactivation of the *IKK $\beta$*  gene results in a phenotype similar to that observed with p65 inactivation: the mice die from massive liver apoptosis at E12.5-E14.5 (Li, Z., et al., 1999; Li, Q. et al., 1999; Tanaka et al., 1999; Beg et al., 1995). This embryonic lethality is thought to be due to the inability of liver cells to survive TNF-induced apoptosis in the absence of NF- $\kappa$ B (Beg and Baltimore, 1996; Liu, Z.G., et al., 1996; Wang et al., 1996; Van Antwerp et al., 1996), mice that lack both p65 and TNF activities are viable and have an apparently normal liver (Doi et al., 1999). *IKK $\beta$* <sup>-/-</sup> mice are deficient in both p65 and p50 activity and it appears that the loss of IKK $\beta$  leads to a more severe reduction in NF- $\kappa$ B activity than the loss of p65 alone (Horwitz et al., 1997). The over-expression of a catalytically inactive form of IKK $\beta$  prevented cytokine-induced NF- $\kappa$ B activation. Also, the over-expression of IKK $\beta$  resulted in the activation of NF- $\kappa$ B (Woronicz et al., 1997). All this reaffirms IKK $\beta$  as the primary component in NF- $\kappa$ B activation.

Double knockout mice for *IKK $\alpha$*  and *IKK $\beta$*  die at E12. This is thought to be due to a large degree of liver apoptosis (Israel, 2000). Approximately 70% of these mice showed neural tube closure to be incomplete, suggesting that NF- $\kappa$ B has a role in vertebrate embryonic neurodevelopment. Previous null mutations in the Rel family members in mice did not result in a similar phenotype (Israel, 2000), though this may be due to the effects of redundancy in these mice.

### 1.4.2c IKK $\gamma$

The third known component of the IKK complex is a non-enzymatic sub-unit called IKK $\gamma$  or NEMO (Di Donato et al., 1997; Rothwarf et al., 1998; Mercurio et al., 1999). It is a 48kDa protein and is required for the activation of the IKK complex by upstream components of the NF- $\kappa$ B cascade. IKK activation requires an intact IKK $\gamma$  subunit (Karin, 1999-review). The N-terminal region of IKK $\gamma$  is responsible for the interaction with the other IKK subunits and it exhibits a greater affinity for IKK $\beta$  than IKK $\alpha$  (Rothwarf et al., 1998; Yamaoka et al., 1998; Mercurio et al., 1999; Chu et al., 1999). Inactivation of the IKK $\gamma$  gene results in embryonic fatality at E12.5-E13 due to severe liver damage caused by apoptosis (Rudolph et al., 2000). *IKK $\gamma$*  knockout cells are completely deficient in IKK and NF- $\kappa$ B activation in response to IL-1, TNF and endotoxin (Yamaoka et al., 1998), implicating IKK $\gamma$  as an essential component for IKK and NF- $\kappa$ B activation. In *IKK $\gamma$ <sup>-/-</sup>* cells there is a complex containing IKK $\alpha$ :IKK $\beta$

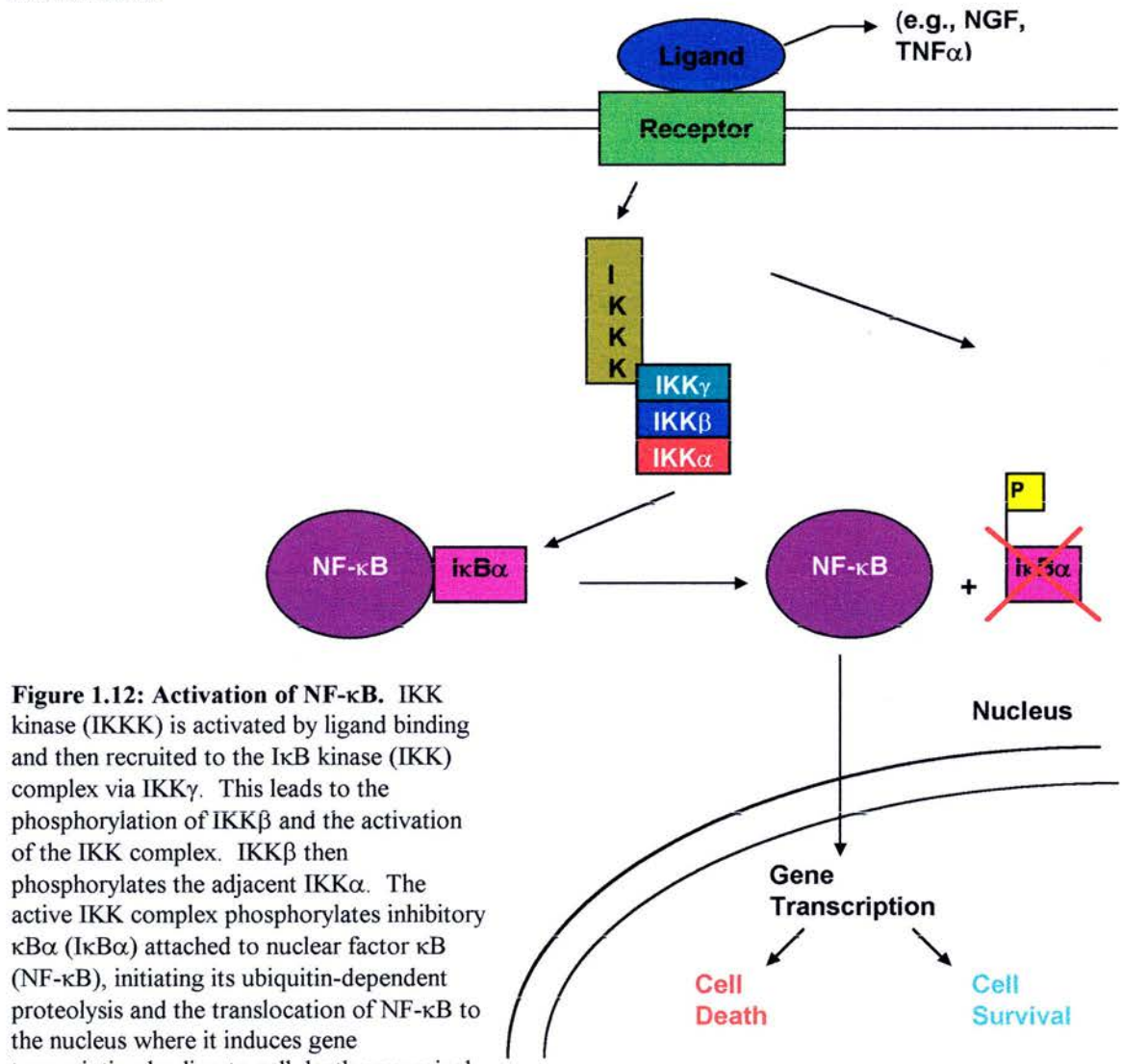
heterodimers, although their apparent molecular mass is approximately half of that of the normal complex and the complex does not respond to any tested stimuli (Yamaoka et al., 1998). IKK complexes that contained a mutant of IKK $\gamma$  in which the C-terminal was deficient were unresponsive to TNF, IL-1 and endotoxin stimulation (Rothwarf et al., 1998). This indicates that under these circumstances IKK $\gamma$  is absolutely required for NF- $\kappa$ B activation and suggests that the C-terminal region of IKK $\gamma$  is necessary for the recruitment of upstream activators.

#### **1.4.2d The Regulation of IKK Activity**

A three state model has been proposed to describe the control of IKK activity (reviewed by Karin 1999). Initially IKK kinases are activated by upstream stimuli and they are then recruited to the IKK complex via IKK $\gamma$ . This leads to the phosphorylation of IKK $\beta$  and the activation of IKK. Through trans-autophosphorylation the activated IKK $\beta$  subunit can phosphorylate the adjacent subunit (either IKK $\alpha$  or IKK $\beta$ ), as well as other inactive IKK complexes. The active IKK complexes then phosphorylate the I $\kappa$ B proteins attached to NF- $\kappa$ B, initiating their ubiquitin-dependent proteolysis and the activation and translocation of NF- $\kappa$ B (figure 1.12). At the same time the activated IKK $\beta$  and IKK $\alpha$  subunits undergo C-terminal autophosphorylation. This operates as a device such that when nine or more of the C-terminal serine residues are phosphorylated the enzyme attains a low activity state. This allows for the inactivation of IKK by



phosphatases when the upstream signal has been inactivated. As the C-terminal phosphorylation sites are adjacent to the intrinsic activator domain they might exert their negative influence on kinase activity by altering the conformation and affecting its interaction with the kinase domain. This method of regulation explains why activation of IKK is highly transient as the process is initiated by the phosphorylation of IKK $\beta$  but after only eight subsequent phosphorylation events on the C-terminal, the complex is deactivated.



**Figure 1.12: Activation of NF- $\kappa$ B.** IKK kinase (IKKK) is activated by ligand binding and then recruited to the I $\kappa$ B kinase (IKK) complex via IKK $\gamma$ . This leads to the phosphorylation of IKK $\beta$  and the activation of the IKK complex. IKK $\beta$  then phosphorylates the adjacent IKK $\alpha$ . The active IKK complex phosphorylates inhibitory  $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ ) attached to nuclear factor  $\kappa$ B (NF- $\kappa$ B), initiating its ubiquitin-dependent proteolysis and the translocation of NF- $\kappa$ B to the nucleus where it induces gene transcription leading to cell death or survival.



### 1.4.3 The Induction of NF- $\kappa$ B Activation

A number of approaches have suggested that some MAPKKKs, including NIK (which has been implicated in NF- $\kappa$ B activation by most stimuli) (Woronicz et al., 1997; Ling et al., 1998; Lin et al., 1998; Karin and Delhase, 1998; Nakano, et al., 1998; Nemoto et al., 1998) and MEKK1 (Karin and Delhase, 1998; Nakano et al., 1998; Nemoto et al., 1998; Lee et al., 1998; Mercurio and Manning, 1999), might be involved in the activation of the IKK complex in response to specific stimuli, particularly TNF, through the phosphorylation of the two kinase subunits (Israel, 2000). NIK is suggested to represent one of many routes to IKK activation by specific stimuli. It was first identified by its association with TRAF2 and it has been seen potently to activate NF- $\kappa$ B when over-expressed (Malinin et al., 1997; Ling et al., 1998). In spite of this, analysis of NIK-deficient mice indicates that NIK does not participate in IKK activation in response to either TNF or IL1.

Two studies suggest that Akt kinase is also involved in activation of the IKK complex. The first concludes that both Akt and NIK are required for TNF activation of NF- $\kappa$ B (Ozes et al., 1999). The second suggests that Akt is involved in IL-1 mediated NF- $\kappa$ B activation through p65 subunit phosphorylation (Sizemore et al., 1999). Akt can contribute to TNF $\alpha$ -induced NF- $\kappa$ B transcriptional activity (Madrid et al., 2001), but not to IKK activation (Delhase et al., 2000). This indicates that Akt is involved in activating NF- $\kappa$ B downstream from the IKK complex.

Another protein kinase of the MAPKKK family, TPL-2 is known to stimulate the protein-mediated degradation of the NF- $\kappa$ B precursor protein, p105, resulting in NF- $\kappa$ B activation (Belich et al., 1999). TPL-2 can also activate IKK (Lin et al., 1999) and thus much of the activation of NF- $\kappa$ B in cells expressing TPL-2 may be due to its induction of I- $\kappa$ B $\alpha$  degradation. The exact role of TPL-2 in the activation of NF- $\kappa$ B is still unclear.

Thus a number of proteins have been suggested as activators of the IKK complex. It is still uncertain what the specific role of each of these is in activating IKK and what determines which protein is active in a given cell.

NF- $\kappa$ B can be activated by a number of extracellular stimuli leading to various intracellular responses. Binding of both TNF $\alpha$  and neurotrophins to their cell membrane receptors have been found to induce NF- $\kappa$ B activation.

## 1.5 The Role of Tumour Necrosis Factor in Apoptosis

Tumour necrosis factor (TNF) is a pleiotropic pro-inflammatory cytokine produced by macrophages, monocytes, lymphocytes, keratinocytes and fibroblasts, in response to inflammation, infection, injury and other environmental challenges (Baud and Karin, 2001). It was first identified as a factor that induced rapid haemorrhagic necrosis of transplantable tumours (Carswell et al., 1975). A number of TNFs exist of which the predominant two are TNF $\alpha$  and TNF $\beta$ . These bind to two receptors TNFR1 and 2, both of which are expressed on all somatic cell types except red blood cells. TNFR1 is related to the p75 receptor of the neurotrophin family (Marsters et al., 1992) and it is by this receptor that the TNF-induced effects in most cells are mediated (Heyninck and Beyaert, 2001). Genes for this TNF $\alpha$  receptor have been demonstrated to be constitutively expressed on neurons and oligodendrocytes (reviewed by Szelenyi, 2001), indicating that TNF $\alpha$  may contribute to the normal functioning of the brain. The permanent presence of the TNF $\alpha$  receptor causes neural cells to be sensitive to TNF $\alpha$  even at low levels (Wong, et al., 1996; Wong and Licinio, 1994). In the CNS, TNF $\alpha$  is believed to be primarily produced by microglia and astrocytes, although it is also produced by fibroblasts (Arnett et al., 2001; Baud and Karin, 2001). TNF $\alpha$  has also been observed to be synthesised by neurons (Breder et al., 1993). This production of TNF $\alpha$  in the CNS suggests that it has a functional role in the nervous system.

TNF $\alpha$  has been found to either promote or repress cell death depending upon the environment in which it is acting. For instance, TNF $\alpha$  promotes the survival of

osteoclasts *in vitro* (Lee et al., 2001) but has been proposed to be involved in mediating apoptosis of osteoclasts *in vivo* in joint destruction (Redlich et al., 2002). In order to bring about cell death, TNF $\alpha$  binds to TNFR1. The cytoplasmic death domain of TNFR1 then interacts with an adaptor protein TRADD which in turn interacts with a further adaptor protein FADD. FADD recruits and activates pro-caspase 8 which triggers activation of the cell death machinery (Ashkenzai and Dixit, 1998). The intracellular domain of TNFR1 contains an 80 amino acid domain, which is responsible for the induction of such cell death pathways (Heyninck and Beyaert, 2001). TNFR2 lacks this death domain but synergistically enhances TNFR1-induced cytotoxicity (Grell et al., 1999). To promote cell survival, TNF $\alpha$  binding to TNFR1 initiates a signalling cascade that ultimately leads to activation of the transcription factor NF- $\kappa$ B. In response to TNF $\alpha$ , I $\kappa$ B (and other inhibitory subunits of NF- $\kappa$ B) is phosphorylated by the IKK complex, resulting in its ubiquitination, degradation and the nuclear translocation of NF- $\kappa$ B (Karin and Ben-Neriah, 2000). NF- $\kappa$ B then induces the expression of a variety of anti-apoptotic genes including caspase-8-c-FLIP which can interact with FADD and pro-caspase 8, and thus inhibit apoptosis ( Baud and Karin, 2001).

Thus the primary determinant of the dual roles of TNF $\alpha$  may well be the NF- $\kappa$ B pathway. It has been suggested that NF- $\kappa$ B is induced by TNF $\alpha$  under certain circumstances leading to cell survival and inhibited under others with concurrent promotion of alternative pro-apoptotic signals (Kaltschmidt et al., 1999). TNF $\alpha$  has also been found to have a biphasic effect in neurons recovering from injury. It exerts a

damaging effect in the early post-traumatic (ischaemic) period, where deficits in memory retention were shown to be lower in *TNF $\alpha$* <sup>-/-</sup> mice as compared to their wildtype counterparts. In contrast to this, the absence of TNF $\alpha$  during the post-injury period may be deleterious as TNF $\alpha$  -deficient mice were seen to have increased levels of cortical tissue loss at this stage, when compared to wildtype mice (Scherbel et al., 1999). Differences in NF- $\kappa$ B activity may partially account for regional differences observed in response to TNF $\alpha$  under identical conditions (Galasso et al., 2000).

Further activators of NF- $\kappa$ B activity are members of the neurotrophin family of proteins. These have also been observed to have both neuroprotective and neurotoxic effects, depending upon the circumstances present.

For a more detailed discussion of TNF $\alpha$  activity see the introduction to chapter 4.

## 1.6 The Neurotrophic Hypothesis and Neurotrophins

### 1.6.1 The Neurotrophic Hypothesis

A number of extracellular stimuli play vital roles during the development and upkeep of the nervous system. In vertebrates an assortment of neurotransmitters and soluble neurotrophic factors are known to have significant influences on neuronal cell physiology. The actions of these factors are mediated by the binding of specific transmembrane receptors. Their effects include the stimulation of survival, proliferation and differentiation of developing neuroblasts and adaptive plasticity responses in the mature nervous system (Segal and Greenberg, 1996). This is demonstrated by nerve growth factor (NGF), which has a number of functions. For example, it is a differentiating factor for neural crest derived sensory neurons, sympathetic neurons and basal forebrain cholinergic neurons (Davies, 1994). It also acts as a proliferative factor for striatal neuroepithelial precursors, a regulator of process outgrowth and neuropeptide and neurotransmitter synthesis and possibly as an activity-dependant organiser of the visual cortex (reviewed in Eide et al., 1993; Davies, 1994; Averbuch, 1994; Thoenen, 1995; Lewin and Barde, 1996; Maina et al., 1998, Kalcheim and Gendreau, 1998). The characterisation of the intrinsic signal transduction pathways stimulated by the binding of the extrinsic factors to these receptors is beginning to elucidate how such diverse cellular responses as proliferation, differentiation and apoptosis can be induced in a

single cell type by varying the nature of the initial stimulus and how a given extrinsic factor can induce different responses depending on the cell type.

The “Neurotrophic Hypothesis” suggests that substances are present in the target field of developing neurons upon which these neurons are dependant for survival. These neurotrophic factors are present in limiting concentrations so that those neurons that do not get an adequate supply die by apoptosis (Cowan et al., 1984; Davies 1988a and b; Purves, 1988; Barde, 1989).

### **1.6.2 Nerve Growth Factor**

This hypothesis was based on studies of the first neurotrophin isolated, Nerve Growth Factor (NGF) (Cohen, 1960). Some of the earliest experiments in examining the effects of NGF were those in which early limb bud ablation in chick embryos was seen to result in the death of all the motoneurons that would normally have innervated the limb, whereas grafting an extra limb bud saved some of the 50% of neurons that would usually have died (Hamburger and Levi-Montelcini, 1949; Levi-Montelcini and Angeletti, 1968; reviewed by Oppenheim, 1991). NGF was initially isolated from a fraction of snake venom (Cohen and Levi-Montelcini, 1956), and was subsequently found to be abundant in the murine submandibular salivary gland (Cohen, 1960). The murine NGF amino acid sequence was determined (Angeletti and Bradshaw, 1971) leading to the cloning of the murine NGF cDNA (Scott et al, 1983) and the human,

bovine and chicken *ngf* genes (Ullrich et al., 1983; Meier et al., 1986, Ebendal et al., 1986). This showed NGF to be highly conserved throughout evolution, indicating its physiological importance.

Biologically active NGF consists of two hydrophobically linked glycosylated peptide chains produced by dibasic proteolytic cleavage of a 307 amino acid precursor polypeptide (Berger and Shooter, 1977; Scott et al., 1983; Edwards et al., 1988). Each chain contains 118 amino acids including six highly conserved cysteine residues (Greene and Shooter, 1980). In its crystal structure the monomer consists of a 'cysteine knot' of three interchain disulphide bonds and two anti-parallel pairs of  $\beta$ -strands connected by  $\beta$ -hairpin loops, creating a large flat surface that is covered in the dimer by the corresponding surface of the second monomer (Mc Donald et al., 1991). Other structurally related members of the neurotrophin family were later described. These include brain derived neurotrophic factor (BDNF) (Barde et al., 1982), Neurotrophin-3 (NT-3) (Ernfors et al., 1990a; Hohn et al., 1990; Jones and Reichardt, 1990; Maisonpierre et al., 1990a,b), NT4/5 (Berkemeier et al., 1991; Ip et al., 1992) and NT-6 (Gotz et al., 1994). All members of the neurotrophin family contain the highly conserved cysteine knot region by which they are identified. Tissue culture studies using explants or dissociated neuron-enriched cultures of sensory and sympathetic neurons indicate that each neurotrophin supports the survival of different, albeit overlapping, central and peripheral neuronal populations *in vitro* (Davies, 1994).



Experimental manipulation of NGF availability to sympathetic and certain sensory neurons of the developing murine embryo lead to the detection of its trophic (survival promoting) activity (reviewed by Eide et al., 1993). Neurons that are responsive to NGF include those of the DRG (Winick and Greenberg, 1965; Levi-Montelcini and Angeletti, 1968; Greene, 1977b; Barde et al., 1980), paravertebral sympathetic ganglia (Levi-Montelcini and Angeletti, 1968; Varon, 1975; Chun and Patterson, 1977; Greene, 1977a), the sensory neurons of the nodose ganglia (Forgie et al., 2000) and the murine trigeminal ganglion (Davies et al., 1981; Davies and Lumsden, 1984). Depriving these neurons of NGF leads to a decrease in neuronal survival, whereas if given at critical stages of development, the injection of exogenous NGF increases the survival of NGF target neurons, e.g., sympathetic neurons (Levi-Montelcini and Angeletti, 1963, 1968; Menesini-Chen and Levi-Montelcini, 1978, Johnson et al., 1980; Hamburger et al., 1981). This, combined with the finding that sympathetic and sensory neurons deprived of NGF die by apoptosis, further validated NGF's role as a neurotrophic factor (Deckwerth and Johnson, 1993; Crowley et al., 1994; Edwards and Tolkovsky, 1994). The application of anti-NGF antibodies results in the elimination of up to 85% of developing DRG neurons *in vitro* (Johnson et al., 1980), as well as a significant number of sympathetic neurons (Levi-Montelcini and Booker, 1960). *In vivo* evidence of a role for NGF in regulating neuronal survival comes from studies of *ngf* knockout mice that show reduced numbers of sympathetic and certain sensory neurons (Crowley et al., 1994; Davies, 1994; Lewin and Barde, 1996). As many as 70-80% of sensory neurons in

the DRG and the trigeminal ganglion are dependant on NGF during development (Lewin and Barde, 1996) and approximately 80% of the normal complement of DRG neurons are lost in *ngf* knockout animals. In addition, 70% of trigeminal ganglion and 95% of SCG neurons are eliminated (Crowley et al., 1994; Birling and Price, 1995). This indicates the importance of NGF in supporting developing neurons *in vivo*. The cell loss in the DRG of mice homozygous for null mutations in NGF is restricted to a subset of neurons that convey nociceptive and thermoceptive information and as a result these neurons fail to respond to noxious mechanical stimuli (Crowley et al., 1994).

Thus, the neurotrophic hypothesis is substantiated by this *in vitro* and *in vivo* evidence along with other experimental evidence (Chun and Patterson, 1977; Hamburger et al., 1981; Davies and Lindsay, 1985; Levi-Montalcini, 1987; Hartikka and Hefti, 1988; Barde, 1989; Albers et al., 1994; Davies, 1994). Another validation of the neurotrophic hypothesis is the fact that many NGF synthesising tissues have been demonstrated to be the target tissues of NGF responsive neurons (Korsching and Thoenen, 1983; Heumann et al., 1984; Shelton and Reichardt, 1984; Korsching et al., 1985). Additionally the final innervation density of the target regions is determined by the level of NGF synthesised (Harper and Davies, 1990) and the onset of NGF production in target tissues coincides with the initiation of target field innervation (Davies et al., 1986a; Korsching and Thoenen, 1988; Harper and Davies, 1990). Overall, NGF has clearly been demonstrated to be a target derived neurotrophic factor for sympathetic neurons and certain populations of neural crest-derived sensory neurons (Chun and Patterson, 1977; Gorin

and Johnson, 1980; Hamburger et al, 1981; Davies and Lindsay, 1985; Levi-Montelcini, 1987; Hartikka and Hefti, 1988).

### **1.6.3 Brain Derived Neurotrophic Factor**

BDNF was the second neurotrophin to be discovered (Barde et al., 1982). It is a 12kDa protein that is processed from a precursor polypeptide to a mature 119 amino acid glycosylated protein. BDNF was originally isolated from the porcine nervous system and has subsequently been cloned in the human, mouse and rat (Hofer et al., 1990; Jones and Reichardt, 1990; Maisonpierre et al., 1990b). It is 50% homologous to NGF, including the cysteine knot region (Leibrock et al., 1989). Biologically active BDNF forms a disulphide linked homodimer (Radziejewski et al., 1992). BDNF supports proprioceptive, enteroceptive and placode derived cutaneous sensory neurons (Davies et al., 1986a, b) and dopaminergic neurons of the substantia nigra (Hyman et al., 1991). It is the cutaneous sensory neurons of the ventrolateral region of the trigeminal ganglion that are supported by BDNF (Davies et al., 1986b). The enteroceptive neurons of the nodose ganglion contain a major sub-population of BDNF-supported neurons and a smaller sub-population of NT-3 dependent neurons (Hohn et al., 1990; Davies et al., 1986b; Lindsay et al., 1985a,b). In order for trigeminal neurons to survive in culture at the stage when their axons begin to grow (E10) the presence of BDNF is required (Enokido et al., 1999; Paul and Davies, 1995; Buchman and Davies, 1993). This requirement of certain trigeminal neurons for BDNF switches to NGF at later stages of

development and in the transition period the neurons respond to both neurotrophins (Enokido et al., 1999; Paul and Davies, 1995). Several populations of placode-derived sensory neurons do not undergo this switch in responsiveness. These include the ventrolateral section of embryonic chicken trigeminal ganglion (Buj-Bello et al., 1994). The onset of BDNF dependence in early sensory neurons is controlled by an intrinsic timing mechanism (Vogel and Davies, 1991, 1993) and BDNF mRNA is expressed in the peripheral trigeminal territory prior to the arrival of the earliest sensory axons (Arumae et al., 1993; Buchman and Davies, 1993). BDNF treatment has been demonstrated to decrease developmental cell death of DRG and nodose neurons from chick embryos (Hofer and Barde, 1988) and it is required for the maintenance of survival of nodose neurons during development (Davies et al., 1993; Lindsay et al., 1985b). The administration of BDNF to quail embryos during the period of cell death decreased the number of nodose neurons undergoing cell death (Hofer and Barde, 1988). Also, BDNF increases the survival of sensory and motoneurons after axotomy (Koliatsos et al., 1993; Vejsada et al., 1995; Yan et al., 1992) and enhances axonal regeneration of adult sensory neurons in vitro (Lindsay, 1988). Innervation density is also raised when BDNF is over-expressed in the target field of pre-ganglionic sympathetic neurons (Causing et al., 1997). These findings support the role of BDNF as a trophic factor in these regions.

BDNF mRNA expression is very low in the developing hindbrain, suggesting that the periphery is the primary source of neurotrophins for developing trigeminal neurons

(Wilkinson et al., 1996; Arumae et al., 1993; Buchman and Davies, 1993). In *bdnf* knockout mice there are severe deficits in cranial and spinal sensory neurons, including a 50% reduction in trigeminal neurons (Ernfors et al., 1994; Jones et al., 1994). BDNF has been shown to enhance the maturation of dorsal root ganglion (DRG) neurons by an autocrine mechanism (Wright et al., 1992). If BDNF activity is inhibited the number of surviving sensory neurons in the DRG is decreased by 35% (Acheson et al., 1995). DRG sensory neurons do not receive synaptic contacts from the periphery so BDNF produced by these cells apparently does not serve as target derived trophic support for an innervating neuronal population. It should be noted that these neurons receive multiple synapses from the central nervous system and signals via these may be effecting sensory neuron survival. Since some sensory neurons are known to express the BDNF receptor, TrkB, it has been proposed that BDNF is also acting in an autocrine or paracrine manner to support either these sensory neurons or neighbouring neurons respectively (Acheson et al., 1995; Wright et al., 1992; Ernfors et al., 1990a; Korsching, 1993). This mechanism of action differs from the classic pathway for neurotrophins that is utilised by BDNF to support the survival of neuronal populations.

The deviation from the classical neurotrophin pathway seen with sensory neurons lead to the suggestion that BDNF may be being transported anterogradely towards the targets of their axonal processes (Ernfors et al., 1990a), in contrast to the retrograde transport used for target derived neurotrophic support. It was found that BDNF is transported by sensory neurons towards both peripheral and central targets and that it is not simply

released along the nerve but transported to the target (Zhou and Rush, 1996). Thus, in the peripheral nervous system BDNF is utilised as both a classical target derived neurotrophin and an anterograde source of neurotrophin to neuronal target. The function of anterogradely transported BDNF is presently unknown but it has been suggested that it is involved in a number of processes, including the sensation or transmission of pain, synaptic transmission, inflammation, the response to denervation or to provide trophic support for TrkB expressing cells in the targets (reviewed by Tonra, 1999).

The neurotrophins produce their effects by binding to one or both of the two neurotrophin receptors, a discussion of which will follow.

## 1.7 The Neurotrophin Receptors

### 1.7.1 The Tyrosine Kinase (Trk) Receptors

Neurotrophins bind specifically to two types of transmembrane glycoprotein receptor. The first of these is the high affinity Trk (tropomyosin-related kinase) family of receptors, which are specific for the neurotrophins. The Trk family is composed of three related receptors TrkA, TrkB and TrkC (reviewed in Barbacid, 1995). It was demonstrated that neurotrophins assist transphosphorylation of Trk tyrosine kinases (Kaplan et al., 1991a,b; Klein et al., 1991a,b; Soppet et al., 1991). Neurotrophins also elicit a proliferative response from cell lines (Cordon-Cardo et al., 1991; Glass et al., 1991; Lambelle et al., 1991; Loeb et al., 1991; Squinto et al., 1991) and embryonic neurons (Allsopp et al., 1993b) transfected with Trk cDNAs. This indicates that Trk receptors play an important part in neurotrophin signalling. Neurotrophins bind differentially to the three receptors, TrkA is the preferred receptor for NGF and also binds NT-3 /5 (Bothwell, 1995; Hempstead et al., 1991; Kaplan et al., 1991a,b; Klein et al., 1991a). TrkB binds BDNF and NT-3/4/5 (Ip et al., 1992; Berkemeier et al., 1991; Glass et al., 1991; Klein et al., 1991b, 1992; Soppet et al., 1991; Squinto et al., 1991) and TrkC binds NT-3 (Lamballe et al., 1991). Analysis of germ-line mutations in each member of the neurotrophin and Trk families found that the presence of both the neurotrophin and its associated Trk receptor are essential in the development of the peripheral nervous system and also that the different neurotrophins and their receptors

support specific sub-populations of neurons (Concover and Yancopoulos, 1997). The description of TrkA as an NGF receptor followed the demonstration that non-neuronal cells transfected with TrkA acquired NGF binding activity and this binding stimulated intracellular signal transduction pathways (Allsopp et al., 1993b). The same has been shown to be true for cultured nodose and spinal motor neurons (Xu et al., 1994). Also the *trkA* gene was found to be highly expressed in NGF-dependent neurons in the mouse embryo by *in situ* hybridisation (Martin-Zanca et al., 1990).

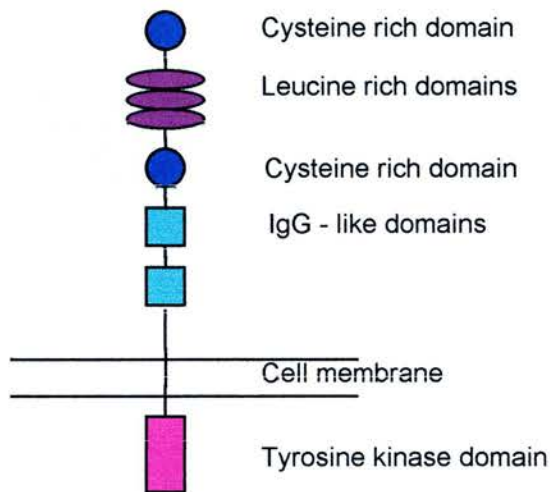
The Trk proto-oncogene locus was originally isolated from a human colonic carcinoma biopsy (Martin-Zanca et al., 1986; Martin-Zanca et al., 1989). The proto-oncogene encodes two tyrosine protein kinase isoforms of 790 and 796 amino acid residues known as TrkA and in this oncogene tropomyosin is fused to the kinase domain of the Trk (Martin-Zanca et al., 1989; Meakin et al., 1992; Barker et al., 1993; Horigome et al., 1993). The Trk proteins have a tripartite structure with an extracellular ligand recognition domain that contains two immunoglobulin-like regions that form a growth factor binding pocket, with the second region being responsible for binding.

Extracellularly there are also a series of cell adhesion motifs of three tandem leucine rich regions flanked by two cysteine clusters in their amino terminal (Urfer et al., 1998).

Trks also have a single transmembrane domain and a tyrosine kinase containing cytoplasmic domain for signal cascade initiation (see figure 1.13). The tyrosine kinase is thought to be activated by allosteric dimerisation (Eide et al., 1993-review).



Structural analysis has indicated that each neurotrophin binds to its given Trk receptor in the same conserved manner (reviewed in Kaplan and Miller, 2000).



**Figure 1.13: Representation of TrkA.** The receptor includes an extracellular ligand recognition domain that contains two immunoglobulin-like modules and an intracellular tyrosine kinase domain. Neurotrophins bind resulting in the dimerisation of the Trk receptor and activation of the tyrosine kinase domain.

### 1.7.1a Signalling Mechanisms Responsible for Trk Receptor Activity

Neurotrophin binding to Trk receptors results in receptor dimerisation, internalisation, retrograde transportation to the soma and kinase activation. *In vivo*, sensitive RNA and protein assays have demonstrated that NGF protein and mRNA are present in targets of sympathetic and sensory neurons and that in the somas of these neurons NGF protein but not mRNA is present, indicating that there is retrograde transport from the target areas (reviewed by Korsching, 1993). There are ten conserved tyrosines in the cytoplasmic domain of each Trk receptor, three of which constitute an autoregulatory loop. The phosphorylation of this loop further activates the kinase and the phosphorylation of the other amino acids promotes signalling by creating docking sites for adaptor proteins that

couple the receptors to intracellular signalling cascades (reviewed in Kaplan and Miller, 2000; Pawson and Nash, 2000). In some retinal ganglion neurons and spinal motoneurons, the TrkB receptor is apparently mainly sequestered in intracellular vesicles and a second messenger signal such as cAMP or Ca<sup>2+</sup> is necessary for efficient insertion of receptors into the surface membrane (Meyer-Franke et al., 1998). Transfer of Trk receptors to various membrane compartments has been demonstrated to regulate the efficiency of Trk interaction with the adaptor proteins and the subsequent activation of intracellular signalling cascades (York et al., 2000; Saragovi et al., 1998; Qian, et al., 1998). The ability of Trk receptors to activate specific signalling pathways is regulated by membrane trafficking as certain adapters and signalling molecules are localised to specific membrane compartments (Saragovi et al., 1998). In fact it has been demonstrated that the activation of certain signalling pathways is dependent upon the endocytosis of the neurotrophin-receptor complex (Zhang, Y. et al., 2000; York et al., 2000). Binding of NGF is followed by its internalisation and transport to the cell body (Hendry et al., 1974a, b; Campenot, 1977; Johnson et al., 1978). This indicates that Trk-mediated signalling is regulated by the specificity and kinetics of membrane transport.

Trk-receptor-mediated signalling has largely different consequences in different cells. In certain neurons, ligand binding of Trk receptors promotes survival (Huang and Reichardt, 2001; Sofroniew et al., 2001; Reichardt and Farinas, 1997). In other cells neurotrophins acting via these receptors induce mitosis, differentiation or apoptosis (Kim et al., 1999). Analysis of mice deficient in one or more SHC homologues

demonstrates that signalling pathways differ significantly between neuronal sub-populations (Sakai et al., 2000).

Essentially all peripheral sensory neurons express one of the Trk receptors at some stage during development and mutants deficient in these receptors lack specific sub-populations of these neurons, for example *trkA* knockout mice are deficient in nociceptive and thermoceptive neurons, whereas TrkC deficient mice lack proprioceptive neurons (reviewed in Huang and Reichardt, 2001; Reichardt and Farinas, 1997). In mice deficient for TrkB approximately 30-35% of the DRG neurons were lost (Klein et al., 1993). This is similar to the number of DRG neurons lost in BDNF deficient mice (Acheson et al., 1995) and BDNF has been found to act via TrkB to enhance the survival of retinal neurons during development (reviewed by Frade et al., 1999). Mice with null mutations in the *trkA* gene show the same neuronal deficiencies as those found in the *ngf* knockout mice (Lewin and Barde, 1996; Davies, 1994). They show loss of more than 90% of sympathetic ganglion neurons and 70% of trigeminal ganglion and DRG neurons at P0 (postnatal day 0) (Smeyne et al., 1994). Also the death of sympathetic neurons is significantly increased in the SCG of TrkA deficient mice (Fagan et al., 1996). This implies that it is TrkA that mediates the anti-apoptotic effects of NGF on developing neurons (see figure 1.15). The phenotypic defects seen in both NGF and TrkA deficient mice result from increased loss of NGF-dependent neurons including differentiated sympathetic neurons, as demonstrated by increased

levels of pyknotic nuclei in the sympathetic ganglia of mutants during the phase of naturally occurring cell death (Davies, 1994).

Three proteins have been identified by their specific binding to the phosphorylated Trk receptor. These are Phospholipase C  $\gamma$  (PLC $\gamma$ ), SHC and phosphatase inositol-3 kinase (PI3'K) (Dikic et al., 1995; Obermeier et al., 1993, 1994; Raffioni and Bradshaw, 1992; Soltoff et al., 1992; Vetter et al., 1991).

PLC $\gamma$  binds to the phosphorylated Trk receptor, is phosphorylated and catalyses the formation of diacylglycerol and inositol trisphosphate from phosphoinositol-4,5-bisphosphate. These second messengers stimulate protein kinase C and lead to raised cytoplasmic calcium due to release of intracellular stores. This is thought to result in cytoskeletal rearrangement and neurite outgrowth (reviewed by Patapoutian and Reichardt, 2001; Klesse and Parada, 1999).

SHC is an adapter protein, involved in the activation of the G-protein, ras and its subsequent signalling cascade. The ras pathway has been implicated in neurotrophin signalling in sensory and sympathetic neurons. Ras function blocking fragments prevent NGF from promoting sensory and sympathetic neuron survival (Borasio et al., 1993; Nobes and Tolkovsky, 1995). Activated ras can also promote the survival of these neurons in the absence of NGF *in vitro* (Borasio et al., 1989, 1996; Nobes et al., 1996). Furthermore the expression of dominant negative ras inhibits sensory neuron survival in

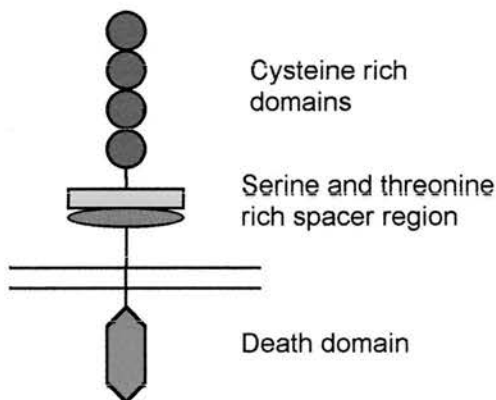
the presence of NGF and the expression of a constitutively active form of ras is sufficient for sensory neuron survival in the absence of neurotrophins (Klesse and Parada, 1998). This implicates the SHC activated ras pathway in the stimulation of NGF-induced cell survival.

The interaction of PI3'K with the Trk receptor is unclear. PI3'K has been found to complex with the activated Trk receptor although it is uncertain whether it associates directly (Raffoni and Bradshaw, 1992; Soltoff et al., 1992). PI3'K and its downstream effector the serine-threonine kinase Akt have been implicated in the mediation of cell survival (Dudek, et al., 1997; Hemmings, 1997; Kennedy et al., 1997; Kulik et al., 1997; Marte and Downward, 1997) and the inhibition of PI3'K activity in both sensory and sympathetic neurons by chemical inhibitors blocks neurotrophin induced survival (Crowder and Freeman, 1998; Klesse and Parada, 1998). Although in contrast, microinjection of activated forms of PI3'K or Akt inhibit neuron survival when NGF is present (Crowder and Freeman, 1998). Thus the role of PI3'K in neurotrophin signalling is unclear.

Thus the interaction of different proteins with Trk receptors may mediate a variety of different cellular functions of NGF.

### 1.7.2 The p75 Neurotrophin Receptor

The other transmembrane glycoprotein receptor that binds neurotrophins is p75. p75 is a common receptor for all the NGF family of neurotrophins (Radeke et al., 1987). The human *p75* gene was isolated using gene transfer assays followed by immunological detection of the transfected NGF receptor-expressing cells (Chao et al., 1986). The gene encodes a glycoprotein with a transmembrane domain consisting of 427 amino acids. 399 of these go to form the mature protein (Large et al., 1989; Radeke et al., 1987; Johnson et al., 1986). The p75 protein has a molecular weight of 75kDa, as indicated by its name and it was cloned from human melanoma cells (Johnson et al., 1986). A cluster of basic amino acids is responsible for the binding of ligands to the negatively charged p75. It is a tripartite structure consisting of four cysteine-rich extracellular domains, a short serine- and threonine-rich spacer region, and a cytoplasmic domain (Eide et al., 1993) (see figure 1.14).



**Figure 1.14: Representation of p75.** Includes the extracellular cysteine rich binding domains and the intracellular 'death domain'. On neurotrophin binding various intracellular proteins are recruited to the intracellular domain initiating a signalling cascade.

p75 caused confusion due to its relatively low affinity for NGF compared to TrkA, although importantly p75 does bind other neurotrophins with virtually exactly the same affinity with which it binds NGF (reviewed by Barrett, 2000). This binding of all the neurotrophins to p75 is not due the conservation of a single p75 binding domain on the neurotrophins. Different neurotrophins use residues at different spatial locations to bind to p75 (Ryden et al., 1995). This indicates that p75 binding of multiple neurotrophins serves a particular function although it is as of yet uncertain what that function is. It has been hypothesised that the presence of p75 on neurotrophin responsive cells may either assist the response of Trk receptors to their particular neurotrophin or that it may confer specificity on the Trk proteins (reviewed by Barrett, 2000). p75 has been shown to act as a co-receptor for the Trk tyrosine kinases and also to signal independently (Bothwell, 1996). A number of alternative roles for p75 in the regulation of the cell survival/death pathway have been proposed.

### **1.7.2a Cellular Responses to the p75 Receptor**

In neurotrophin-responsive neuronal populations, p75 and Trk receptors are often co-expressed, especially in the vertebrate peripheral nervous system. The p75 receptor has been found to promote TrkA activity (Horten et al., 1997; Barker and Shooter, 1994; Verdi et al., 1994), by increasing the rate of NGF binding to high-affinity binding sites by twenty five fold (Mahadeo et al., 1994) (see figure 1.18). In addition to this there is an increased rate of internalisation (Mahadeo et al., 1994). NGF responsiveness and the



ability to form high affinity sites are dependent on the relative levels of p75 and TrkA receptors (Verdi et al., 1994; Benedetti et al., 1993). Neuronal cell lines that express both TrkA and p75 receptors show enhanced TrkA autophosphorylation resulting in a faster differentiative response to NGF (Verdi et al., 1994). In *p75* knockout mice there are decreased levels of sensory and sympathetic innervation (Davies et al., 1993; Lee et al., 1992, 1994a) consistent with, but less severe than the phenotypes exhibited by NGF, BDNF, NT-3 and TrkA, TrkB and TrkC deficient mice (Snider, 1994). Sensory neurons from *p75* deficient embryonic trigeminal ganglion and neonatal sympathetic neurons require a four fold higher NGF concentration to promote their survival than wild type neurons. Although these neurons are fully capable of responding through TrkA when *p75* is absent they do so with significantly lower sensitivity (Lee et al., 1994b; Davies et al., 1993). Sympathetic neurons from neonatal *p75* knockout mice needed higher concentrations of NGF for survival than those of the wildtype at earlier developmental stages (Lee et al., 1994b). Also the inhibition of *p75* with a monoclonal antibody reduced TrkA activation (Barker and Shooter, 1994). All this infers that *p75* enhances the sensitivity of neurons expressing TrkA to NGF. Neuronal sensitivity is enhanced by increasing high-affinity TrkA binding sites and enhancing TrkA autophosphorylation in response to NGF (Davies et al., 1993; Eide et al., 1993; Lee et al., 1994a; Verdi et al., 1994). This was thought to occur either by *p75* forming a complex with TrkA that modifies ligand binding or by *p75* 'concentrating' NGF within the TrkA local environment. Responsiveness to NGF and the ability to form high affinity sites are dependent on the relative levels of *p75* and TrkA receptors present on the cell surface



(Casaccia-Bonnet et al., 1998-review). Other explanations of how p75 modulates Trk receptor function have been put forward. One theory suggests that the high-affinity state is due to p75 presentation of the ligand to the TrkA receptor (Barker and Shooter, 1994). Another predicts that a high affinity binding interaction is produced by the independent association of the ligand with p75 and TrkA (Chao and Hempstead, 1995). In this model conformational alterations in the receptors occur to facilitate ligand binding, as indicated by ligand-independent clustering and interactions between the two receptors (Ross et al., 1996). This is consistent with the hypothesis that p75 enhances TrkA function.

There is a further hypothesis that p75 acts as a constitutively active pro-apoptotic receptor. It was found that immortalised neural cell lines that over-expressed p75 had increased levels of cell death in response to serum withdrawal. The proposed mechanism of cell death was ligand independent apoptosis mediated by p75 and negated by the binding of NGF (Rabizadeh et al., 1993). This route of apoptosis was supported by the observation that following growth factor withdrawal in PC12 cells high levels of p75 expression enhanced susceptibility to cell death and the rate and extent of apoptosis was directly related to the level of p75 expression (Barret and Georgiou, 1996).

Likewise, the down regulation of p75 expression in neonatal DRG sensory neurons resulted in increased survival in the absence of NGF (Barret and Bartlett, 1994).

Marked increases in neuronal death during the development of sensory and sympathetic ganglia were found in transgenic mice expressing only the p75 intracellular domain

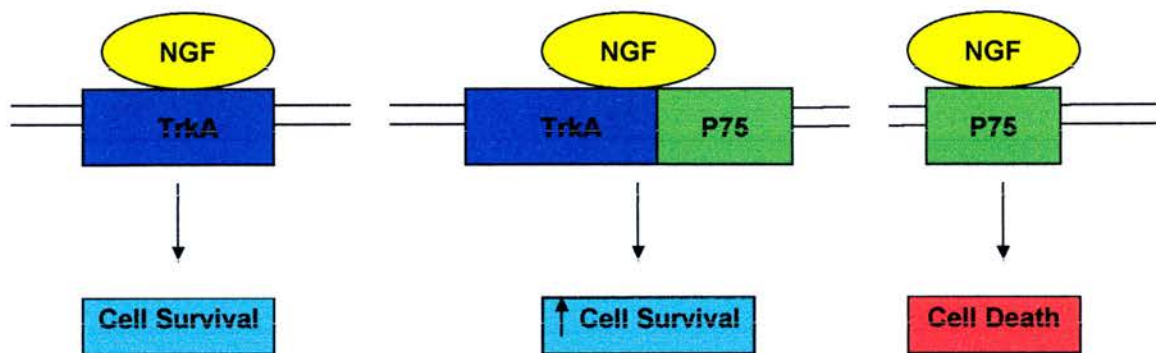
(Majdan et al., 1997), which were thus unable to bind NGF. Also cell death was increased in cells expressing p75 but not TrkA in the absence of NGF and a mutant PC12 cell line that did not express p75 had reduced levels of apoptosis (Rabizadeh and Bredesen, 1994).

Ligand dependent cell death is another proposed model for p75 activity. It is suggested that in cells co-expressing p75 and Trk, it is the Trk signal that predominates leading to cell survival, whereas in cells that lack specific Trk receptors each of the neurotrophins bind to p75, inducing cell death. Although there is only evidence from NGF responsive neurons to support the later part of this theory to date. During the development of the spinal cord and murine retina, which do not express TrkA, NGF binding to p75 initiates a death cascade (Frade and Barde, 1999, 1998a, b; Barrett, 2000). Accordingly, both anti-NGF antibodies and those that inhibit NGF binding by p75 caused a decrease in the number of these neurons undergoing apoptosis (Frade et al., 1996). Also detailed analysis of NGF and p75 deficient mice has shown that there is a decrease in the magnitude of cell death in these regions during development (Frade and Barde, 1999). In the developing chick isthmo-optic nucleus NGF induced apoptosis in TrkB and p75 expressing neurons, whereas BDNF and NT-3 stimulated survival (Von Bartheld et al., 1994). In these cells, antibodies against p75 inhibited NGF-induced cell death (Von Bartheld et al., 1996). It has also been demonstrated that cultured embryonic TMN neurons are killed in response to p75 binding of NGF, whereas BDNF produced a survival effect via TrkB receptors on these cells (Davey and Davies, 1998).

Motoneurons subjected to axotomy or nerve crush injury also showed increased cell death when treated with NGF (Sendtner et al., 1998; Terrado et al., 1998) and p75 has been known to be involved in the mediation of cell death following nerve injury for some time (Moix et al., 1991; Lee et al., 1995; Rende et al., 1995). Other cells shown to express p75 but not TrkA during development have been found to undergo cell death in response to NGF binding (see figure 1.15). These include DRG neurons (Barrett and Bartlett, 1994), neurons of the retina (Frade et al., 1996), cholinergic forebrain neurons (Yeo et al., 1997), Schwann cells (Carter et al., 1996; Khursigara et al., 1999) and oligodendrocytes (Casaccia-Bonnet, 1996). The apoptotic response in these cells was seen to require p75 binding of NGF as anti-p75 antibodies reversed the effect.

Both *in vitro* and *in vivo* studies have demonstrated p75 to be an inducer of programmed cell death (Bredesen and Rabizadeh, 1997; Carter and Lewin, 1997). It was observed that when early Schwann cells were exposed to NGF, there was a significant increase in apoptosis, which did not occur in p75 knockout mice (Soilu-Hanninen et al., 1999). Another study has supported the role of NGF as a mediator of cell death via p75, in motoneurons during embryonic development. Local administration of NGF to motoneurons reduced their survival to 2%, but the cell death was not enhanced in the same manner in p75-deficient mice (Weise et al., 1999). Also induced transgenic over-expression of p75 lead to increased apoptosis of motoneurons after axotomy of the facial nerve (Majdan et al., 1997). Further vindication came from a study which showed that exogenous NGF enhanced motoneuronal apoptosis by 37%

(Sedel et al., 1999). This pro-apoptotic effect was prevented by the addition of anti-p75 antibody as was the apoptosis observed after nerve injury (Ferri et al., 1998). This effect was not seen in response to the addition of other neurotrophins, (e.g., BDNF, NT3 and NT4/5). All this indicates that p75 can mediate NGF-induced cell death both during development and following nerve injury (Coulson et al., 2000), with the extent of neuronal death being dependent on the level of p75 expression. In these studies, p75 only mediated apoptosis when Trk receptors were not expressed, inactive or sub-optimally activated (see figure 1.15). This indicates that Trk receptor activation can silence p75 apoptotic signalling. Robust Trk activation inhibited p75-mediated death of sympathetic (Bamji et al., 1998) and trigeminal mesencephalic sensory neurons (Davey and Davies, 1998) and the expression of exogenous TrkA in oligodendrocytes (Yoon et al., 1998) or TrkB in sympathetic neurons (Atwal et al., 2000) inhibited NGF- and BDNF-induced apoptosis, respectively. During the postnatal period of naturally occurring sympathetic neuronal apoptosis deficiencies in p75 dramatically reduced apoptosis (Bamji et al., 1998), whereas a lack of TrkA resulted in the death of the majority of sympathetic neurons (Smeyne et al., 1994). This indicates that developmental apoptosis is to some extent due to constitutive receptor-mediated death signals that must be inhibited by sequestration of the appropriate pro-survival factor.



**Figure 1.15: Summary of actions of NGF in cells expressing different combinations of NGF receptors.** In cells expressing only TrkA NGF binding induces cell survival. If p75 is co-expressed with TrkA this cell survival is enhanced, but if p75 is expressed alone NGF binding induces apoptosis.

### 1.7.2b Signalling Mechanisms Responsible for p75 Receptor Activity

p75 shares a high degree of homology with the TNF super-family of receptors which includes the TNF and Fas receptors. All of these receptors have a death domain, which is a region of approximately 75 amino acids at or near the C-terminus. In many of the proteins containing it this region is involved in the initiation of apoptosis. The extracellular homology is based on the expression of a variable number of cysteine-rich loop regions, which are involved in ligand binding. It should be noted however that p75 differs from most other members of this family of receptors in several important respects. It is a soluble rather than membrane-bound factor, its ligand does not trimerise on binding, and it is not highly expressed in cells of the immune system or tumour cells (reviewed by Barrett, 2000). The only protein involved in TNFR1 and Fas signalling that has been unequivocally linked to p75 signal transduction is NF- $\kappa$ B. FADD does not have any affinity for the intracellular domain of p75, despite their shared death domains

(Coulson et al., 1999). In fact none of the known death domain proteins have been found to interact with p75 (Nichols et al., 1998) and nuclear magnetic resonance (NMR) has shown p75 conformation to be subtly different from other members of the TNF receptor family. p75 is the only member of the TNFR1 family in which the death domain does not self-associate *in vitro* (Liepinish et al., 1997). A number of other proteins not associated with apoptosis have been found to contain cytoplasmic domains homologous to the death domain (Feinstein et al., 1995). Thus, it has been proposed that there are two subtypes of death domain. Subtype 1 is the established death inducing death domain of TNFR1 and Fas, whereas p75 and other proteins containing death domains of unknown significance are classified as subtype 2. Truncated p75 that lacks the death domain has been shown to induce apoptosis (Coulson et al., 1999), indicating that p75 is still involved in inducing cell death but not through its death domain. The death domain may conceivably have a role in the regulation of apoptosis.

A protein of the TRAF family, TRAF6 was found to bind to the intracellular domain of p75 and the association was facilitated by high concentration of NGF (Khursigara et al., 1999). TRAF6 is expressed in neural tissues (Ishida et al., 1996). The binding site for p75 was mapped to the juxtamembrane region of the p75 cytoplasmic domain (Khursigara et al., 1999). This region has been implicated in signalling by a study in which microinjection of a p75 cDNA lacking the whole cytoplasmic domain expressed in sensory neurons of neonatal mice did not induce apoptosis, whereas both full-length and truncated versions containing the juxtamembrane region did (Coulson et al., 1999).



Deletion of the death domain did not reduce apoptosis, but deletion of the juxtamembrane region prevented it (Coulson et al., 1999). This implicates the juxtamembrane region and not the death domain in the induction of the cell death pathway by p75.

In contrast to this, a recent report has suggested that cell death signalling through the death domain of p75 is possible. In immortalised striatal neurons p75 was found to induce apoptosis through its death domain in a NGF independent manner. A p75 construct deficient in the death domain dominantly interfered with p75 signalling, implying that receptor multimerisation is required for this process. FADD, TRADD and apical caspase 8 were found not to be involved in this process although caspases 3,6 and 9 were activated (Wang et al., 2001). This indicates that p75 signals for cell death through a similar pathway as the other death receptors but that it uses different adaptors and apical caspases.

Further proteins have been proposed to interact with p75 and be involved in its induction of apoptosis. A protein called NADE (p75NTR-associated cell death executor) has been demonstrated to specifically bind to the cell death domain of p75. The co-expression of NADE and p75 induced caspase -2 and -3 activities and the fragmentation of nuclear DNA in oligodendrocytes, PC12, and other cell populations, whereas in the absence of p75, NADE failed to induce apoptosis, indicating that its activity is important but not sufficient for p75 mediated apoptosis. NADE activity was seen in response to NGF but

not any other neurotrophin (Mukai et al., 2000). 14-3-3 was seen to bind NADE *in vivo* and the transient expression of a mutant form of 14-3-3 which still associated with NADE, inhibited NGF-dependent p75/NADE mediated apoptosis (Kimura et al., 2001). This suggests that 14-3-3 plays an important part in the modulation of this pathway. A p75 binding protein called NRAGE (neurotrophin receptor interacting MAGE homologue) has been identified by hybrid analysis. NRAGE was found to bind to p75 *in vivo* and *in vitro*, and it associates with the plasma membrane when p75 binds NGF. It inhibits the interaction between p75 and TrkA and TrkA over-expression eliminates NRAGE mediated, NGF-dependent apoptosis, indicating that these proteins bind to p75 exclusively. NRAGE over-expression facilitates cell cycle arrest and allows NGF-dependent cell death in sympathetic neuronal precursors (Salehi et al., 2000). This indicates that NRAGE contributes to p75-mediated apoptosis. NRIF is a ubiquitously expressed protein that interacts with p75. Mice deficient in NRIF showed a deficit in embryonic retinal apoptosis (Casademunt et al., 1999). This deficit was similar to that found in the *ngf*<sup>-/-</sup> and *p75*<sup>-/-</sup> mice (Frade et al., 1996; Frade and Barde, 1999). This indicates that NRIF is involved in the p75-mediated apoptotic response to NGF. There is a large part of the p75 signalling pathway that has yet to be elucidated and a number of proteins may be involved in the p75 response to neurotrophin binding.

A number of signalling mechanisms have been proposed as being initiated in response to p75 activity. It was found that ceramide is released by hydrolysis of the phospholipid sphingomyelin in the cell membrane. Intracellular ceramide levels have been found to



be raised twofold after TNF stimulation in cells expressing TNFR1 (Wiegmann et al., 1994) and Fas stimulation of lymphoblastoid cell lines (Tepper et al., 1995). Because of this it was proposed that ceramide is involved in the transduction of TNFR1 and Fas stimulated responses, including apoptosis. In the last few years the route by which TNF and Fas activate apoptosis machinery has been defined in detail (as described previously) and has been found not to include or require ceramide. It is possible that ceramide is activated collaterally to the main signalling pathways and can exert a modulatory influence. Ceramide has a number of targets including ceramide-activated protein kinase (CAPK), a raf kinase (Zhang et al., 1997). Raf is a kinase suppressor of ras, which is involved in one of the key second messenger pathways and is activated by Trk proteins. Ras is often associated with responses such as cell proliferation and can inhibit apoptosis (Nobes and Tolkovsky, 1995; Yan and Greene, 1998). NGF has been shown to raise ceramide levels in T9 glioma cells, which express p75 but not Trk A (Dobrowsky et al., 1994) and NGF, BDNF, NT-3 and NT-4 induced sphingomyelin hydrolysis in fibroblasts transfected with p75 but not expressing Trk receptors (Dobrowsky et al., 1995). NT-3, but not NGF, induced sphingomyelin hydrolysis in PC12 cells, suggesting that the presence of TrkA signalling inhibited p75-dependent sphingomyelin hydrolysis in response to NGF. In oligodendrocytes expressing p75 but not TrkA, NGF induced ceramide generation and was accompanied by cell death (Casaccia-Bonnet et al., 1996). A large body of evidence has also emerged against ceramide generation being the sole route of apoptosis mediation. The increased intracellular concentrations of ceramide that follow receptor activation do not always

result in cell death and apoptosis may also be inhibited in certain sympathetic neurons (Nair et al., 2000) and motor neurons (Irie and Hirabayashi, 1999) deprived of NGF following ceramide treatment. This combined with the demonstration that Bcl-2 acts downstream of ceramide accumulation preventing cell death (Zhang et al., 1996), implies the possibility that ceramide does not function as the sole endogenous activator of apoptosis. Both ceramide and the ceramide analogues C2- and C6-ceramide significantly increased the survival of sensory neurons from neonatal mice (Ping and Barrett, 1998). But this survival effect of ceramide was reversed by ceramidase inhibition suggesting that it is ceramidase-mediated conversion of ceramide to sphingosine that leads to increased survival in sensory neurons. When ceramidase is inhibited, sphingosine is not formed and the ensuing apoptosis may have been due to ceramide accumulation which under normal circumstances would have been metabolised to sphingosine (Ping and Barrett, 1998). This indicates that ceramide is unlikely to induce physiological cell death *in vivo*.

There have been a number of reports associating the signalling protein JNK to apoptosis and p75 activity. Knockouts of *jnk-1*, *-2* and *-3* support a role for these kinases in apoptosis. The *jnk-1/-2* double knockout affected developmental apoptosis with decreases in cell death in the hindbrain and increases in the forebrain (Kuan et al., 1999). The *jnk-3* knockout showed reduced glutamate neurotoxicity and associated apoptosis in the hippocampus (Yang et al., 1997). JNK was seen to be essential for apoptosis in motoneurons and the neuroblastoma cell line PC12 following growth factor

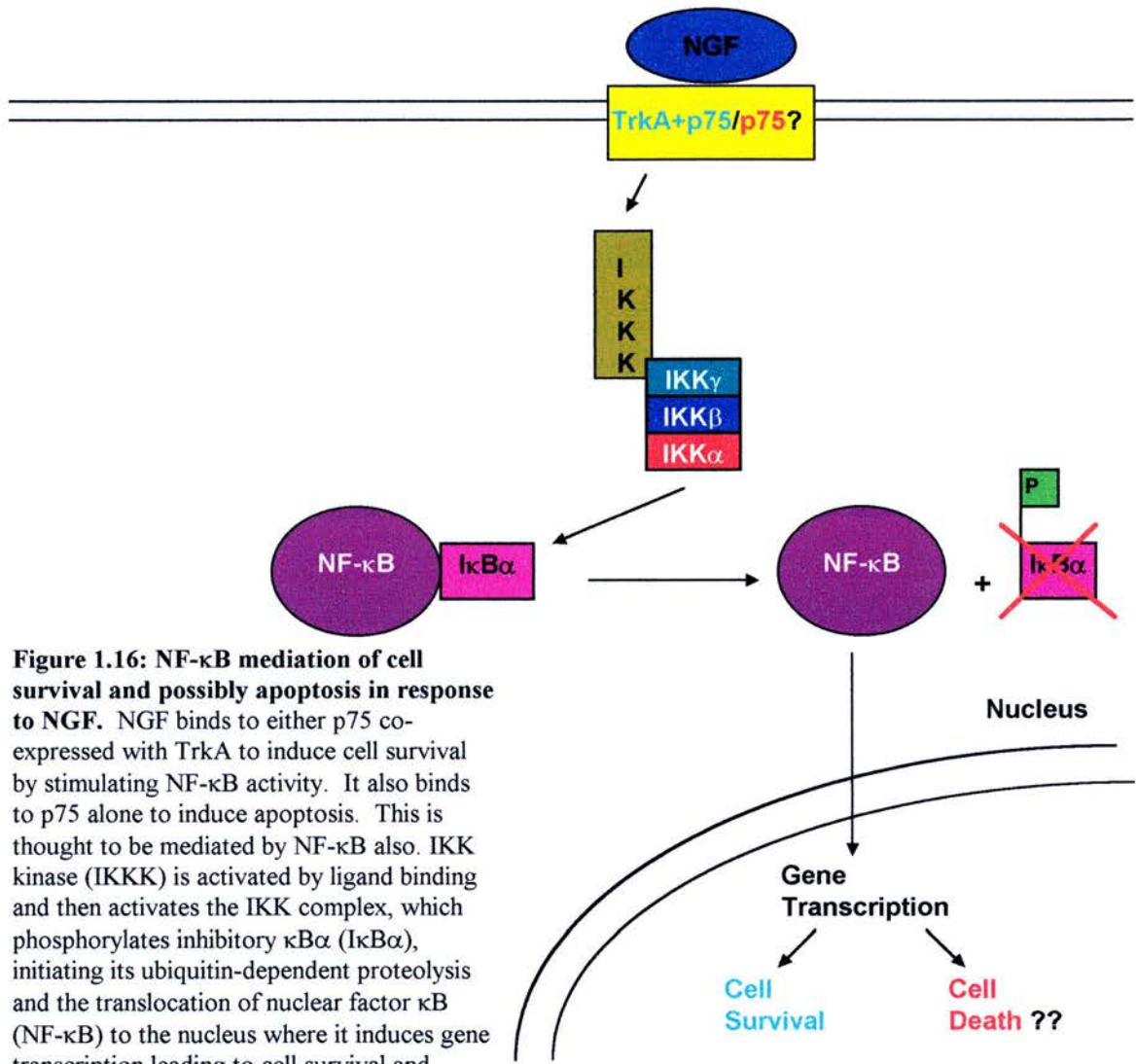
deprivation (Maroney et al., 1998). In neonatal rat oligodendrocytes that express p75 and not TrkA, binding of NGF to p75 induces JNK activation and apoptosis. Also NGF-induced apoptosis in oligodendrocytes was inhibited by an alkaloid that blocks the JNK pathway (Yoon et al., 1998). In these cells when TrkA expression was introduced retrovirally NGF treatment resulted in the suppression of JNK activity and prevented p75-mediated apoptosis (Yoon et al., 1998). However, in adult human oligodendrocytes NGF did not induce JNK activity or apoptosis (Ladiwala et al., 1998). In sympathetic neurons, which express both p75 and TrkA NGF did not induce JNK activity, however BDNF induced both JNK activity and apoptosis in the presence of NGF. As these cells do not express TrkB this indicates that it was a p75 mediated process (Bamji et al., 1998). The inability of NGF to over-ride JNK activation and prevent BDNF-induced apoptosis in cells expressing TrkA is not consistent with the finding that NGF binding to transfected TrkA prevented JNK activation and apoptosis in oligodendrocytes (Yoon et al., 1998). However very high levels of BDNF were required to induce apoptosis in the presence of low concentrations of NGF. In PC12 cells undergoing NGF withdrawal, JNK activity occurred prior to cell death (Xia et al., 1995). These findings suggest that JNK activation may be a common feature of apoptosis induced by either NGF withdrawal or p75 signalling. However, after NGF withdrawal a number of other factors are influential and there remains controversy as to whether JNK activity is central to determining cell death.

The JNK-p53-bax pathway has been found to be activated in sympathetic neurons both by p75 activation and by NGF withdrawal (Aloyz et al., 1998). JNK-mediated activation of c-jun has been shown to be vital for NGF withdrawal induced apoptosis of sympathetic neurons (Davies R.J., 1999; Ellers et al., 1998; Ham et al., 1995; Estus et al., 1994). p53 is also important for both p75-mediated and NGF withdrawal-induced sympathetic neuronal death. The over-expression of p53 is sufficient to cause the apoptosis of sympathetic neurons when NGF is present (Slack et al., 1996). The levels of p53 were increased when sympathetic neurons died in response to either p75 activation or NGF withdrawal and if the increase in p53 was inhibited then the apoptosis was prevented (Aloyz et al., 1998). It was demonstrated also that sympathetic neurons from p53 deficient embryos have enhanced survival in NGF-deficient culture (Vogel and Parada, 1998). In *p53*<sup>-/-</sup> mice sympathetic neuron apoptosis was delayed (Aloyz et al., 1998). It was found that the activation of JNK pathway lead to increased levels of p53 and p53-dependent apoptosis of sympathetic neurons (Aloyz et al., 1998). This indicates that the JNK-p53-bax pathway may be highly involved in p75 mediated apoptosis. Although the mechanism by which p75 induces this pathway is still uncertain. In sympathetic neurons, the activation of TrkA inhibits the JNK-p53 pathway via ras (Mazzoni et al., 1999) and exogenous TrkA inhibits JNK activation in oligodendrocytes and PC12 cells (Yoon et al., 1998).

The activation of NF- $\kappa$ B is generally considered as an anti-apoptotic signal that is found in TNFR1 signalling in response to TNF $\alpha$ . The binding of NGF to p75 can also lead to

the nuclear translocation and activation of NF- $\kappa$ B. This was first demonstrated in rat schwann cells which express p75 but not TrkA (Carter et al., 1996). It was also shown that NGF can cause NF- $\kappa$ B activation in PC12 cells which are transfected with p75 (Carter et al., 1996). This activation was not seen in cells from p75 deficient mice and was not induced by the binding of either BDNF or NT-3. NF- $\kappa$ B was activated in rat schwannoma cells that express p75, but not TrkA in response to NGF exposure (Gentry et al., 1998). It was found that p75 caused the activation of NF- $\kappa$ B in sensory neurons (Kimpinski et al., 1999). This p75-mediated activation of NF- $\kappa$ B is not inhibited by co-expression of active TrkA (Yoon et al., 1998), suggesting that NF- $\kappa$ B activation is part of a pro-survival pathway that is mediated by p75 and collaborates with Trk. In cultured sympathetic neurons and PC12 neurons which both co-express TrkA and p75NTR, NGF has been shown to stimulate NF- $\kappa$ B (Wood, 1995; Maggiwar et al., 1998) and apoptosis resulted from the inhibition of NF- $\kappa$ B in both these cell types (Tagliatela et al., 1997; Maggiwar et al., 1998), indicating that it is important for NGF-mediated survival. NF- $\kappa$ B activation has also been seen to inhibit TNF-induced cell death. It also has a protective effect against the action of various genotoxic agents in lymphoid and fibroblast cell line (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996) and against oxidative stress-induced apoptosis in hippocampal neurons (Mattson et al., 1997). p75 activation of NF- $\kappa$ B has been proposed to mediate NGF induced cell survival in trigeminal neurons, which express TrkA (by Hamanoue et al., 1999).

In neonatal rat oligodendrocytes p75 activation of NF- $\kappa$ B induces cell death (Yoon et al., 1998). This was not seen in adult human oligodendrocytes where NGF binding to p75 results in NF- $\kappa$ B activation and nuclear translocation but not apoptosis (Ladiwala et al., 1998). p75-mediated, NF- $\kappa$ B activation has also been suggested to be the route of transduction of the cell death signal induced by NGF in rat Schwann cells. Thus it is thought that NF- $\kappa$ B has both pro-apoptotic (Schneider et al., 1999) and anti-apoptotic (Mattson et al., 1997; Tagliabatella, et al., 1997; Maggiwar et al., 1998) activities, depending on the cell type and whether or not TrkA is expressed (see figure 1.16). NF- $\kappa$ B can be seen to have dual functions as a mediator of both cell survival and cell death depending upon the cell type and the extracellular stimuli that bind to certain receptors on the cell surface.



**Figure 1.16: NF- $\kappa$ B mediation of cell survival and possibly apoptosis in response to NGF.** NGF binds to either p75 co-expressed with TrkA to induce cell survival by stimulating NF- $\kappa$ B activity. It also binds to p75 alone to induce apoptosis. This is thought to be mediated by NF- $\kappa$ B also. IKK kinase (IKKK) is activated by ligand binding and then activates the IKK complex, which phosphorylates inhibitory  $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ ), initiating its ubiquitin-dependent proteolysis and the translocation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) to the nucleus where it induces gene transcription leading to cell survival and possibly cell death.



## 1.8 Hypotheses

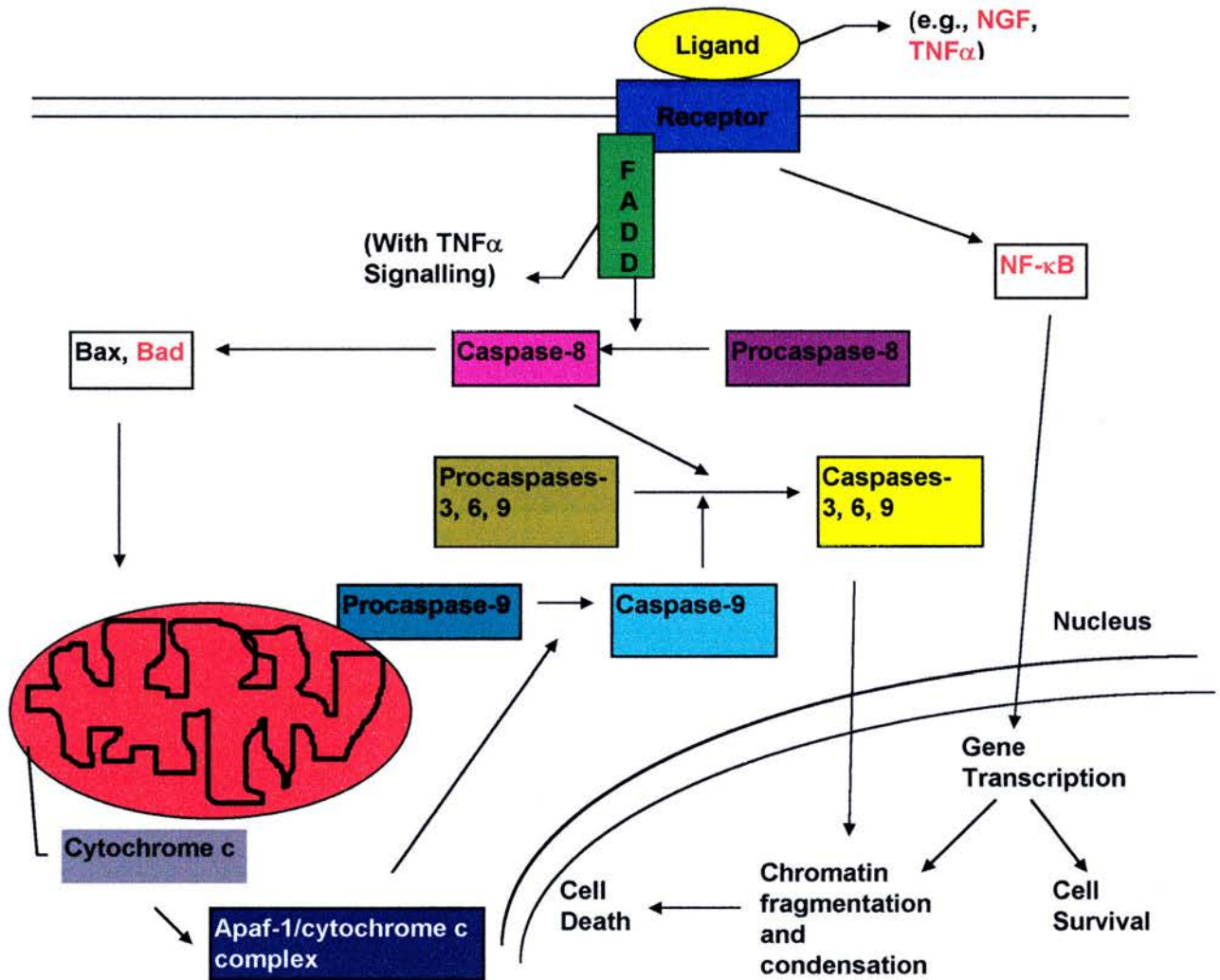
In this thesis I will examine the universality of the finding that NF- $\kappa$ B mediates NGF induced cell survival in neurons that express TrkA (Hamanoue et al., 1999). I will also investigate the hypothesis that NF- $\kappa$ B is mediating apoptosis induced by NGF binding to p75 in the retina and spinal cord (Frade and Barde, 1999), two regions in which TrkA is not expressed.

There is much evidence that TNF $\alpha$  can have both pro- and anti-apoptotic effects within the nervous system. Although there is growing knowledge of the damaging and protective effects of TNF $\alpha$  in cytotoxic states, little is known about the effects of TNF $\alpha$  on neuronal survival during the development of the nervous system. For this reason I will examine the involvement of TNF $\alpha$  in the regulation of cell death and survival during development.

Bad is known to be widely expressed in the peripheral nervous system (Merry and Korsmeyer, 1994), suggesting a role for it in the nervous system. The over-expression of Bad in NGF-supported sympathetic neurons induced apoptosis (Hamner et al., 2001). The role of Bad in the cell death cascade in peripheral neurons suggested by these



findings remains to be fully elucidated. I examined the hypothesis that Bad is involved in mediating cell death during the development of these areas. See figure 1.17.



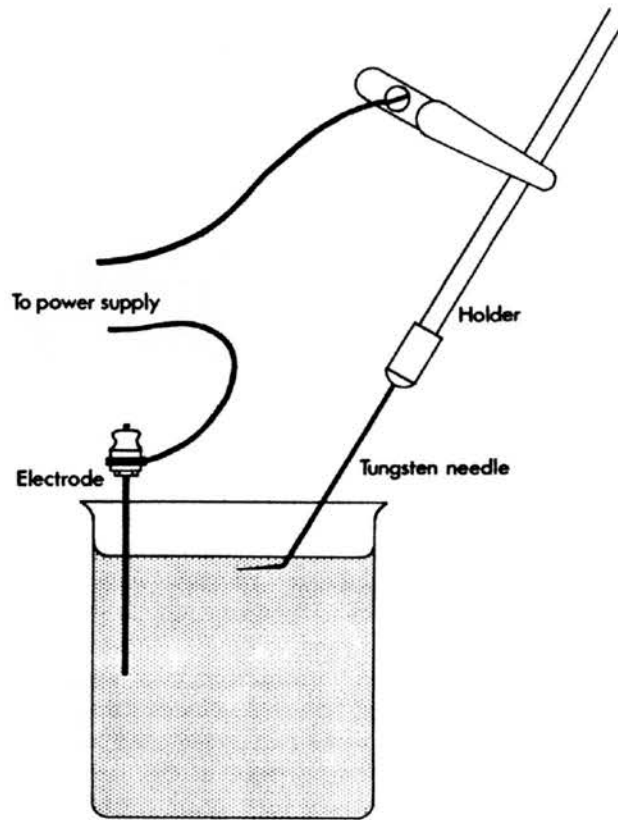
**Figure 1.17: Overview of cell death and survival pathways during development.** Ligand binding (e.g., NGF or  $\text{TNF}\alpha$ ) leads to the activation of  $\text{NF-}\kappa\text{B}$  mediated cell survival, Bcl-2 family controlled release of mitochondrial components and the induction of caspase mediated cell death or Death Domain (e.g., TRADD and FADD) induction of the caspase cascade and ultimately cell death. Components under examination in this thesis are highlighted in red.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Dissection Techniques

#### 2.1.1 Instruments

A number of instruments were used in the course of each dissection including toothed forceps, straight and curved watchmaker's forceps, and fine scissors. Instruments were sterilised by flaming after soaking in 100% analar grade ethanol. Tungsten needles were used for the final stages of dissection and to remove adherent mesenchymal tissue and nerves from ganglia. Tungsten needles were made from 0.5mm diameter tungsten wire which was cut to 3 to 5cm lengths and the terminal 1cm was bent at an angle of approximately  $60^{\circ}$ . This end was immersed for a few minutes in 0.5M potassium hydroxide and a 3 to 12v AC current passed through the wire as shown in figure 2.1. The tungsten was electrolytically etched away to form a taper from the bend to the tip of the needle. To make the point sharp, the needle was held vertically in the solution for the last 30 seconds (figure 2.1). After the needle was made it was washed in water to remove the alkali. For dissection, the needles were held in chuck-grip platinum wire holders and sterilised by flaming. Flaming also strengthens the needle.



**Figure 2.1: Making tungsten needles** (from Davies, 1995)

### **2.1.2 Isolation of Mouse Embryos**

The species used was *Mus musculus* with a congenic background strain of CD1 or in the case of the TNF transgenic colony C57. The collection and fixation of samples were carried out as quickly as possible to minimise tissue degradation. For embryo collection, the mother was killed by cervical dislocation, or exposure to carbon dioxide in a rising concentration followed by cervical dislocation, at the required stage of gestation. The precise stage of development of the embryos was determined by the

criteria of Theiler, (1972). The neck was dislocated gently as using too much pressure forces blood into the embryos which can cause problems in later procedures.

The uterine horns were removed from the abdomen of the mother and transferred to a petri dish containing fresh phosphate buffered saline (PBS) for histology or L15 medium for tissue culture. Embryos embryonic day 15 (E15)<sup>4</sup> and older were removed from the uterine horns by making a single continuous incision along the anti-mesometrial border of each uterine horn exposing the embryos within their membranes. These membranes were then removed with fine forceps. Younger embryos were removed by making a small hole in the musculature of the anti-mesometrial border for each embryo. The embryo, including the extra-embryonic membranes, was then extruded through this hole due to contraction of the remaining uterine muscles. Again the membranes were removed using fine forceps.

After removal from the uterine horns the embryos were transferred to fresh PBS or L15, to wash off blood on the outside of the embryos. This reduced the risk of contaminating tissue for genotyping, where appropriate, with maternal blood. A single incision was made using a tungsten needle anterior to the forelimbs in those which the retina, sympathetic and sensory ganglia were to be examined and posterior to the forelimbs in those which the spinal cord was to be studied.

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<sup>4</sup> E0 is defined as the day that the plug is formed, indicating pregnancy.

## 2.2 Transgenic Mice

Mice with a null mutation in the *p65* gene, the *ikk $\alpha$*  gene and the *ikk $\beta$*  gene were received from Michael Karin (the Laboratory of Gene Regulation and Signal Transduction, Dept. Pharmacology, University of California). The colonies of p65, IKK $\alpha$  and IKK $\beta$  mice were derived from a series of heterozygous crosses whilst the mice were in rabies quarantine. At least ten back-crosses were carried out before the mice were used in experiments to ensure purity of strain. The background strain was CD1.

Mice heterozygous for a null mutation in the *ngf* gene and the *tnf $\alpha$*  gene were purchased from Jackson Laboratories. The NGF and TNF $\alpha$  colonies were derived from a series of heterozygous crosses whilst the mice were in rabies quarantine. At least ten back-crosses were carried out before the mice were used in experiments to ensure purity of strain. The background strain of NGF mice was CD1, whereas that of TNF $\alpha$  mice was C57/BL6 (C57). Experiments on TNF $\alpha$  mice with background strains of both CD1 and C57 were compared to ensure that background had no influence on the results. It was found that background strain was not influential.

The NGF/p65 colony was derived from a series of crosses between *ngf* and *p65* heterozygous mice. At least ten back-crosses were carried out before the mice were

used in experiments to ensure purity of strain. The background strain of both NGF and p65 mice was CD1.

Mice with a null mutation in the *bad* gene were received from Stanley Korsmeyer, (Howard Hughes Medical Institute). The Bad colony was derived from a series of heterozygous crosses whilst the mice were in rabies quarantine. At least ten back-crosses were carried out before the mice were used in experiments to ensure purity of strain. The background strain was CD1.

By the constraints of our Home Office project animal licence, the mice could not be genotyped until they were at least 4 weeks old. At this age, the mice were sedated, using halothane and 1.5cm were clipped from the tip of the tail and transferred to a labelled 1.5ml microfuge tube. An ear tag (IMS) was then fitted so that individuals could be identified. Heterozygotes were used for matings both for experiments and to supplement breeding stock. Wild type individuals were eliminated. Homozygous null mutations are not viable in the p65, NGF/p65, IKK $\alpha$ , IKK $\beta$  and NGF colonies. *tnf $\alpha$* <sup>-/-</sup> and *bad*<sup>-/-</sup> mice were not used in this study as heterozygous crosses give wildtype and knockout age matched litter-mates.

All animal work was approved by our institutional animal use committee and by the Home Office.

## **2.2.1 Drawbacks in the Use of Transgenic Mouse Models**

### **2.2.1a Background strain**

The background strain that the transgenic mice are bred into may be influential on the effects that the null mutation has. Extensive study of the null mutations that have been used in these experiments bred using a variety of background strains may reveal different results. The need for extensive matings of mice heterozygous for the null mutations to achieve a pure strain with all the different available background strains precludes the inclusion of this kind of background check in this study.

### **2.2.1b Redundancy**

One of the main problems encountered when using transgenic mouse models is the possibility of redundancy of genes that belong to the same family or parallel pathways in the system under examination. Two genes can be defined as redundant if each can partially or totally substitute the function of the other (Thomas, 1993). Even if only the clearly established cases are considered genetic redundancy is a common phenomenon (Thomas, 1993). This redundancy may be at the level of (a) a functional redundancy of another member, (b) an increase in the expression of a related member or (c) a change in catalytic activity. It is difficult to determine whether proteins have been up-regulated to compensate for the removal of the activity of the gene that has been knocked out

(Shastry, 1998). Alternatively the removal of a certain gene may remove an inhibitory action of its protein product on certain other proteins leading to the activation of processes that would normally be under inhibitory control. To an extent it is difficult to be certain whether the effects seen in mice with null mutation are due to the direct effects of the gene that has been knocked out or whether indirect or compensatory effects are influential. A fairly rigorous test for functional redundancy is to determine whether there are additional abnormalities in the double mutant when compared with each single mutant (Thomas, 1993).

Also a given gene often has multiple functions. Thus, simple inactivation of a gene has limitations in demonstrating its complete spectrum of functions. For some genes an early lethal phenotype prevents the examination of its later roles, for others, the phenotype affects multiple cellular events, hindering the study of individual functions.

Since a single gene alteration affects the expression of many other genes it is possible that a simple gene knockout may alter the entire developmental program of an organism. The knockout mice do not reveal which of these genes are affected and hence the interpretation could be misleading. Thus, in knocking out a gene in order to evaluate the consequences to the organism of not having the gene product, it may be found that those consequences are complex and affect multiple pathways.



Also with knockout mice the proteins under examination are knocked-out not just at the given age of interest but at all stages of development as inactivation of the gene is limited to irreversible alteration of the chromosomal DNA. Thus, it is possible that the effects seen are not due to the removal of the protein at the stage under examination but that it is due to the influence of earlier effects of the removal of the protein's activity. A way of overcoming this is the use of conditional knockouts. This is a strategy that combines homologous and site specific recombination and allows the creation of conditional genome alterations by controlling their onset, frequency, spatial location and tissue/cell type specificity (Lobe and Nagy, 1998). Conditional knockouts can be used in adult animals or late in development in order to bypass embryonic lethal phenotypes such as that of p53. Alternatively cells in the nervous system may be specifically targeted for null mutation in the genes of interest thus eliminating the lethal effects of null mutation in other areas, (e.g., in the liver of p53 knockout mice).

Despite the drawbacks the identification of selected mutations in whole animals is the most informative way to begin clarifying and separating the steps by which biological processes are carried out. Also the need to understand processes that occur only in complex organisms, such as the development of the nervous system, means that analysis must be pursued in those organisms. The mouse is an ideal mammal for transgenic study as it is small, prolific, and is a very good analogue for most human biological processes.

Alternative methods of examining these systems are over-expression analysis and western blot analysis for the examination of downstream signalling pathways.

Unfortunately at the ages of interest the cells were too small for microinjection and there is not enough tissue in the sensory and sympathetic ganglia therefore these techniques were not possible.

## 2.3 Genotyping

### 2.3.1 DNA Extraction

Tissue samples taken at the time of dissection and following tail tip removal were digested and DNA isolated using the Nucleospin™ C+T kit. This method allows genomic DNA to be prepared from cells and tissues. Lysis is achieved by incubation of the sample material in a proteinase K/SDS solution. Appropriate conditions for binding of DNA to the silica membrane in the tissue columns are created by the addition of chaotropic salts and ethanol lysate. The binding process is reversible and specific to nucleic acids. Contaminators are removed by subsequent washing with two different buffers. Pure genomic DNA is finally eluted under low ionic strength conditions in a slightly alkaline elution buffer.

The DNA was isolated from the tail and limb tissue left over after dissection of samples. Digestion was achieved by the addition of 180µl of T1 buffer (50mM Tris HCl pH8.0; 10mM EDTA; 0.1M sodium chloride; 1% sodium dodecyl sulphate) followed by 25µl of proteinase K (50mM Tris HCl pH8, 10mM EDTA, 0.1M NaCl, 1% sodium dodecyl sulphate and 500µg/ml proteinase K per 500µl proteinase K solution) to each sample. The samples were mixed by vortexing and incubated for 3-4 hours in a 56°C waterbath (optimal for enzyme activity). Additional vortexing 3-4 times per hour aided complete lysis. After lysis, the samples were centrifuged for approximately 10 seconds, 300µl of

double distilled water (ddH<sub>2</sub>O) were added to each sample and the samples were again centrifuged for 5 minutes in order to pellet residual tissue. 200µl of supernatant was removed to a fresh tube and 200µl of B3 solution were added to each sample and the samples were then vortexed to mix the solution. B3 contains chaotropic salts which are activated by incubation in a 70°C waterbath for 10 minutes and allow the binding of DNA to the tissue columns. After a 10 second centrifugation, 210µl of 100% ethanol were added to each sample, the samples were vortexed and centrifuged again. Ethanol also contributes to the binding of DNA to the tissue columns. Each sample was removed into a filter-spin column, which was placed in a catch tube. These were then centrifuged for 30 seconds at 6000g to draw the liquid through the filter. The catch tubes were emptied and 500µl of wash buffer (WB) solution were added to the filter-spin columns. The contents of the catch tubes was discarded and the process was repeated with 600µl of B5 solution. These solutions were buffers that washed the DNA bound to the columns in order to remove any contaminants. The contents of the catch tubes were discarded and the filter-spin column was placed in a fresh 1.5ml Eppendorf tube. 80µl of elution buffer was added to each column and the samples were incubated for 1-2 minutes in a 70°C waterbath to facilitate the elution process. The samples were centrifuged for 5 seconds and PCR was carried out on the filtrate.

### 2.3.2 Fast Prep™ DNA Extraction

This method of DNA extraction was used in order to genotype samples that were to be used for tissue culture so that the ganglia from animals of the same genotype could be pooled in order to give sufficient cells for detailed dose response analysis. Samples were placed in a Fast-prep tube (Calbiochem) containing sand granules and yellow ball. A single yellow ball was placed on top of this and 1ml of CLS-TC solution was added to each tube. Samples were run at a speed of 4.5rpm for 30 seconds in the Fast prep machine. This lyses the tissue through a combination of mechanical and chemical means. They were then centrifuged at 13000rpm for 10 minutes to pellet any remaining masses of tissue. 300µl of the supernatant, which contained the DNA fraction, was removed to a new 1.5ml Eppendorf tube. 300µl of binding matrix was added to this and the contents of the tube were mixed by inversion. Samples were incubated on the bench for 5 minutes, after which the tubes were centrifuged at 13000rpm for 10-20 seconds. The supernatant was removed and discarded and the DNA remained in the pellet. The samples were again centrifuged at 13000rpm briefly and the remaining supernatant was removed. The pellets were resuspended in 500µl of SEWS-M solution by vortexing in order to remove any bound proteins into the supernatant. The samples were centrifuged at 13000rpm for 10-20 seconds. The supernatant was removed and discarded as described previously and the pellet was resuspended in 100µl of DES solution. Samples were incubated for at least 3 minutes on the bench to elute the DNA from the binding

matrix. Following this they were centrifuged at 14000rpm for 1 minute. The supernatant was transferred to a new 1.5ml Eppendorf for use in the PCR reaction.

### **2.3.3 Polymerase Chain Reaction**

The PCR technique provides direct access to defined segments of genomic DNA for analysis, including the determination of the DNA sequences of amplified targets. (Innis et al., 1990).

To produce the knockout mice used in this project, a segment of the chosen genes was replaced by a neomycin-resistance gene cassette by homologous recombination in embryonic stem cells. In addition to preventing production of functional proteins, this allows selection of embryonic stem cells carrying the transgene. The polymerase chain reaction (PCR) can be used to distinguish which of the two possible alleles mice carry (i.e. wild type or neomycin cassette disrupted). With the exception of TNF $\alpha$  and Bad a multi-prime PCR reaction was used to allow identification of both wild type and mutated alleles in a single PCR reaction.

For the p65 samples a reaction mixture that was sufficient for ten reactions was made up using 20 $\mu$ l of 10x reaction buffer, 10 $\mu$ l of 5mM dNTPs (Pharmacia), 1.3 $\mu$ l of HS Taq thermostable DNA polymerase (Hot star, Qiagen), 140 $\mu$ l of ddH<sub>2</sub>O, 10 $\mu$ l of 25mM

magnesium chloride and 3µl of each primer. The primers were RelAKO (5' AAA TGT GTC AGT TTC ATA GCC TGA AGA ACG<sup>3'</sup>) which was unique to the *p65*-deficient allele, RelAWT (5' AAT CGG ATG TGA GAG GAC AGG<sup>3'</sup>) which was unique to the wildtype allele and RelAU (5' CCT ATA GAG GAG CAG CGC GGG<sup>3'</sup>) which was common to both alleles. 19µl of this was added to 1µl of each DNA sample. A drop of mineral oil was placed on the surface of this as a seal and the PCR programme was run (See Appendix 1A for the schedule). In this reaction one primer was located in the gene coding sequence and was common to both alleles. For detection of the wild type allele, a second primer was located in the gene coding region replaced by the Neo gene in the mutant. A third primer was employed that bound to the Neo gene, to detect the mutant allele. The use of these primers together gave amplification of one single PCR product for wild type animals, another unique single PCR product for knockout animals, and both PCR products for heterozygotes, as they carry both alleles. The PCR products were of different sizes and were easily resolved by agarose gel electrophoresis.

For the IKKα samples a reaction mixture that was sufficient for ten reactions was made up using 20µl of 10x reaction buffer, 10µl of 5mM dNTPs, 1.5µl of Taq thermostable DNA polymerase (Hot star, Qiagen), 62.5µl of ddH<sub>2</sub>O, 3.8µl of Q solution and 50µl of 25mM magnesium chloride. 0.2µl of the primers for the *ikkα*-deficient allele, αko1 (5' CAA CAT TAA ATG TGA GCG AG<sup>3'</sup>) and αko2 (5' GGA ACA TTG CAG TAT TTG G<sup>3'</sup>), 0.2µl of the primers for the wildtype, αwt1 (5' ACA ATA CAT AAA ATA ATT GAT<sup>3'</sup>), and αwt2 (5' TGA AAT GTT TCC ACT ACC ATT<sup>3'</sup>) were added. 19µl of this

was added to 1µl of each DNA sample. A drop of mineral oil was placed on the surface of this as a seal and the PCR programme was run (see appendix 1B for schedule). The wildtype samples will show a reaction product, following gel electrophoresis, for  $\alpha$ wt1 and 2 but not for  $\alpha$ ko1 and 2. The reverse is true for the *ikk $\alpha$* -deficient mice and heterozygotes will have reaction products from primers for *ikk $\alpha$* -deficient allele and the wildtype.

For the IKK $\beta$  samples a reaction mixture that was sufficient for ten reactions was made up using 20µl of 10x reaction buffer, 10µl of 5mM dNTPs, 3µl of Taq thermostable DNA polymerase (Hot star, Qiagen), 142.5µl of ddH<sub>2</sub>O and 15µl of 25mM magnesium chloride. 3µl of the common primer, P1 (5' AGT CCA ACT GGC AGC GAA TA 3'), 3µl of the primer for the *ikk $\beta$* -deleted allele P2 (5' CAA CAT TAA ATG TGA GAG AG 3') and 3µl of the primer for the wildtype, P3 (5' CTA TGC TAT TCA TTC CCG GG 3') were added. 19µl of this was added to 1µl of each DNA sample. A drop of mineral oil was placed on the surface of this as a seal and the PCR programme was run in a similar manner as the p65 samples (see appendix 1C for schedule). The genotypes were determined as described for p65.

For the NGF samples a reaction mixture that was sufficient for ten reactions was made up using 20µl of 10x reaction buffer, 10µl of 5mM dNTPs, 1.3µl of HS Taq thermostable DNA polymerase (Hot star, Qiagen), 72µl of ddH<sub>2</sub>O, 40µl of 25mM



magnesium chloride, 38µl of Q solution and the three primers. 1.6µl of NGF1 (5' AAA TGT GTC AGT TTC ATA GCC TGA AGA ACG3') for the *ngf* null mutant allele was added, 5µl of NGF2 (5' AAT CGG ATG TGA GAG GAC AGG3') for the wildtype allele and 2.4µl of NGFC (5' CCT ATA GAG GAG CAG CGC GGG3') which is common for both alleles. 19µl of this mixture was added to 1µl of each DNA sample. A drop of mineral oil was placed on the surface of this as a seal and the PCR programme was run in a similar manner as the p65 samples (See Appendix 1D for the schedule). The genotypes were determined as described for p65.

For the NP65 samples both p65 and NGF reactions were carried out in duplicate on each of the DNA samples to ascertain their genotypes.

For the TNFα samples a reaction mixture that was sufficient for ten reactions was made up using 20µl of 10x reaction buffer, 10µl of 5mM dNTPs, 1.5µl of HS Taq thermostable DNA polymerase (Hot star, Qiagen), 145µl of ddH<sub>2</sub>O, 10µl of 25mM magnesium chloride and 2µl of the two primers. The primers were David (5' GCG TCC AGC TGA CTA AAC ATC CTT3') and Toby (5' ACCACTAGTTGGTTGCTTTGAGAT3'). 19µl of this mixture was added to 1µl of each DNA sample. A drop of mineral oil was placed on the surface of this as a seal and the PCR programme was run (See Appendix 1E for the schedule). The primers are common to both wildtype and *tnfα* null mutant alleles but a size difference between the

two alleles is apparent following gel electrophoresis. The PCR products of different sizes were easily resolved by agarose gel electrophoresis.

For the *Bad* samples a reaction mixture that was sufficient for ten reactions was made up using 20 $\mu$ l of 10x reaction buffer, 10 $\mu$ l of 5mM dNTPs, 1 $\mu$ l of HS Taq thermostable DNA polymerase (Hot star, Qiagen), 10 $\mu$ l of 25mM magnesium chloride and two primers. To detect the wildtype allele 3.2 $\mu$ l of both primers 1(5'CCT ATA GAG GAG CAG CGC GGG<sup>3'</sup>) and 2 (5'CCT ATA GAG GAG CAG CGC GGG<sup>3'</sup>) were added along with 102 $\mu$ l of ddH<sub>2</sub>O, 38 $\mu$ l of Q solution. To detect the knockout allele the primers added were 3 (5'AAA TGT GTC AGT TTC ATA GCC TGA AGA ACG<sup>3'</sup>) and 4 (5'AAT CGG ATG TGA GAG GAC AGG<sup>3'</sup>) and 140 $\mu$ l of ddH<sub>2</sub>O was added. 19 $\mu$ l of each of these were added to 1 $\mu$ l duplicate samples of each DNA extract. A drop of mineral oil was placed on the surface of this as a seal and the PCR programme was run (See Appendix 1F for the schedule). For each sample two separate reactions were set up, one containing the primers unique to the knockout (3 and 4) and one containing primers unique to the wildtype (1 and 2). Thus the wildtype samples will show a reaction product following gel electrophoresis, in the reaction containing 1 and 2 but not the reaction containing 3 and 4. The reverse is true for the *bad*-deficient mice and heterozygotes will have reaction products from both reactions.

The basic PCR reaction consisted of 34-36 cycles, following an initial 15 minute step at 95°C to activate the HS Taq thermostable DNA polymerase. Cycle conditions were: 1 minute at 94°C (denature), followed by 1 minute at 55-64°C (temperature specific to each reaction) (primer annealing), followed by 1 minute 30 seconds at 72°C (elongation). There was also a final elongation stage of 10 minutes at 72°C. Samples were then cooled to 4°C. Variations on this programme were used for each of the different genes under examination. Specific reaction schedules for each of the genes can be seen in appendices 1A to 1F. For each individual animal, two reactions were carried out to safeguard against wrong genotyping. If two consistent genotypes were not found then the reactions for that individual were repeated to obtain consistent results. Positive and negative controls were also carried out in duplicate for each reaction. (The positive control is a heterozygote sample, the negative control was a sample that was put through the DNA extraction procedure but that contained no tissue). The products of the reaction were visualised on an agarose gel containing ethidium bromide.

#### **2.3.4 Gel Electrophoresis**

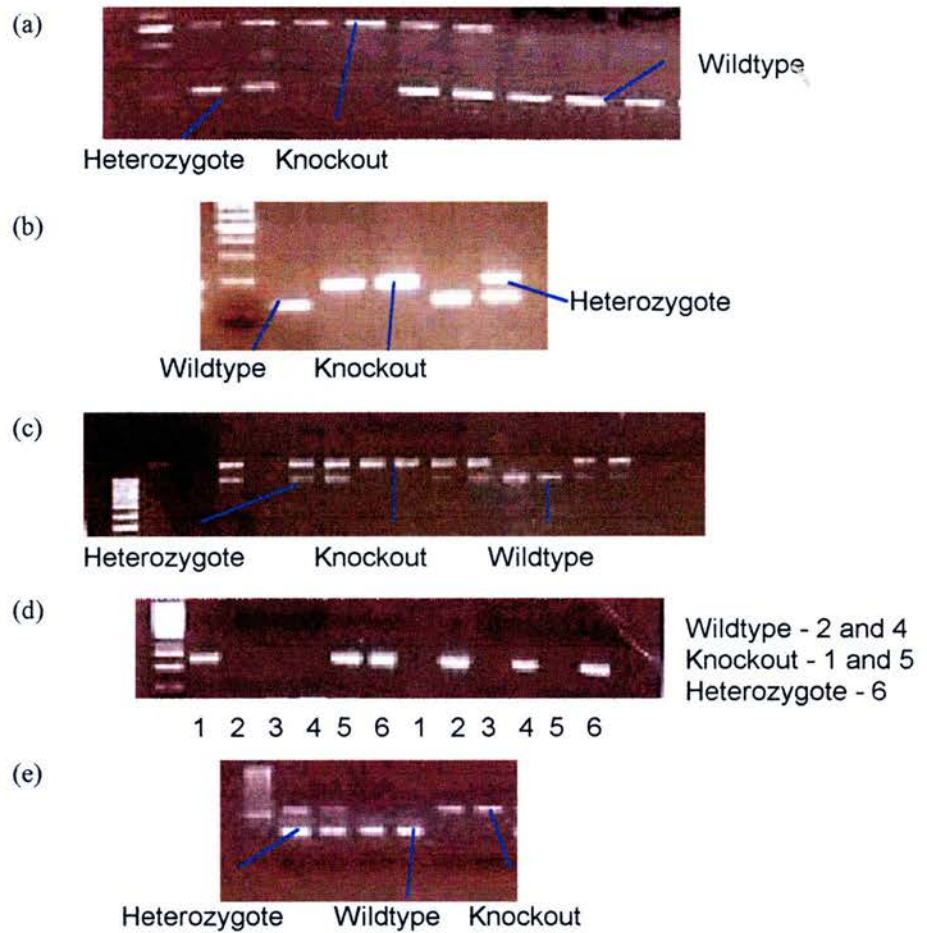
DNA, which is negatively charged at neutral pH, will migrate from the cathode to the anode when a potential difference is applied across the gel. The rate of migration of linear duplex DNA molecules is inversely proportional to  $\log_{10}$  their molecular weight.

It is also affected by the agarose concentration of the gel, DNA conformation (i.e. linear, circular, or supercoiled), the voltage applied across the gel and the composition and ionic strength of the electrophoresis buffer. Tris-acetate electrophoresis buffer (TAE) was used for agarose gel electrophoresis. This was prepared as a 50x stock (2M Tris HCl pH8.0, 0.05M EDTA pH 8.0, 6% concentrated glacial acetic acid) which was then diluted to a 1x solution for use.

To cast an agarose gel, the desired amount of ultra pure agarose (Gibco) was completely melted in the TAE, in a microwave. The percentage of agarose used to make up the gel depends on the samples to be run on it. IKK $\alpha$  and  $\beta$ , NGF, Bad and TNF samples were run on 2% gels whereas, p65 samples were run on 3% gels (this was as the products of this reaction are small and close together). Ethidium bromide was added to the gel mixture to a final concentration of 0.5 $\mu$ g/ml. Ethidium bromide binds to the DNA during electrophoresis and fluoresces in UV light, allowing DNA bands to be seen on the gel. When the molten gel had cooled to around 60 $^{\circ}$ C, it was poured into a gel-casting plate whose ends had been sealed with masking tape, the well-forming comb was put in position, and the gel was allowed to set. The set gel was placed in the electrophoresis tank and covered with ethidium bromide containing TAE buffer and the comb removed. The DNA samples were mixed with 6x gel-loading buffer (40mM EDTA; 20% glycerol; 0.1% Bromophenol Blue (or Orange G, in the case of p65) and loaded onto the gel. Orange G is used in p65 reactions as bromophenol blue migrates at a similar rate to the reaction products thus obscuring them. A molecular weight standard was loaded into the first well to allow the size of the DNA bands to be measured. A voltage of 1-5v/cm was applied across the tank and the gel was left to run

for approximately 1 hour. After electrophoresis was complete, the genotypes could be ascertained and a permanent record of the gel made by photographing it on the UV transilluminator.

The genotypes were determined by the presence of either or both DNA bands. In the majority of cases the product for the knockout allele is heavier for p65 at 550 base pairs (550bp), IKK $\alpha$  (600bp), NGF (520bp) and TNF (1500bp), therefore the band appears higher up the gel. The lighter wildtype products, p65 (390bp), IKK $\alpha$  (400bp), NGF (210bp) and TNF (1200bp) ran further on the gel. The heterozygote samples were recognised by the presence of both bands. Examples of gels demonstrating samples of all three genotypes from each of the reactions can be seen in figure 2.2. In the case of IKK $\beta$  this was reversed such that the wildtype product was larger at 2000bp as compared to the 1900bp knockout product. Products of both Bad 1 and 2 and Bad 3 and 4 were 690bp but they were run separately as described previously.



**Figure 2.2: Agarose gels.** Gels show examples of wildtype, knockout and heterozygote genotypes for (a)NGF, (b) P65, (c)TNF, (d)Bad, and (e)IKK $\alpha$  .

## **2.4 Histology**

Samples were dissected with a single incision made using a tungsten needle anterior to the forelimbs of the embryos in which the retina, sympathetic and sensory ganglia were to be examined and posterior to the forelimbs in which the spinal cord was to be studied. Samples were coded to avoid observer bias in the experiments. They were then fixed, processed, embedded, sectioned and mounted.

### **2.4.1 Fixation**

The aim of fixation is to:

- (i) Confer chemical stability on the tissue.
- (ii) Harden the tissue to facilitate further handling.
- (iii) Halt enzyme autolysis.
- (iv) Halt bacterial putrefaction.
- (v) Enhance later staining techniques (i.e. act as a mordant - a corrosive substance that fixes dyes).
- (vi) Fix components of the tissue in position, in as near their normal form as possible.
- (vii) It may also prepare the tissue for the later process of infiltration of an embedding medium by making the cells more permeable.

Some tissue constituents will be destroyed and leached out or simply distorted with any fixative. The process is therefore a compromise and it is important to:

- (i) Fix tissue promptly.
- (ii) Manipulate the tissue as little and as gently as possible.
- (iii) Cut samples small enough to allow fairly rapid penetration as fixatives penetrate tissues at different rates.
- (iv) Use a fixative in a buffered salt solution for the best preservation of intracellular detail (particularly for electron microscopy).
- (v) Vary fixation time according with tissue type, size and water content.

#### **2.4.1a Neutral Buffered Formalin**

Neutral buffered formalin (NBF)(BDH) is a reasonably fast fixative that fixes proteins in position by cross-linking them. It is a good general fixative for cytological detail. The nature of the salt solution in NBF makes it an extremely good fixative for neuronal tissue. The protein cross-linkage makes it an unsuitable choice for most immunohistochemistry (IHC) with the exception of transferase mediated dUTP biotin nick end labelling (TUNEL) staining which is best done after NBF or paraformaldehyde fixation. It was for this procedure that the samples were fixed in NBF. Cresyl fast violet (CFV) staining was also carried out on samples fixed in NBF. E12 samples were fixed for 24 hours and E14.5 were fixed for 48 hours in NBF at room temperature.



### 2.4.1b Carnoy's Fluid

Carnoy's is a rapid fixative. It contains ethanol, which fixes the tissue by coarsely coagulating proteins and precipitating nucleic acids and glycogen. Acetic acid counteracts the potential for shrinkage caused by the ethanol and again precipitates DNA.

Carnoy's Fluid (1L):  
600ml ethanol  
300ml chloroform  
100ml glacial acetic acid

Carnoy's is an excellent fixative for immunohistochemistry and there is no need for antigen retrieval techniques when using it. It is also good for cresyl fast violet staining particularly at younger ages. Sections for pyknotic counting were fixed in Carnoy's and stained with CFV. It should be noted that non-neuronal cells stain up a very dark purple which gives the section a very different appearance to neuronal cells. Sections for Islet-1,  $\beta$ III tubulin and PCNA staining were also fixed in Carnoy's. Both E12, E14.5 and E16 samples were fixed for 30 minutes at room temperature, whereas E18 and P1 samples were fixed overnight at 4°C.

## 2.4.2 Processing

The paraffin wax, used to embed the samples is insoluble in both alcohol and water but not other substances such as chloroform, xylene and toluene. Of these, chloroform was used as an intermediate in the embedding process. None of these clearing agents are water soluble so all water must be removed from the tissue prior to embedding - this is known as dehydration and was done using a graded alcohol series. Chloroform is used routinely as a clearing agent in the lab. The reason for this is that chloroform bleaches the tissue allowing the cresyl fast violet to definitively stain Nissl substance. Other clearing agents, e.g. xylene do not have this effect and so can severely compromise the cresyl fast violet staining. The processing schedule depended on several factors:

- (i) the fixative used.
- (ii) the age of the tissue.
- (iii) purity of the alcohols in the graded series.

After processing, the samples were taken through a series of three waxes to infuse the tissue fully with wax prior to embedding. (See Appendix 2 and 3 for schedules)

### **2.4.3 Embedding**

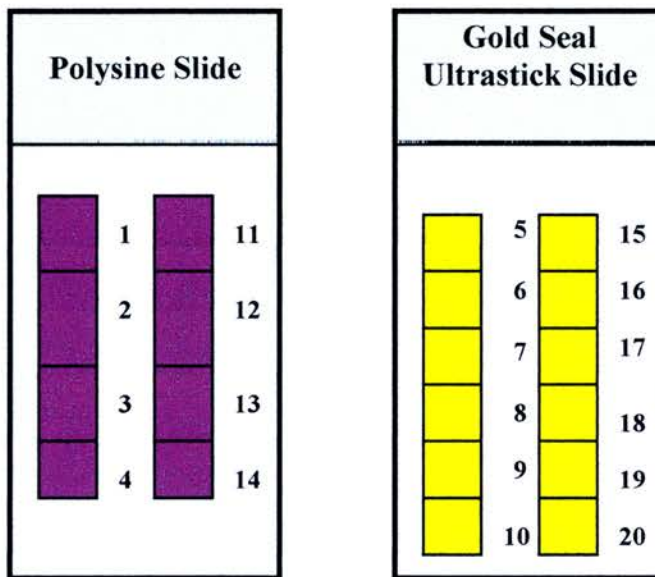
Embedding is the blocking of the tissue into (what will be) a solid wax block with enough wax surrounding the tissue to support it during sectioning. Samples were embedded with the top of the head to the bottom of the base mould and sitting upright such that the neck was pointing upwards, so that cross sectional planes can be obtained. The tissue was embedded in paraffin wax after three changes in filtered paraffin wax at 56°C.

### **2.4.4 Sectioning**

Sections were cut using a rotary microtome and routinely sections were cut at 8µm. After sectioning, the ribbons of sections were floated out on a water bath to flatten them for mounting onto slides. Sections for cresyl fast violet staining were mounted onto polysine coated slides.

A slightly different technique was used to cut sections for immunohistochemistry and TUNEL staining. Firstly, four sections were cut and mounted onto a polysine-coated slide for cresyl fast violet staining. This was done so that the slides containing sections for IHC/TUNEL staining could be determined. Next, a ribbon of six sections was cut and this was mounted onto a Gold Seal Ultrastick slide for

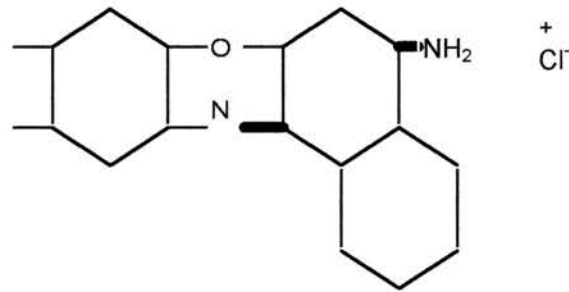
immunohistochemistry/TUNEL. The reason for this is as there is a tendency for the immunohistochemistry/TUNEL procedure to detach the sections from the polysine slides. The next ribbon was another ribbon of four, which was mounted on the polysine slide followed by a six for the gold slide and so the pattern was continued (see Figure 2.3).



**Figure 2.3: Mounting pattern for sections for immunohistochemistry /TUNEL analysis.** Four sections are mounted on the polysine slide for CFV staining and six sections are mounted on the gold seal ultrastick slide for IHC staining.

## 2.4.5 Staining

### 2.4.5a Cresyl Fast Violet



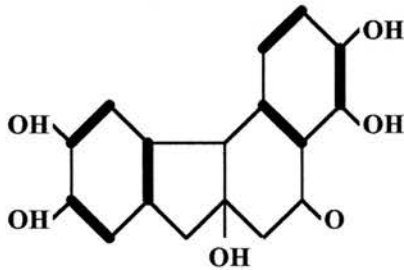
**Figure 2.4: Cresyl Fast Violet.** Chemical structure of the basic dye cresyl fast violet.

— = single bond  
= = double bond

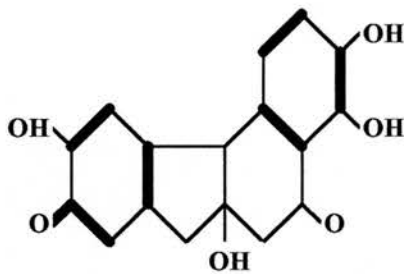
Cresyl fast violet is a basic dye that exists as a mixture of two or more dyes based on the above structure (figure 2.4) but which vary in their alkyl groups. It is a metachromatic dye staining nucleoli dark violet, Nissl substance lighter violet and cartilage pink. The colour conferring part of the dye is basic and is attracted to basophilic tissue constituents such as RNA and DNA. The concentration of the cresyl violet acetate solution used was dependent on the age of the tissue. For young embryos a dilute solution of 0.001% was used whilst for older embryos stronger solutions of up to 0.1% were required. cresyl fast violet was dissolved in ddH<sub>2</sub>O to the concentration required. It was then heated at 36°C in a water bath for 2 hours and finally filtered before use. The exact strength required

varied between batches of stain so each bottle was best optimised on a few test slides to establish the approximate concentration.

#### 2.4.5b Haematoxylin



**Figure 2.5:**  
**Haematoxylin.** Chemical structure of the dye haematoxylin.  
— = single bond  
— = double bond



**Figure 2.6: Haematein.**  
Chemical structure of haematein.  
— = single bond  
— = double bond

Although it is common practice to use haematoxylin as the source of dye, it is not itself the actual dye. During the preparation of staining solutions, the haematoxylin (figure 2.5) is converted into haematein (figure 2.6). This is usually accomplished with chemical oxidising agents, but is sometimes accomplished by atmospheric oxygen over time. Sodium iodate is the most commonly used oxidising agent for this purpose (0.2g will oxidise 1g haematoxylin). Others are mercuric oxide, potassium permanganate and

iodine. Haematein may also be referred to as *haematoxein*. Haematein is not incorporated directly into most staining solutions because it continues to oxidise in solution and forms non-staining products. The quality of haematoxylin is usually higher and more consistent than the quality of haematein, and solutions made with it are more easily standardised.

This dye is usually used in conjunction with a mordant, the two commonest being aluminium (as ammonium or potassium alum), or a ferric salt (ferric chloride or iron alum). Other mordants are used much less frequently but include chrome alum and phosphotungstic acid. The tissue component most frequently demonstrated is nuclear chromatin using an aluminium mordant in the Haematoxylin and Eosin method. Using ferric salts as the mordant, it is also used for acid resistant nuclear staining, the demonstration of muscle striations and numerous other elements. With phosphotungstic acid it can demonstrate fibrin, muscle striations and some glia fibres. For this study the mordant used was aluminium. 1g of haematoxylin was mixed with 20g of aluminium potassium sulphate and 10ml of alum. This was then heated to boiling in 200ml ddH<sub>2</sub>O, on a heated stirring plate. 0.5g of aluminium oxide was then added and the solution was cooled. Once cold 8ml of glacial acetic acid were added.

Prior to staining, xylene was used to clear the sections of wax and to act as an intermediary between the wax and the alcohol series used to rehydrate the tissue prior to

staining. The sections were rehydrated by passing them through a graded alcohol series and finally distilled water. The sections were then stained. It should be noted that the length of time for which sections require to be stained varies greatly depending on the age of the samples. After staining, the sections were rinsed with distilled water acidified with 3 drops of glacial acetic acid per 250ml, passed quickly into 96% alcohol plus glacial acetic acid (as before) to differentiate. Differentiation time varied between a few dips and 20 minutes depending on the age of the tissue and the intensity of staining desired. After the differentiation sections were placed in 96% alcohol, slides were left slightly over-stained and dehydrated further in 100% alcohol. The next differentiation step was carried out in chloroform and it was here that the residual stain came out of the nucleus whilst the staining in the nucleoli intensified. The aim was to achieve a clear nucleus with dark nucleoli and nissl staining in the cytoplasm. The chloroform was washed out and any remaining water was removed with two changes of 100% alcohol. The sections were then cleared with two changes of xylene and mounted using DPX as a mounting medium to permanently adhere the coverslips to the slides.

#### **2.4.6 TUNEL Staining**

TUNEL staining was used to determine the presence of apoptotic neurons. It was carried out using the TdT-FragEL™ DNA fragmentation Detection Kit (Calbiochem). This was a non-isotopic system for labelling DNA breaks in apoptotic nuclei. Deoxynucleotidyl transferase (TdT) end-labelled the free 3'-OH groups generated at the



end of the classical DNA ladders produced by apoptotic endonucleases and catalysed the addition of biotin-labelled and unlabelled deoxynucleotides. Biotinylated nucleotides were detected using a streptavidin-horseradish peroxidase (HRP) conjugate. Diaminobenzide tetrahydrochloride (DAB) then reacted with the labelled sample to produce an insoluble coloured substrate at the site of DNA fragmentation (see figure 2.7). Counterstaining with methyl green can aid the morphological evaluation and characterisation of normal and apoptotic cells, but was not used as the stain was found to overpower the TUNEL stain. Sections that had been fixed in NBF were cleared in xylene and rehydrated through a series of alcohols. They were then permeabilised using 1% proteinase K in 10mM Tris. Following this they were placed in quench (1% hydrogen peroxide in methanol) to inactivate endogenous peroxidases, washed with PBS and from here placed in TdT equilibration buffer. The sections were labelled by incubation at 37°C for two and a half hours. The reaction was terminated using stop buffer. Detection involved the application of blocking buffer, followed by 1x peroxidase streptavidin conjugate solution, which labels the site of DNA fragmentation. The chromogen DAB was then applied as this reacts with the labelled sample to generate an insoluble substrate at the site of DNA fragmentation. The DAB was rinsed off and a 0.5% copper sulphate, 0.85% sodium chloride mix was used to intensify the staining. The sections were dehydrated through a series of alcohols, cleared using xylene and mounted with DPX. (See Appendix 4 for staining schedule).

### 2.4.7 Immunohistochemistry

Immunohistochemical analysis was carried out on sections of the spinal cord, retina, SCG, trigeminal and nodose ganglia. Islet-1 staining demonstrated the levels of motoneurons present in the spinal cord,  $\beta$ III tubulin allowed quantification of neurons in the SCG, trigeminal and nodose ganglia and PCNA staining demonstrated the levels of proliferating cells in the SCG, trigeminal and nodose ganglia.

Immunohistochemical methods are based on immuno-enzymatic reactions using either mono- or poly-clonal antibodies to detect tissue antigens. The use of an indirect method increased the sensitivity of the immunohistochemical reaction. The primary antibody that recognised the antigen of choice was raised in a given species - this antibody was not labelled in any way. This was detected after binding of a labelled secondary antibody raised against the immunoglobulin of the species that donated the primary antibody. This method had several advantages over direct methods:

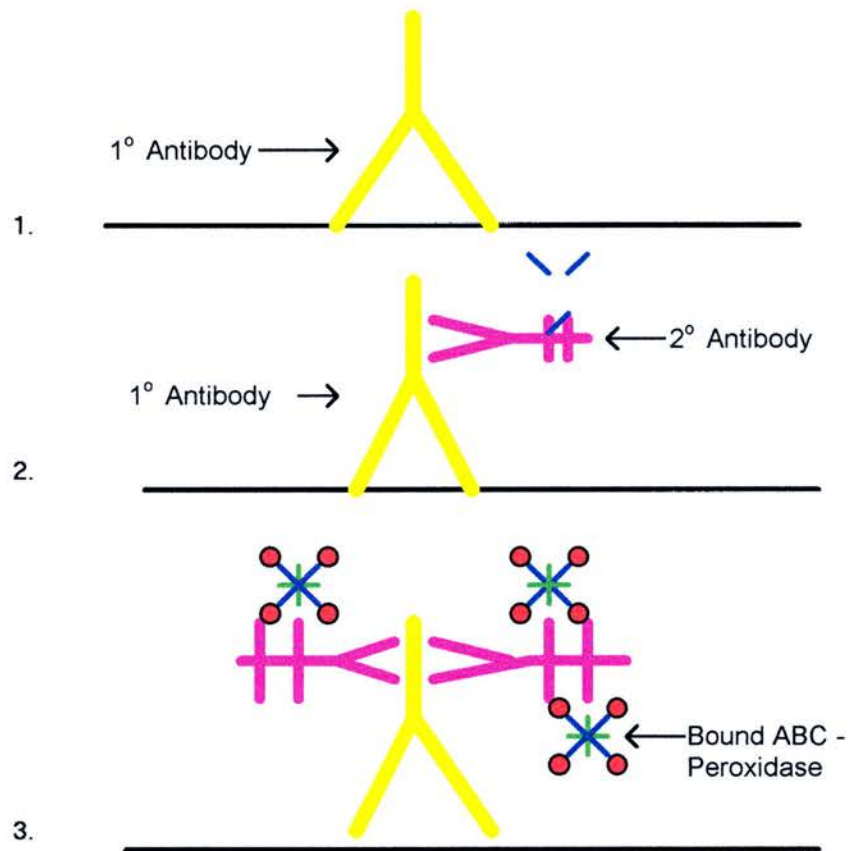
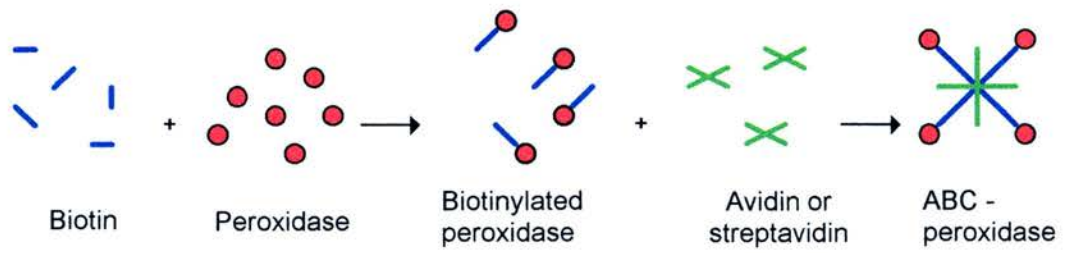
1. Anti-immunoglobulin G (IgG) sera are usually hyperimmune and of very high affinity.
2. At least two labelled anti-IgGs could bind to each primary antibody increasing the sensitivity of the reaction and therefore the detectability of the antigen.
3. It was more economical as one labelled second-layer antibody could be used to detect any number of first-layer antibodies to different antigens provided they had all

been raised in the same host species. Further layers were added to further increase sensitivity, i.e., the ABC™ technique (Vector).

The immunohistochemical method used was the avidin-biotin method as it has high levels of sensitivity, relatively short incubation times and low background staining. The anti-peroxidase and streptavidin-alkaline phosphatase methods could also have been used. Avidin is a 68kDa glycoprotein with an extremely high affinity ( $10^{15}M^{-1}$ ) for the small molecular weight vitamin, biotin. Due to the fact that this is greater than one million times higher than the affinity of the antibody for most antigens, there is essentially irreversible binding. Avidin has four binding sites for biotin and therefore most proteins, including antibodies and enzymes, can be conjugated with several molecules of biotin. The immunoperoxidase procedure based on these properties used for localising antigens employed unlabelled primary antibody, followed by biotinylated secondary antibody and finally a pre-formed avidin and biotinylated horseradish peroxidase macromolecular complex (Hsu et al., 1981). This is termed the ABC™ technique (Vector) (See Figure 2.7).

It was this IHC procedure that was used for the Islet-1,  $\beta$ III-tubulin staining and PCNA. For the Islet-1 staining the chromagen used was VIP as this had been found to stain Islet-1 positive cells clearly as pink/purple structures. Other chromogen systems are available, (e.g. DAB, NovaRED and Vector SG) and for both  $\beta$ III-tubulin staining and PCNA staining the chromogen used was DAB at a concentration of 1mg/ml (a tablet of 12 $\mu$ l of

30% H<sub>2</sub>O<sub>2</sub>/urea was added to 15ml of PBS), as this stains neurons as dark brown structures with pale brown stained cytoplasm and dividing cells as dark brown structures respectively . (See Appendix 4 for procedural schedule). Staining was carried out on three samples of each genotype.



**Figure 2.7: The ABC Technique**

- 1.) Unlabelled primary antibody binds to the antigen.
- 2.) This is followed by the binding of a biotinylated secondary antibody.
- 3.) A pre-formed avidin and biotinylated horseradish peroxidase macromolecular complex then binds to the secondary antibody and it can then be detected. (Adapted from Polak and Van Noorden, 1997)

### **2.4.7a Islet-1 Staining**

Islet-1 is a member of the LIM family of transcription factors that are specifically expressed in the nuclei of motoneurons. Islet-1 is specifically expressed in all somatic motoneuron subclasses and is thought to be required for the specification of features common to the development of all motoneurons (Tsuchida et al., 1994). Islet-1 has been found to be expressed in all brainstem and spinal cord motoneurons and targeted deletion of Islet-1 in mice results in the absence of motoneurons along the entire axis of the brainstem and spinal cord (Pfaff et al., 1996). immunohistochemical staining for Islet-1 was used to identify motoneurons in the spinal cord.

The tissue was fixed, processed, embedded, sectioned and mounted as described previously. The sections were cleared in xylene, rehydrated and washed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was quenched using 1% hydrogen peroxide in methanol. The sections were rinsed in PBS followed by incubation with 10% goat serum plus 0.5% Triton X-100 in PBS to reduce non-specific binding and incubated at 4°C overnight with anti-Islet-1 antibody (received from Oliver Schmidt, Dept. Preclinical Vet. Sci., University of Edinburgh) diluted 1:2 in PBS. The sections were rinsed with PBS and labelled using biotinylated secondary antibody diluted 1:200 in PBS for 3 hours at room temperature followed by further rinses with PBS and incubation for 30 minutes at room temperature with an avidin/biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC kit; Vector Laboratories).

Motoneurons were visualised using a vector VIP substrate kit (Vector Labs) which produced an intense pink/purple reaction product. For staining schedule see appendix 5.

#### **2.4.7b $\beta$ III Tubulin Staining**

Immunohistochemical staining for  $\beta$ -tubulin class III was used for the positive identification of neurons in tissue section (Memborg and Hall, 1995; Moskowitz and Oblinger, 1995; Lee and Pixley, 1994; Easter et al., 1993; Moody et al., 1993).

The tissue was fixed, processed, embedded, sectioned and mounted as described previously. The sections were cleared in xylene, rehydrated and washed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was quenched using 1% hydrogen peroxide in methanol. The sections were rinsed in PBS followed by incubation with 10% horse serum plus 0.5% Triton X-100 in PBS to reduce non-specific binding and incubated in a humidified chamber overnight at 4°C with anti-neuron-specific  $\beta$ -tubulin class 3 antibody (Sigma) diluted 1:10,000 in PBS. The sections were rinsed with PBS labelled using biotinylated secondary antibody diluted 1:200 in PBS for 3 hours at room temperature followed by further rinses with PBS and incubation for 30 minutes at room temperature with an avidin/biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC kit; Vector Laboratories). The substrate used for the peroxidase reaction was 1 mg/ml DAB (Sigma) in Tris-buffered saline (TBS).



The DAB reaction was enhanced using 0.5% copper sulphate in 0.85% sodium chloride. Neurons were recognized by pale brown cytoplasmic staining, surrounded by darkly stained tissue. Sections were then counter-stained with haematoxylin. This was done in order to stain the nucleoli, which can be used as reference point for stereological analysis (See figure 2.8). For staining schedule see appendix 6.



**Figure 2.8: Photomicrograph of trigeminal ganglion and retina stained with  $\beta$ III Tubulin dye.**



### **2.4.7c Proliferating Cell Nuclear Antigen (PCNA) Staining**

Sections were stained for the presence of the proliferating cell nuclear antigen (PCNA), in order to examine neuroblast proliferation. Anti-PCNA antigen recognizes the acidic non-histone auxiliary protein of DNA polymerase  $\delta$ , PCNA. PCNA, also known as cyclin, is essential for DNA replication and synthesis. In many normal tissues, PCNA-expression is limited to the proliferative cells (Start et al., 1992). Because expression of PCNA is not restricted to dividing neuronal cells, the sections were double stained for  $\beta$ III tubulin to positively identify all cells of neuronal lineage. For staining schedule see appendix 7.

The tissue was fixed, processed, embedded, sectioned and mounted as described previously. The sections were cleared in xylene and rehydrated before quenching in 3% hydrogen peroxide in methanol for 20 minutes. Non-specific antibody binding was blocked in 10% horse serum, 0.5% Triton X-100 in PBS before incubation with anti-PCNA monoclonal antibody (Sigma) diluted 1:1000 in blocking buffer for 1 hour at room temperature. The cells were then labelled using biotinylated secondary antibody (1:200) and avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC Kit, Vector Labs). The substrate used for the reaction was vector VIP substrate kit (Vector Labs). The sections were then incubated with mouse anti- $\beta$ III tubulin antibody (Promega) diluted 1:5000 in blocking buffer overnight at 4°C. The cells were then labelled using biotinylated secondary antibody (1:200) and avidin,

biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC Kit, Vector Labs). The substrate used for the peroxidase reaction was 1 mg/ml DAB (Sigma) in Tris-buffered saline (TBS). The DAB reaction was enhanced using 0.5% copper sulphate in 0.85% sodium chloride. Proliferating neurons were recognized by dark brown staining.

## **2.5 Cell Quantification**

### **2.5.1 Pyknotic neurons in the Retina and Spinal Cord**

Sections of the retina of both eyes were examined at 40X magnification. The numbers of pyknotic nuclei were counted in ten 8 $\mu$ m section of serial sections at the level of the optic nerve/disc for each eye. This is the area in which the majority of apoptosis occurs (Frade et al., 1999). Sections of the spinal cord were examined at 40X magnification. To determine the level of cell death in the spinal cord the mean number of pyknotic neurons were counted per 8 $\mu$ m serial section at a defined level of cervical spinal cord. Twenty sections were examined per animal. Counts were done blind so that the genotypes of the samples were not known while the counts were being made, thus avoiding observer-bias.

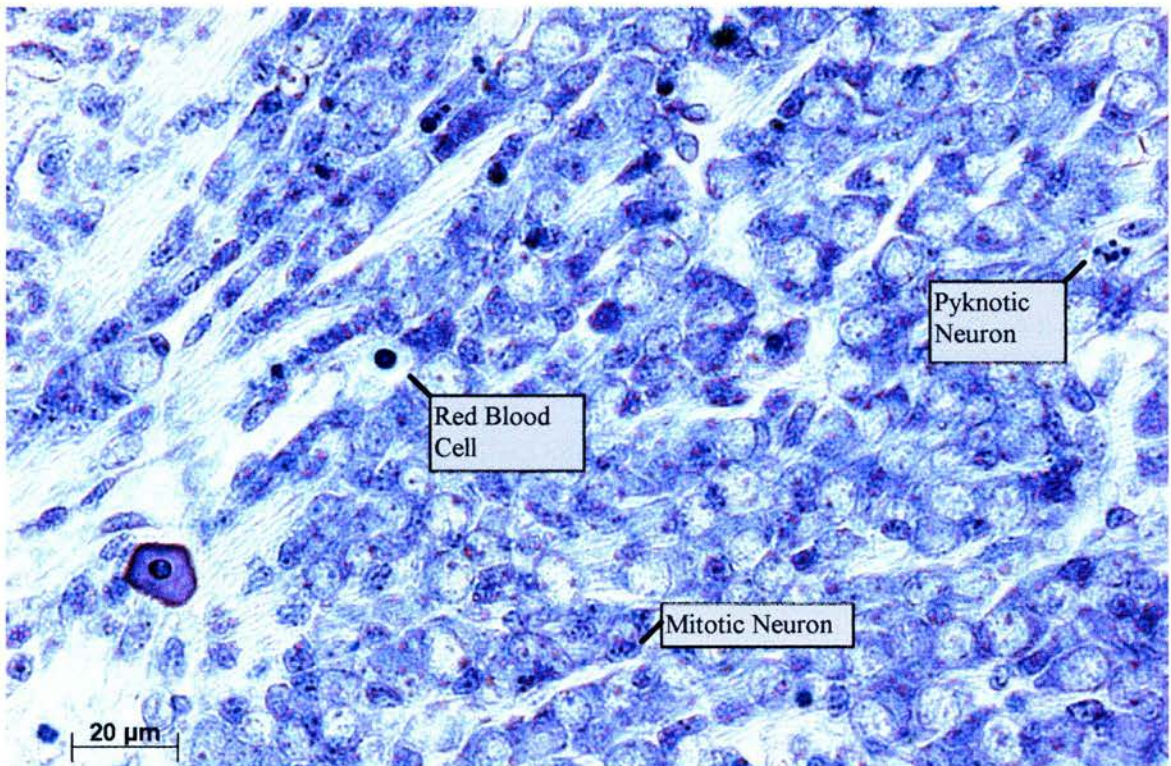
Pyknotic neurons were recognised by virtue of the large purple masses of clumped chromatin surrounded by a membrane and possessing a pale lilac cytoplasm. They were

readily distinguished from red blood cells and mitotic cells according to the criteria seen in table 2.1.

**Table 2.1: Morphological appearance of cell types that show up with CFV staining.**

Type of Cell	Nucleus	Cytoplasm
Pyknotic Neuron	Large purple blebs of clumped chromatin	Pale lilac
Red Blood Cells	One single large purple sphere	Clear - unstained
Mitotic Cells	Chromatin strands visible – pattern varies in accordance with point in cell division	Clear or pale lilac

Examples of some of these cell types can be seen in figure 2.9.



**Figure 2.9: Section showing pyknotic neurons, mitotic neurons and red blood cells.** Pyknotic neurons are seen as large purple blebs of clumped chromatin in pale lilac cytoplasm. The chromatin strands can be distinguished in the mitotic neurons and red blood cells are seen as large purple spheres in clear cytoplasm. All of these could be seen in the retinal and spinal cord sections examined

## 2.5.2 Motoneurons in the Spinal Cord

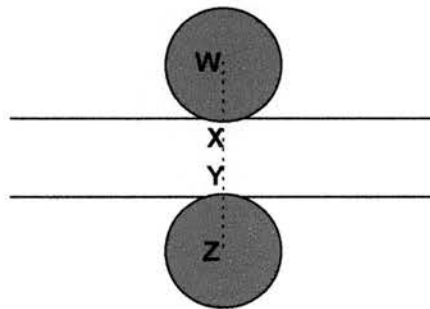
Motoneuron numbers in the spinal cord were quantified by locating the first DRG and thereby ascertaining the level of the first cervical section of the spinal cord. Having located this the numbers of Islet-1 positive cells in the spinal cord from the level of cervical segment 3 to 5 of samples could be quantified stereologically and the mean number per cervical segment could be ascertained. It should be noted that as Islet-1 stains nuclei and not nucleoli therefore nuclei must be counted and Abercrombie's formula for the correction of bias must be applied. This is a formula to correct for the bias introduced by counting nuclei in histological section. The number of nuclei visible in a microtome section can easily be counted, but not all of the objects thus counted are whole nuclei. Some must be fragments of nuclei, because some nuclei lie partly within the section examined, partly within an adjacent section. It is impossible to tell consistently which are fragmentary, which whole. 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. Thus it the nuclear points rather than nuclei in a give section must be counted, such that, a nuclear-point cannot overlap two adjacent sections, and the number of nuclear-points in a section can therefore be extrapolated to the number in any volume of tissue without error, and can serve for exact comparison of nuclear population in different tissues. If  $P$  is the average number of nuclear points per section,  $A$  is the crude count of number of nuclei seen in



the section,  $M$  is the thickness (in  $\mu\text{m}$ ) of the section, and  $L$  the average length (in  $\mu\text{m}$ ) of the nuclei, then:

$$P = A \times M / (L + M) \quad (\text{Abercrombie, 1946})$$

The derivation of this equation is explained in figure 2.10. It means that those nuclei whose nuclear-points are outside the section but which are nevertheless represented, by fragments, inside the section, have their nuclear-points extending through a volume of tissue of (on the average) the thickness of the mean nuclear length. Since microtome sections are commonly roughly the same thickness as the nuclear length, the error in extrapolating from  $A$  to the number of nuclei per unit volume of tissue may often reach 100%. Gross errors of extrapolation can only be avoided, if we can obtain the true density of nuclear (nuclear-point) populations of tissues. We need therefore a method of obtaining the number of nuclear points in a microtome section and must use Abercrombie's correction.



**Fig. 2.10 Diagrammatic representation of the explanation of Abercrombie's correction.** This shows a side view of a microtome section which lies between the two lines. Two spherical nuclei of equal radius are located that they touch the surfaces of the sections at  $X$  and  $Y$ . Any nucleus of this radius will be represented in the section if its centre lies anywhere between points  $W$  and  $Z$ . Since we can assume a random distribution of nuclei between these two points, the proportion of all the nuclei represented in the section whose centres lie within the section is given by  $XY/WZ$ , that is section thickness/(section thickness + nuclear diameter). The argument applies statistically to nuclei of any size or shape (Abercrombie, 1946).

The error of overestimation will in practice seldom exceed 10%. The 10% overestimation of population density is negligible for most purposes, and it contrasts satisfactorily with the overestimation resulting when no correction is made for nuclear size. If we are primarily interested in comparing the density of population of two tissues, we are on safer ground still, since the error in both densities is in the same direction (Abercrombie, 1946).

There are some problems with the use of Abercrombie's correction. The first is that the method is biased (Abercrombie, 1946). Systematic errors will arise if the size, shape, and orientation of cells are not taken into account. The second problem is the difficulty of determining an appropriate value for *object height* to enter into the equation.

Abercrombie's impractical solution was to measure the diameter of cells in sections cut precisely at right angle to the remainder of the tissue. In practice, this is almost never done. Instead, cell diameter is substituted for cell height. The third problem is that the equation is based on the assumption that the knife cleanly cuts through cells in its path. However, if the blade pushes through the tissue, cells will often be pushed aside and remain uncut. When cells are not split, the correction will seriously underestimate cell number and density. Unfortunately, there is no easy way to determine what percentage of cells are cut and what percentage are pushed aside during sectioning (Jones, 1937).

The fourth problem is the sensitivity of Abercrombie's method to variation in the

thickness of individual sections. The volumes of samples counted using Abercrombie's method extend from the upper to the lower surfaces of each section. Consequently, when thickness varies so will the counts. This gives rise to excessive sampling errors. The fifth and final problem is the difficulty of correctly identifying fragments of cells located at the top and bottom of the sections. Small fragments of neurons may be difficult to distinguish from glial cells or pericytes. As a result neurons may be erroneously included or excluded from the count (Williams and Rakic, 1988).

If nucleoli are counted Abercrombie's correction is not required as they are only approximately  $2\mu\text{m}$  in diameter and therefore there is less chance of them appearing in more than one section. The problem of split nucleoli is estimated to result in 2-3% bias and as this occurs in both wildtype and knockout counts the end effects are negligible.

### **2.5.3 Spinal Cord Motoneurons, Pyknotic Neurons, Neurons, and Proliferating Cells in the SCG, Trigeminal and Nodose Ganglia**

The number of pyknotic neurons, neurons and proliferating cells in the SCG, trigeminal and nodose ganglia were estimated using a digital stereology system that uses a combination of the optical dissector and volume fraction/Cavalieri methods (Kinetics Imaging). Correction for split nucleoli was not made as these do not appreciably affect the neuronal estimate (Jones, 1937).

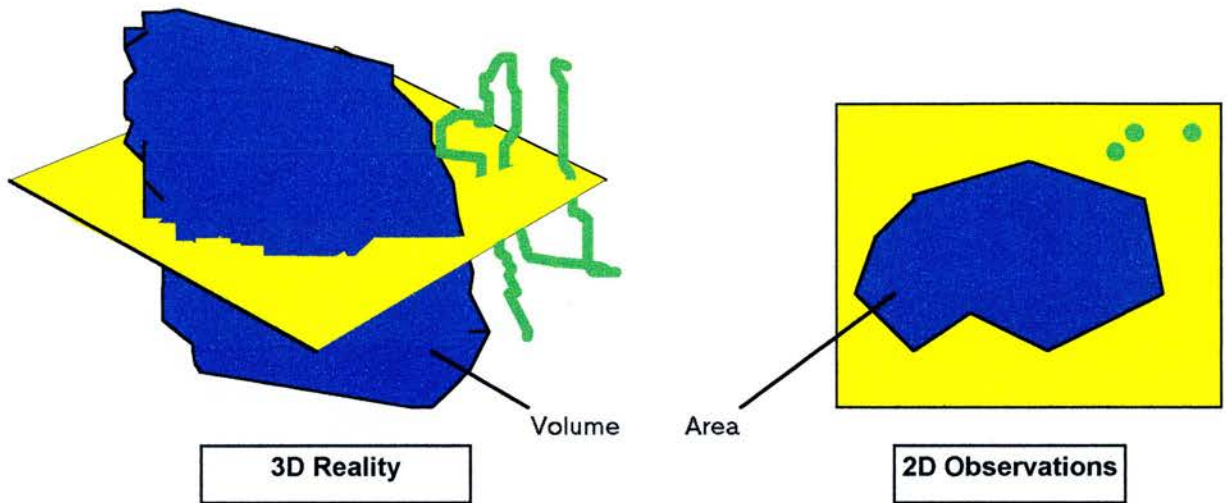


## 2.6 Stereology

Stereology is designed to sample all biological objects of interest in a theoretically unbiased and efficient manner. Sampling begins with unambiguous definition of a reference space, the three-dimensional unit of tissue that contains the biological objects of interest; the second step is sampling the reference space in an efficient manner. Systematic sampling provides an unbiased strategy to optimize sampling of biological tissue for maximum efficiency (Mouton, 2002). For all stereological measurements the neurons of interest (e.g. pyknotic, dividing, etc.) from the ganglion to be investigated must be taken with random position and in general, several samples will be required to obtain a representative estimate of the parameter of interest. The samples are most efficiently obtained using systematic random sampling, i.e., taking a random starting position and a fixed increment for future samples. Random sampling is a key means of avoiding sampling bias. The grid is uniform, such that every part of the specimen (e.g., the ganglion) has an equal chance of being included in the sample before sampling commences. Thus the sample will be representative of the specimen, allowing results of subsequent measurements to be related to the whole specimen. The method is unbiased in part because no underlying assumption is made about the structure being studied. The method relies on systematic uniform random sampling of the specimen, which yields an accurate estimate of the characteristic being sampled. Systemic uniform random sampling is sampling in which a start location for data collection is randomly determined; from that point, sampling is uniform (Mouton, 2002). In a stereology

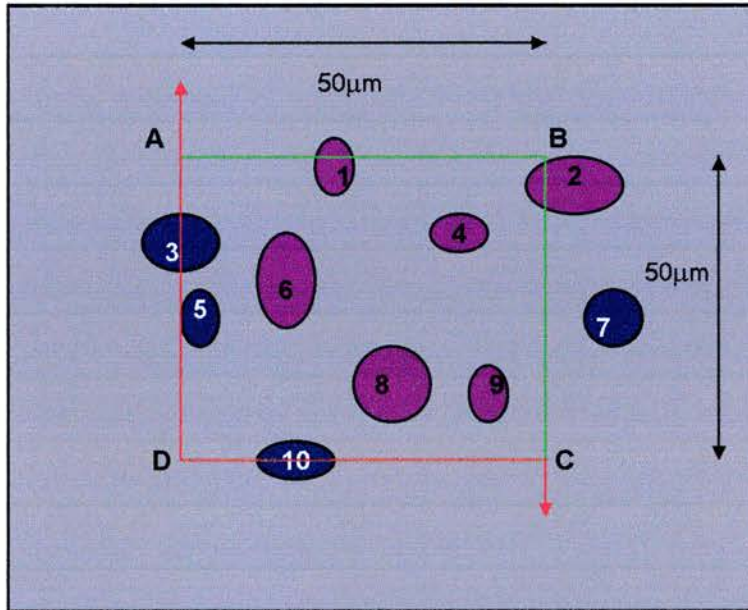
experiment it is typically unnecessary to measure more than one hundred points in an individual sample, if the sampling regime is optimal (Gundersen and Jensen, 1987). The variance of the measurement is very low but higher experimental effects are obtained by examining more individuals.

Cavalieri's principle is used for measuring the reference volume of an object, e.g., a ganglion. The object is sectioned into slices of known thickness ( $8\mu\text{m}$ ). With a random starting position of the first slice, the total area of each section is estimated using the point counting method. A point grid is a set of uniformly spaced points a known distance apart in both the x and y directions. Estimating the volume of a section is a two-step process. First, a point grid is placed at random over the test area; then the grid points that fall on the reference space are counted on all sections. The product of this count and the square of the spacing of the grid points, i.e., the area per point, gives an unbiased estimate of the total reference area. If the distance between the sections is equal then the volume of the object may be estimated by multiplying the distance between sections by the total surface area within the object, throughout the serial sections (see figure 2.11).



**Figure 2.11: A Schematic diagram of the estimation of the volume of a 3D object (e.g., ganglion) from the measurement of the area of 2D sections of that object.** If the distance between the sections is equal then the volume of the object may be estimated by multiplying the distance between sections by the total surface area within the object, throughout the serial sections.

The unbiased probe for estimating total object number per unit volume (numerical density) on tissue sections is the dissector (Sterio, 1984). The dissector is a 3-D sampling probe with defined inclusion and exclusion lines. To perform an object count estimation, the researcher chooses a unique feature of the object (i.e. the nucleolus). The dissector probe is moved systematically through the reference volume. If the particle falls within the dissector and does not hit any exclusion lines, it is counted (see figure 2.12). Because the volume of a dissector is known, the number per volume for a sample can be determined. In a typical case, about 100 to 200 dissectors will be examined with about 1 to 2 objects in each dissector. This level of sampling is normally precise enough to yield an accurate estimate of the number density. The product of the number density and the total volume estimate gives an estimate of the total particle number.



**Fig. 2.12: A schematic diagram of a counting frame.** The counting frame, ABCD, defines the length and width of the counting box-50  $\mu\text{m}$  in both dimensions. If the particle falls within the disector and does not hit any exclusion lines, it is counted (purple), if not (blue) it is excluded. Because the volume of a disector is known, the number per volume for a sample can be determined.

A histological section is a non-representative sample of the cell population and is biased in the height-weighted distribution. For example a cell which is twice as high as another, has double the chance of being in the sample. Also, if a neuron can be estimated to be 30 $\mu\text{m}$  in diameter it is likely to appear in approximately 4 consecutive sections as the sections are only 8 $\mu\text{m}$  thick, thus it is possible that it may be counted a number of times producing a falsely large number of neurons in total. For this reason haematoxylin staining was done following  $\beta\text{III}$  tubulin staining in order to identify the nucleoli, thus providing a specific reference for the neurons in the field, as was the case when counting pyknotic or dividing nuclei. Also by only counting particles which appear in one section of the disector pair and not the other, the method provides a uniform sampling regime for cell counting in 3-D.

It is important to understand that stereology does not predict the exact quantity of a feature in a specimen. Stereology encompasses a set of methods and mathematics for the accurate estimate of the value of a quantity and the standard deviation of that quantity. The calculation of the exact value of a quantity is normally not necessary in research. Given an estimate and standard deviation, researchers can compare results to determine whether quantities are significantly different or the same statistically. For a more in depth discussion of stereological analysis see Howard and Reed, (1998).



## **2.7 Tissue Culture**

### **2.7.1 Dissections**

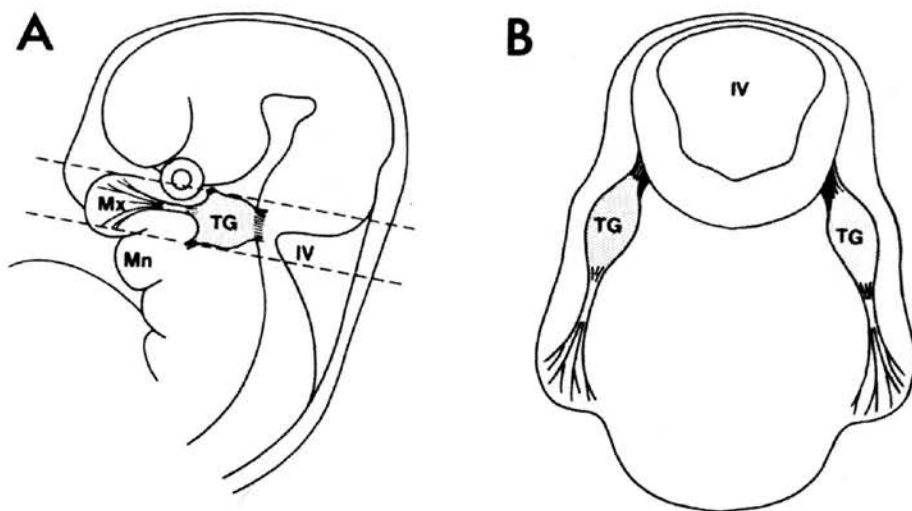
For embryo collection, the mother was killed by cervical dislocation or exposure to carbon dioxide in a rising concentration followed by cervical dislocation. The uterine horns were removed from the abdomen of the mother and transferred to a Petri dish. All procedures were carried out in a laminar flow cabinet using standard sterile technique. All the embryos collected in a 100 mm sterile Petri dish containing filter-sterilised Leibovitz L-15 medium (Gibco). All dissections were also carried out in this medium. Embryos were removed from the uterine horns by making a single continuous incision along the anti-mesometrial border of each uterine horn exposing the embryos within their membranes. These membranes were then removed with fine forceps.

After removal from the uterine horns the embryos were washed by transferring them to fresh L15, to get rid of blood on the outside of the embryos. A single incision anterior to the forelimbs was made using a tungsten needle. If the animal was neonatal, the head was washed in 70% ethanol in order to sterilise it. The required ganglia were then dissected out as described below. In the case of transgenic cultures the remaining tissue was used for DNA extraction and subsequent genotyping by PCR.

### 2.7.1a Trigeminal Ganglion

For embryos older than E13, a pair of forceps was used to steady the embryo whilst the top of the skull was cut off using fine iris scissors in a plane just above the eyes. A second cut was made parallel to the first passing through the mouth (figure 2.13). Two further cuts were made with the scissors, just in front of and just behind the trigeminal ganglion, and the ganglia were then cleaned up using tungsten needles.

The trigeminal ganglion can be seen as two opaque structures in the tissue slice obtained. The ganglia were then cut out of the tissue slice and freed of any adherent mesenchymal tissue using tungsten needles, after which they were transferred to Hank's buffered saline solution (HBSS).



**Figure 2.13: Dissection of the mouse trigeminal ganglion.** Transverse incisions are represented by interrupted lines. Mx= maxillary process; Mn= mandibular process; IV= 4th ventricle; TG= trigeminal ganglion. (From Davies, 1995).

### 2.7.1b Superior Cervical Ganglion

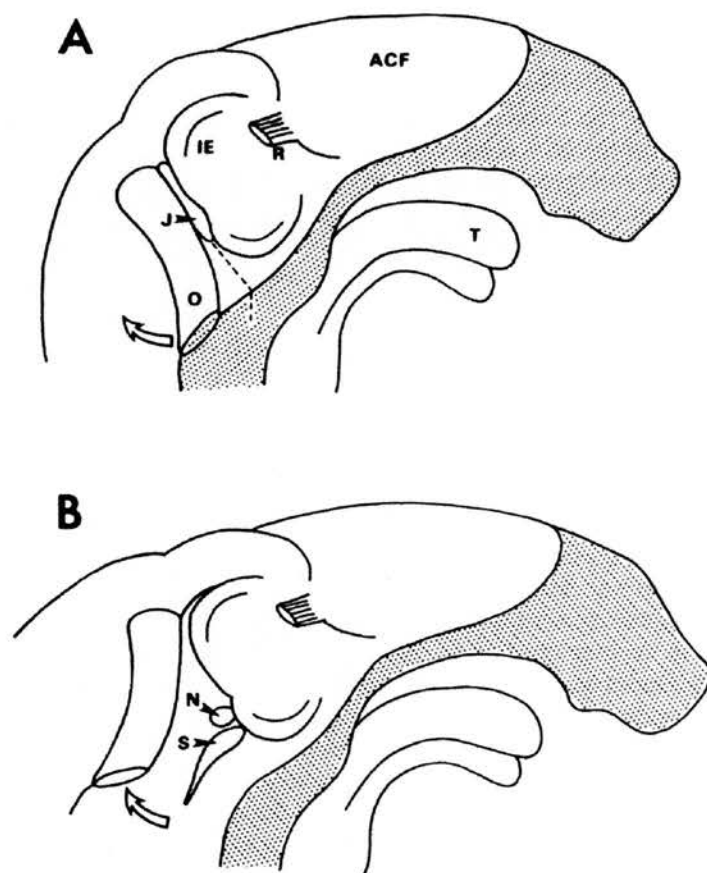
The SCG can be recognised at approximately E13.5 in the mouse, when it contains numerous mitotic precursors. By E15.5 most neurogenesis has occurred (Fagan et al., 1996) and the ganglion is mainly composed of immature sympathetic neurons. Pyknotic profiles can be seen in the ganglia of wildtype animals between E15.5 and P0 (Francis et al., 1999; Fagan et al., 1996).

In order to dissect out the SCG the top of the skull and underlying forebrain were removed by cutting in the plane just above the eye. The head was then cut in half along the sagittal plane using fine scissors. The brain was removed using tungsten needles. The slit-like jugular foramen was opened up with tungsten needles and the occipital bone was removed using fine watchmaker's forceps to reveal the SCG and nodose ganglia lying at the base of this foramen. The SCG can be seen as an opaque structure lying directly adjacent to the branched blood vessel in the tissue slice obtained (figure 2.14). The ganglia were then dissected out of the tissue and freed of any adherent mesenchymal tissue and attached nerves using the tungsten needles, before being transferred to HBSS.



### **2.7.1c Nodose Ganglion**

The top of the skull and underlying forebrain were removed using the same plane of section as described above. The head was then cut in half along the sagittal plane using fine scissors (figure 2.14). The hindbrain was removed using tungsten needles. The slit-like jugular foramen was opened up with tungsten needles to reveal the nodose ganglion lying at the base of this foramen, as with the SCG dissection. The ganglion was then dissected out and cleaned of any attached nerves using the tungsten needles, before it was transferred to HBSS.



**Figure 2.14: Dissection of the mouse superior cervical and nodose ganglia at E14.** A. The incision passing from the jugular foramen (J) to the midline is shown by an interrupted line. The direction in which the large ossified part of the occipital bone (O) should be reflected to open up the jugular foramen after making the previous incision is shown by an arrow. T= tongue; ACF= anterior cranial fossa; R= root of the trigeminal nerve; IE= inner ear. B. The superior cervical ganglion (S) and the nodose ganglion (N) are revealed. (From Davies, 1995)

## 2.7.2 Tissue Dissociation

For the purpose of this thesis, it was important that the tissue be dissociated to single cells prior to plating and low densities of cells were plated. This ensures that any observed effects are not due to paracrine signals passed through intercellular contacts but by direct effects of factors added to the dish. Using a Pasteur pipette, the dissected tissue was transferred to a sterile 10ml conical tube containing 1 to 2ml of calcium- and magnesium- free HBSS. Balanced salt solutions, such as HBSS contain a combination of salts and glucose which control osmotic pressure and pH whilst providing an energy source. Calcium and magnesium-free HBSS was used because calcium and magnesium ions can prevent the trypsin from working. The tissue was centrifuged briefly at 2000rpm, to pellet the ganglia, and the supernatant removed. 900µl of fresh HBSS and 100µl of 1% Worthington's trypsin were added, and the tube transferred to a water bath, at 37°C for the appropriate amount of time depending on the age of the tissue and the ganglia being plated. For SCG, 20 minutes trypsinisation was required at P1. The length of time necessary for trypsinisation decreases with decreasing age of the animal from which the tissue was dissected. The tube was then centrifuged briefly, and as much trypsin solution as possible removed prior to the addition of approximately 10ml of filter-sterilised Ham's F12 medium (Gibco) + 10% heat inactivated horse serum (HIHS) (Gibco). This solution inactivates any remaining trypsin. This wash in F12 +10% HIHS was repeated twice, followed by two washes in 10ml HBSS. In between the washing procedures, ganglia were gently pelleted by 2 minute centrifugations at 2000rpm to facilitate liquid removal. The ganglia were triturated in 1ml HBSS to give a single cell suspension. This was accomplished by taking the tissue up into a flame-

polished siliconised Pasteur pipette, placing the tip against the bottom of the tube, and slowly expelling the contents using firm pressure. This was done 6 to 8 times and the extent of dissociation was checked by examining a drop of the cells under inverted phase contrast microscope. The trituration had to be monitored closely as over-trituration can damage the neurons and detach neurites from their cell bodies. Also the formation of bubbles during trituration was avoided. Samples were then plated.

### **2.7.3 Plating the cells**

Neurons were grown on a laminin/polyornithine substratum in either 35mm diameter plastic Petri dishes (Nunc) or 11mm diameter wells of 4 well tissue culture dishes (Greiner, Stonehouse, UK). To prepare these plates, 1ml of 0.5mg/ml poly-DL-ornithine (Sigma) in 0.5M borate buffer (pH 8.7) was placed on each dish for 12 hours. This changes the hydrophobicity of the dish allowing the laminin to attach. After 12 hours, the polyornithine was aspirated and the dishes washed three times in sterile distilled water before being left to air-dry in a laminar flow hood. 120µl of 10µg/ml laminin (Gibco) in F14 was placed in the centre of each dish and spread with a pipette tip to cover about two-thirds of the dish surface. The dishes were then placed in a CO<sub>2</sub> incubator at 37°C for at least 4 hours. Thereafter, the dishes were washed three times with F12 + 10% HIHS and finally 1ml of F14 with Sato additives (2mM Glutamine, 0.35% bovine albumin (Pathocyte-4, ICN), 60ng/ml progesterone, 16µg/ml putrescine, 400ng/ml L-thyroxine, 38ng/ml sodium selenite, 340ng/ml tri-iodo-thyronine, 60µg/ml penicillin and 100µg/ml streptomycin) was added to each dish. At this point the

neurotrophins (NGF, BDNF) were also added to the culture dish at their required concentrations. Neurotrophins were received as concentrated stocks and were diluted in F12 + 10% HIHS, at pH 5.0, to give a more dilute stock at 1µg/ml. This stock was then used to supplement the dishes for experiments. For example, addition of 10µl of the stock neurotrophins to 2ml of culture medium gave a concentration of 5ng/ml. NGF and BDNF were received from A. Rosenthal, (Rinat Neuroscience Corporation Palo Alto, California).

The dishes were now ready for the cells to be plated. The pooled neuronal fractions were placed in a suitable volume of culture medium and rocked gently within the tube to evenly distribute the neurons within the culture medium. Two lots of 500µl of the cell suspension were added to each culture dish using a circular movement. 4 well dishes were plated at a density of 200-400 neurons per well. The culture medium was not touched by the tip of the pipette as this could transfer small amounts of neurotrophic factors between the dishes. The dishes were then placed in a CO<sub>2</sub> incubator at 37°C.

In the neurotrophin deprivation studies after an initial 12 hour incubation period with saturating concentrations of neurotrophins, NGF for SCG and trigeminal (Kessler and Black, 1980; Davies and Lumsden, 1984; Straznicky and Rush, 1985; Korsching and Thoenen, 1998; Wyatt et al., 1990) and BDNF for nodose neurons (Lindsay et al., 1985b; ElShamy and Ernfors, 1997). The neurons were washed extensively to remove this factor and were subsequently incubated with either no factors, NGF or BDNF, TNF (Calbiochem, Nottingham UK), NGF or BDNF plus TNF $\alpha$ , function blocking anti-TNF

antibody (Upstate Biotechnology, Buckingham, UK), function blocking anti-TNFR1 antibody (Oncogene Research Products, Nottingham, UK), or anti- $\beta$ III tubulin antibody (Promega, Southampton, UK). At this stage the neurons were counted to give an initial count.

To assess cell survival, the number of neurons was counted at six hours after plating to give an initial count and at regular intervals thereafter (e.g., 12, 24 and 48 hours).

Percent survival was calculated relative to the initial hour count. Each dish was centrally positioned over a 12mm by 12mm graticule, comprising 2mm squares and the number of neurons located on the grid counted using an inverted phase contrast microscope at x100. Alternatively the number of cells in each well of the four well dishes was counted. Each condition was studied in triplicate in each experiment for each embryo. Neurons were counted if they had phase-bright cell bodies.

#### **2.7.4 Immunocytochemistry**

Cells were plated in 4-well dishes, each well being of 11mm diameter. This allows plating at high density in a small area thus minimising the quantity of reagents used. After growing cells in culture in a 4% CO<sub>2</sub> incubator at 37°C, for 24 hours, the medium was carefully removed from each well with a pipette and 1.5ml of methanol at -20°C was added to each dish in order to fix the cells. This was followed by a single wash in

PBS (pH 7.2), at room temperature, for 5 minutes. The cells were permeabilised in 0.5% Triton X-100 in PBS for 10 minutes. They were then incubated overnight at 4°C with either anti-mouse TNF $\alpha$  goat polyclonal antibody (L-19, Santa-Cruz Biotechnology, Wembley, UK) or anti-mouse TNFR1 goat polyclonal antibody (Calbiochem, Nottingham, UK) diluted 1:200 in PBS. The cells were rinsed with PBS and incubated with biotinylated anti-goat rabbit antiserum diluted 1:200 in PBS for 3 hours at room temperature followed by further rinses with PBS and incubation for 30 minutes at room temperature with an avidin/biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC kit; Vector Laboratories). The substrate used for the peroxidase reaction was 1 mg/ml DAB (Sigma) in Tris-buffered saline (TBS). TNF $\alpha$  and TNFR1-positive neurons stained up brown.

## 2.8 Statistics

### 2.8.1 The t-Test

The t-test for independent groups (unpaired test) is a powerful test used to compare the difference between two given variables. It calculates the probability of getting the null hypothesis (no difference between samples). The smaller the calculated probability the more reasonable it is to accept that there is a difference. Traditionally a particular value has been set for this 'small probability' beyond which a difference can be accepted. This value is generally 0.05 or 0.01, (but may be less) and is referred to as the 'significance level'. The conventional t-test assumes that the variances (standard deviation squared) in the underlying populations from which the two samples have arisen are equal. This is not always a reasonable assumption, thus there are two versions of the t-test.

The first version is the orthodox t-test which assumes equal variances, the second is an approximate test which uses the t-distribution but does not assume that variances are equal it was the former that was used in this study. The t-test also assumes that the results are normally distributed.

It is possible to predict a difference between samples in either direction or in one specific direction. Generally if there is no prior idea of direction before starting the test



it is best to consider a difference in either direction. This is called a two-tailed test. If looking for a difference in a particular direction a one-tailed test is used. However it is essential to specify the predicted direction in advance, so that if the observed difference was not in the predicted direction it cannot be claimed to represent a genuine difference. For this reason the two-sided t-test tends to be most widely used. For more information see Campbell and Machin (1999).

### **2.8.2 The ANOVA**

The ANOVA (or analysis of variance between groups) can be thought of as an extension of the t-test to an arbitrary number of factors and levels. In this situation multiple t-tests cannot be used because as the number of groups grows, the number of required pair comparisons increases greatly. As this occurs a  $p=0.05$  for any one comparison cannot be considered significant. If  $p=0.05$  is consistently accepted then a type I error (rejection of the null hypothesis erroneously) will occur on average once in every twenty tests. This can be counteracted by setting a more stringent significance level (e.g.,  $p=0.01$ ) but in this case the risk of committing a type II error (accepting the null hypothesis erroneously) increases greatly. The ANOVA overcomes these problems.

The key statistic in ANOVA is the F-test of difference of group means, testing if the means of the groups formed by values of the independent variable (or combinations of

values for multiple independent variables) are different enough not to have occurred by chance. If the group means do not differ significantly then it is inferred that the independent variable(s) did not have an effect on the dependent variable. When the influence of two variables upon the sample mean is being analysed (e.g., age and genotype) the test is a 2-way ANOVA. When only one variable is being considered (genotype) a 1-way ANOVA is used.

Unlike regression, ANOVA does not assume linear relationships and handles interaction effects automatically. Some of its key assumptions are that the groups formed by the independent variable(s) be relatively equal in size and have similar variances on the dependent variable. Like regression, ANOVA is a parametric procedure, which assumes the dependent has a normal distribution for each value category of the independent(s).

The ANOVA test simply indicates the difference between two or more group means but does not say between which. Because of this a post hoc test is required to indicate which means there is a significant difference between. The Tukey test performs pairwise comparisons of the means to see where the significant difference is. For more information see Campell and Machin (1999).

# CHAPTER 3: PRO- AND ANTI- APOPTOTIC ACTIONS OF NF- $\kappa$ B IN THE DEVELOPING NERVOUS SYSTEM

## 3.1 Introduction

NF- $\kappa$ B is a sequence-specific DNA-binding protein complex composed of homo- or hetero-dimers of members of the Rel family. Members of the Rel family of proteins share a conserved DNA-binding and dimerisation domain called the Rel-homology domain (RHD) and are concerned with transcriptionally regulated processes (Bauerle and Baltimore, 1996). The Rel-homology domain-containing proteins are regulated by a mechanism of nuclear-cytoplasmic shuttling (Baldwin, 1996), and it is the RHD that is responsible for dimerisation and DNA binding within the nucleus (Chen et al., 1998). The RHD contains the nuclear translocation signal (NTS), and inhibitors of NF- $\kappa$ B bind to this site (reviewed in Baldwin, 1996). The Rel proteins p65 (Rel A) and p50 are known to heterodimerise to form NF- $\kappa$ B and the transactivation potential of NF- $\kappa$ B is provided by the p65 subunit (reviewed by Miyamoto and Verma, 1995). In most cell types NF- $\kappa$ B is maintained in its inactive form in the cytosol due to its interaction with one of a group of inhibitory proteins. These include I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\gamma$ , I $\kappa$ B $\epsilon$ , p105 and p100. The p50/p65 NF- $\kappa$ B complex is primarily inhibited by I $\kappa$ B $\alpha$  (Thompson et al., 1995; Whiteside et al., 1997). By binding to NF- $\kappa$ B the inhibitors conceal the NTS and retain the protein in the cytoplasm.

In response to various stimuli, including  $\text{TNF}\alpha$ , IL-1 and bacterial endotoxin, the I $\kappa$ B proteins are completely proteolytically degraded by a ubiquitin proteasome pathway. This activates NF- $\kappa$ B, which translocates to the nucleus where it induces the transcription of target genes (Israel, 2000). The regulation of I $\kappa$ B phosphorylation in response to NF- $\kappa$ B activating stimuli may be dependent on a complex of protein kinases called the I- $\kappa$ B kinase (IKK) complex. This is a serine specific complex that phosphorylates the N-terminal regulatory serines of I $\kappa$ Bs. The IKK tripartite complex is formed from I $\kappa$ B kinase - $\alpha$  (IKK $\alpha$ ) and - $\beta$  (IKK $\beta$ ), which are catalytically active subunits (Zandi et al 1997, 1998; Di Donato et al., 1997; Mercurio et al., 1997), together with the regulatory subunit, IKK $\gamma$  (NEMO) (Rothwarf et al., 1998; Yamaoka et al., 1998). The predominant type of IKK is an IKK $\alpha$ :IKK $\beta$  heterodimer that is associated with either a dimer or trimer of IKK $\gamma$  (Rothwarf et al., 1998).

The control of IKK activity can be described by a three state model (reviewed by Karin 1999). Initially IKK kinases are activated by upstream stimuli and they are then recruited to the IKK complex via IKK $\gamma$ . This leads to the phosphorylation of IKK $\beta$  and the activation of IKK. Through trans-autophosphorylation the activated IKK $\beta$  subunit can phosphorylate the adjacent subunit (either IKK $\alpha$  or IKK $\beta$ ), as well as other inactive IKK complexes. The active IKK complexes then phosphorylate the I $\kappa$ B proteins attached to NF- $\kappa$ B, initiating their ubiquitin-dependent proteolysis and the activation and translocation of NF- $\kappa$ B.

NF- $\kappa$ B has been widely demonstrated to act in a pro-or anti-apoptotic fashion depending upon cell type and circumstance (Lezoualc'h and Behl, 1998). It has previously been shown to modulate the survival of NGF-dependent sensory neurons that express both TrkA and p75. In this case NF- $\kappa$ B acts downstream of NGF binding to the p75 receptor (Hamanoue et al., 1999; Gentry et al., 2000). An antibody that inhibits NGF binding to p75 has been demonstrated to abolish NGF activation of NF- $\kappa$ B in cultured trigeminal neurons and to reduce the NGF survival response (Hamanoue et al., 1999). Furthermore the trigeminal neurons of mice that had a null mutation in the p65 subunit of NF- $\kappa$ B were seen to have a depressed survival response to NGF as compared to wildtype embryos. This suggests that NF- $\kappa$ B is involved in the survival response of developing trigeminal neurons to NGF when it binds to p75.

In order to determine the universality of the p65 subunit's involvement in the survival response to NGF binding of p75 in developing neurons that express Trk receptors, I carried out a detailed histological and an *in vitro* examination of the SCG of mouse embryos deficient in p65. The hypothesis proposed here is that if NF- $\kappa$ B is involved in mediating cell survival in these neurons it would be expected for their survival to be decreased when p65 is deficient.

However, NGF binding to p75 does not always elicit a survival response and in certain situations such as during the development of the spinal cord and murine retina (areas

which do not express TrkA), NGF binding to p75 initiates a death cascade (Frade and Barde, 1999,1998; Barrett, 2000). Accordingly, both anti-NGF antibodies and those that inhibit NGF binding by p75 caused a decrease in the number of these neurons undergoing apoptosis (Frade et al., 1996). Also detailed histological analysis of NGF and p75-deficient mice has shown a decrease in the magnitude of cell death in these regions during development in comparison to their wildtype littermates (Frade and Barde, 1999) suggesting that apoptosis is mediated by NGF, acting through p75. Apoptotic nuclei were observed primarily in the mantle zone of the spinal cord and in the region of the optic disc and optic nerve of the retina. This suggests that this cell death could serve the purpose of creating space for axonal growth. It is possible that as with NGF induced cell survival in TrkA expressing neurons described above, NF- $\kappa$ B may be involved in this suggested NGF-induced, p75-mediated apoptosis in the spinal cord and retina.

I wanted to ascertain whether the p65 subunit of NF- $\kappa$ B acts downstream of NGF binding to the p75 receptor in order to bring about cell death in neurons that do not express TrkA, as it does in those that do in order to bring about cell survival. I carried out detailed histological analyses of the E12 spinal cord and E14.5 retina of mouse embryos that were deficient in p65, NGF or both. E12 spinal cords were examined as the period between E10.5 and E13.5 is when commissural axons grow towards the floor plate within the mouse spinal cord (Serafini et al., 1996) and therefore this is the period in which the peak of cell death occurs to create space for the growth of these axons.

The retina was examined in E14.5 embryos as although peak levels of apoptosis were observed between E15.5 and E17.5 (Frade and Barde, 1999), the p53-deficient embryos are non-viable after E14.5. If NF- $\kappa$ B is acting downstream of an NGF-p75 interaction to bring about cell death then a decrease in cell death can be expected in the spinal cord and retina of p53 deficient mice.

Additionally, I carried out the same studies in the spinal cords and retinas of embryos that had targeted null mutations in either the IKK $\alpha$  or IKK $\beta$  subunit of the IKK complex, which is part of the signalling cascade that leads to NF- $\kappa$ B activation (Karin and Ben-Neriah, 2000). This was in order to verify the involvement of the NF- $\kappa$ B pathway in the observed responses to NGF. If NF- $\kappa$ B is involved in inducing cell death in these regions the levels of cell death will be reduced in the spinal cord and retina of IKK $\alpha$  and IKK $\beta$  deficient mice as NF- $\kappa$ B cannot be activated in the absence of these proteins.

## 3.2 Results

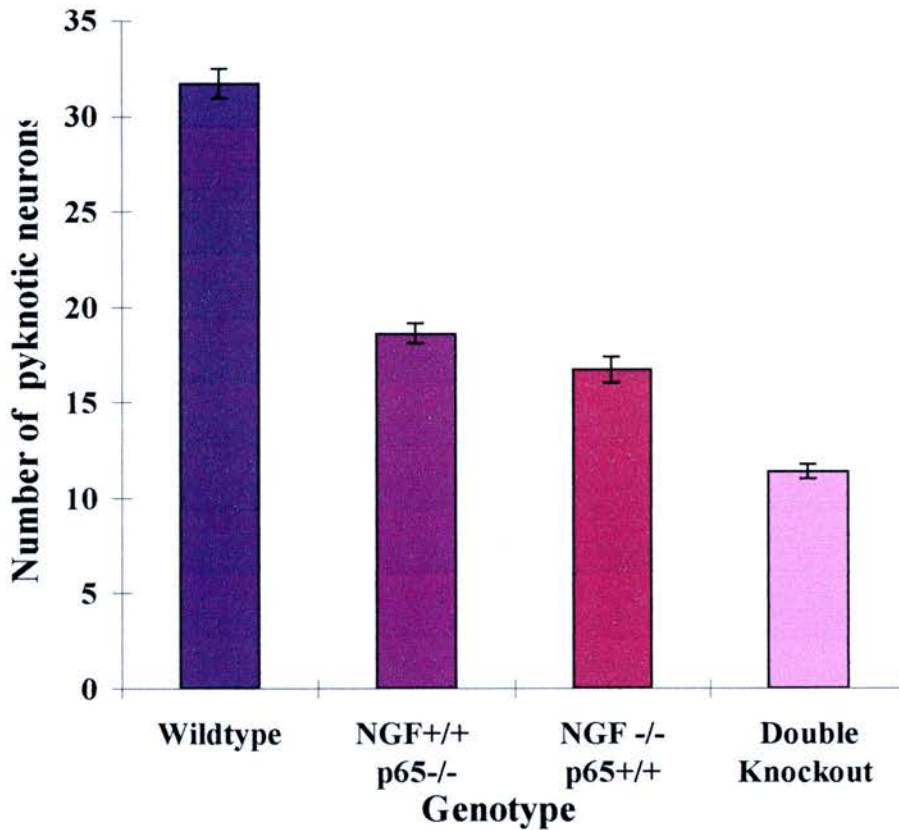
### 3.2.1 p65 and NGF act Independently to Induce Cell Death of Motoneurons in the Spinal Cord

To determine the level of cell death at E12 in the spinal cord I counted the mean number of pyknotic neurons per 8 $\mu$ m serial section at a defined level of cervical spinal cord (C3-5) in embryos that had a specific null mutation in the p65 subunit of NF- $\kappa$ B ( $p65^{-/-}$   $NGF^{+/+}$ ), NGF ( $p65^{+/+}$   $NGF^{-/-}$ ), both alleles ( $p65^{-/-}$   $NGF^{-/-}$ ) or wild type control litter-mates ( $p65^{+/+}$   $NGF^{+/+}$ ). Embryos were fixed, embedded and serially sectioned and stained for cresyl fast violet (CFV). All histology slides were coded so that observer bias could not influence the results. At E12 in the  $p65^{-/-}$   $NGF^{+/+}$  spinal cord there were approximately 47% less dying neurons than in wild type animals ( $p65^{+/+}$   $NGF^{+/+}$ ) (figure 3.1 and table 3.1) (significant,  $p < 0.0001$ , one way ANOVA with Tukey post hoc multiple comparisons test). In the  $p65^{+/+}$   $NGF^{-/-}$  spinal cord there were 41% less dying neurons observed than in the wild type (significant,  $p < 0.0001$ , one way ANOVA with Tukey post hoc multiple comparisons test). The differences between the number of dying neurons in the p65-deficient and NGF-deficient spinal cord were not statistically significant (1 way ANOVA with Tukey post hoc multiple comparisons test). However in the absence of both p65 and NGF there were 64% fewer dying neurons observed in the developing spinal cord than in wild type litter-mates. This difference was highly statistically significant ( $p < 0.0001$ , one way ANOVA with Tukey post hoc multiple comparisons test)



and the reduction in the magnitude of cell death in the spinal cord of p65 and NGF doubly deficient animals as compared to those deficient in either p65 or NGF alone was also significant ( $p < 0.0001$ , one way ANOVA with Tukey post hoc multiple comparisons test).

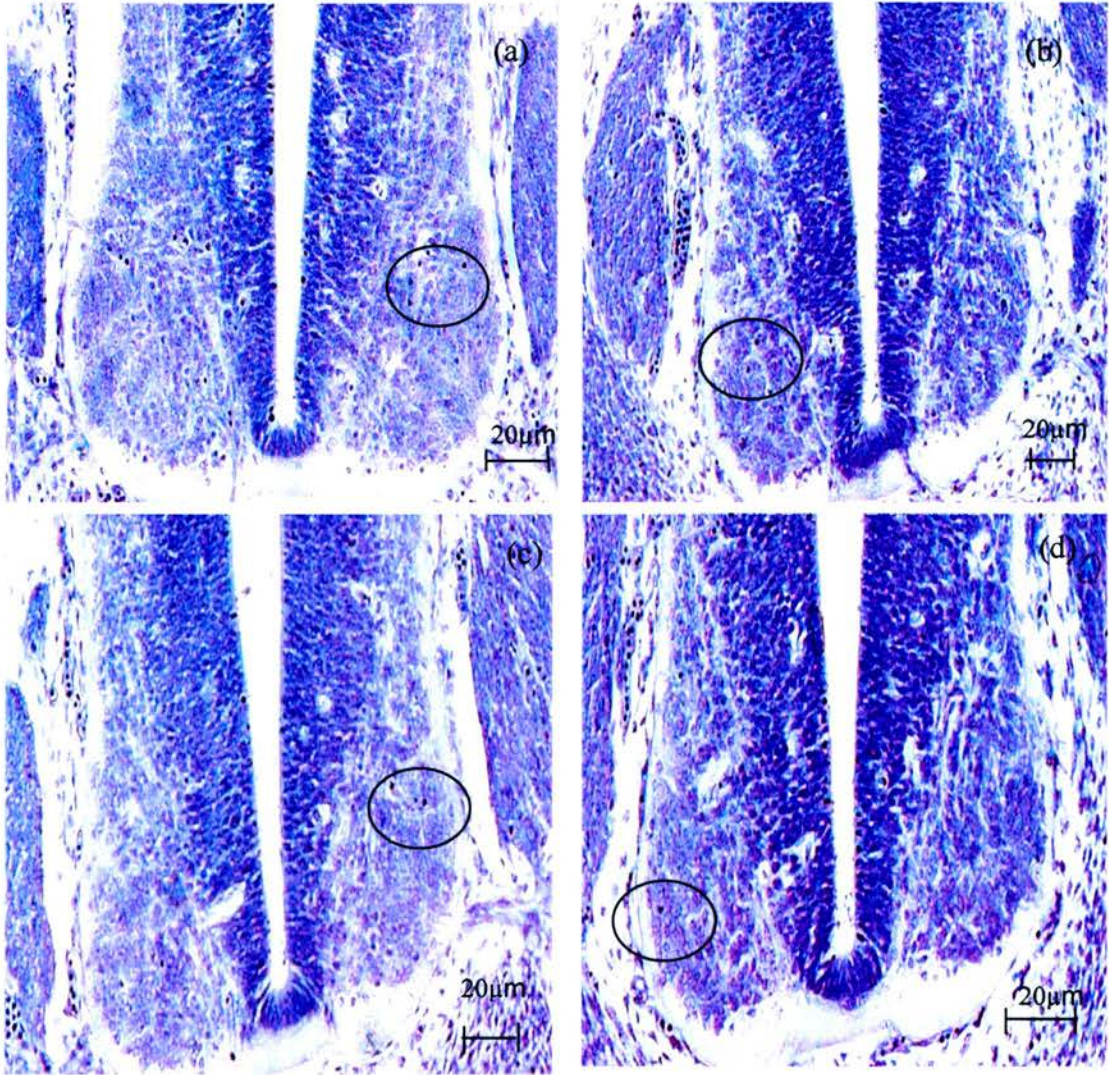
## E12 Spinal Cord: Pyknotic Neurons



**Figure 3.1:** Bar chart showing the number of pyknotic neurons in cross-sections of cervical spinal cord in E12 wildtype,  $p65^{-/-}NGF^{+/+}$ ,  $p65^{+/+}NGF^{-/-}$ , and double knockout embryos. The means and standard errors of 5 mice of each genotype are shown. The number of pyknotic neurons, and therefore cell death, are reduced by 47% and 41% in the spinal cords of p65 deficient and NGF deficient embryos respectively compared to their wildtype littermates. This apoptosis is further decreased in the double knockout, being reduced by 64%.

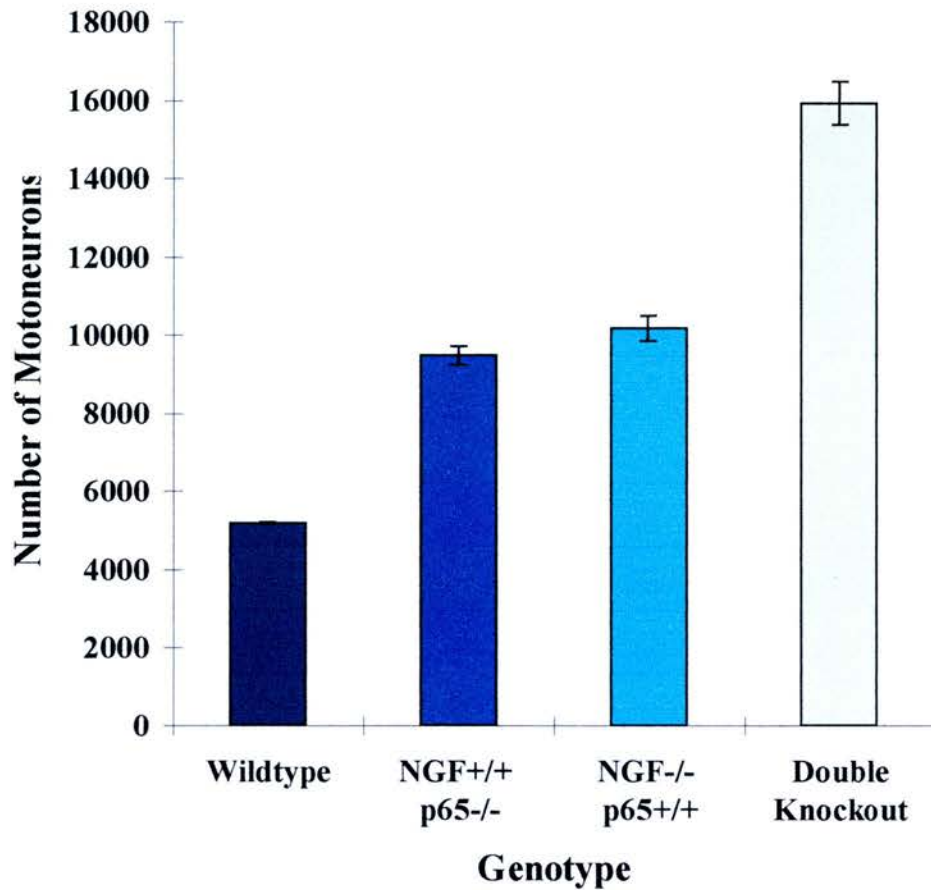
As the pyknotic neurons were primarily seen to be in the region of the ventral horn (see figure 3.2), I hypothesised that they were probably motoneurons. Also it has previously been shown by Islet-1 staining that in the spinal cords of NGF-deficient mice where there are decreases in apoptosis some of the supernumerary cells were motoneurons (Frade and Barde, 1999). In order to determine whether or not the dying cells were motoneurons I carried out an immunohistochemical stain to identify all cells that are positive for the motoneuron-specific marker Islet-1 and used digital stereology to determine the number of motoneurons in a segment of the spinal cord at E12 stretching from C3 to C5. This level was determined by first locating the first DRG and thereby positioning the first cervical segment from which point the third to fifth could be ascertained. In accordance with the differences in the magnitude of cell death, there were approximately 45% and 49% more Islet-1 positive neurons observed in the spinal cord of  $p65^{-/-}NGF^{+/+}$  and  $p65^{+/+}NGF^{-/-}$  mice respectively and 67% more Islet-1 positive neurons in  $p65^{-/-}NGF^{-/-}$  mice as compared to wild type controls (figure 3.3, 3.4 and table 3.1). All results were highly statistically significant ( $p < 0.0001$ , one way ANOVA with Tukey post hoc multiple comparisons test). Some Islet-1 positive cells can be seen to be present in the upper section of the spinal cord these are probably motoneurons in the sympathetic lateral horn (figure 3.4).





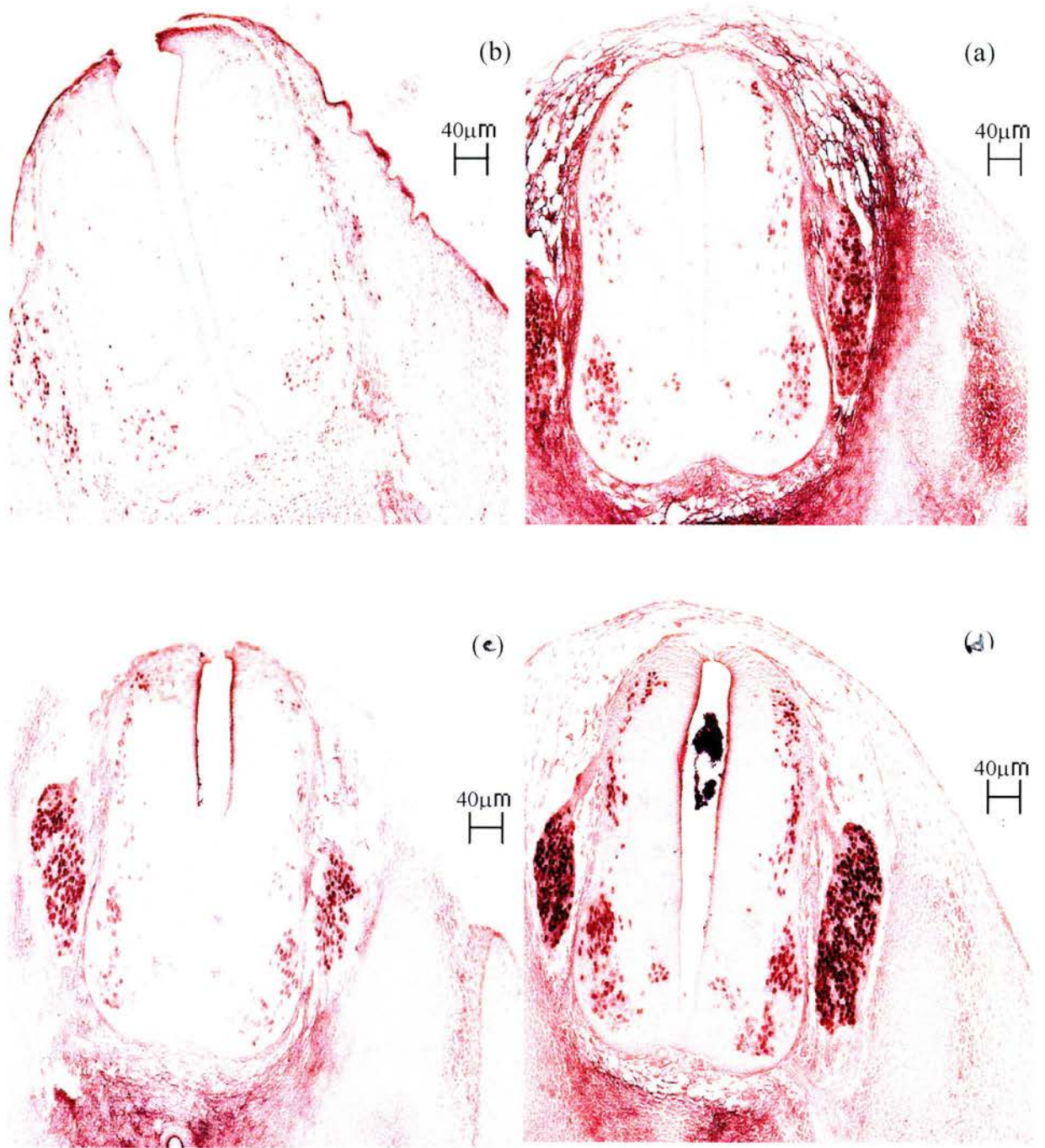
**Figure 3.2: Photomicrograph showing the pyknotic neurons to be primarily in the ventral horn of the E12 spinal cord. The number of pyknotic neurons in the spinal cord decreases from a) wildtype to b) p65<sup>-/-</sup> and c) NGF<sup>-/-</sup> and then further to d) p65<sup>-/-</sup>:NGF<sup>-/-</sup>. Examples of pyknotic neurons are ringed.**

## E12 Spinal Cord: Motoneurons



**Figure 3.3:** Bar chart showing the number of motoneurons in cervical spinal cord segments in E12 wildtype,  $p65^{-/-}NGF^{+/+}$ ,  $p65^{+/+}NGF^{-/-}$ , and double knockout embryos. This was determined by counting the total number of Islet-1 positive cells at the level of C3 to C5. The means and standard errors of 5 mice of each genotype are shown. The number of Islet-1 positive neurons, and therefore motoneurons, are increased by 45% and 49% in the spinal cords of p65 deficient and NGF deficient embryos respectively compared to their wildtype littermates. This apoptosis is further increased in the double knockout, being increased by 67%.





**Figure 3.4: Photomicrograph showing the motoneurons in the E12 spinal cord.** The number of motoneurons in the spinal cord increases from a) wildtype to b) p65<sup>-/-</sup> and c) NGF<sup>-/-</sup> and then further to d) p65<sup>-/-</sup>:NGF<sup>-/-</sup>.

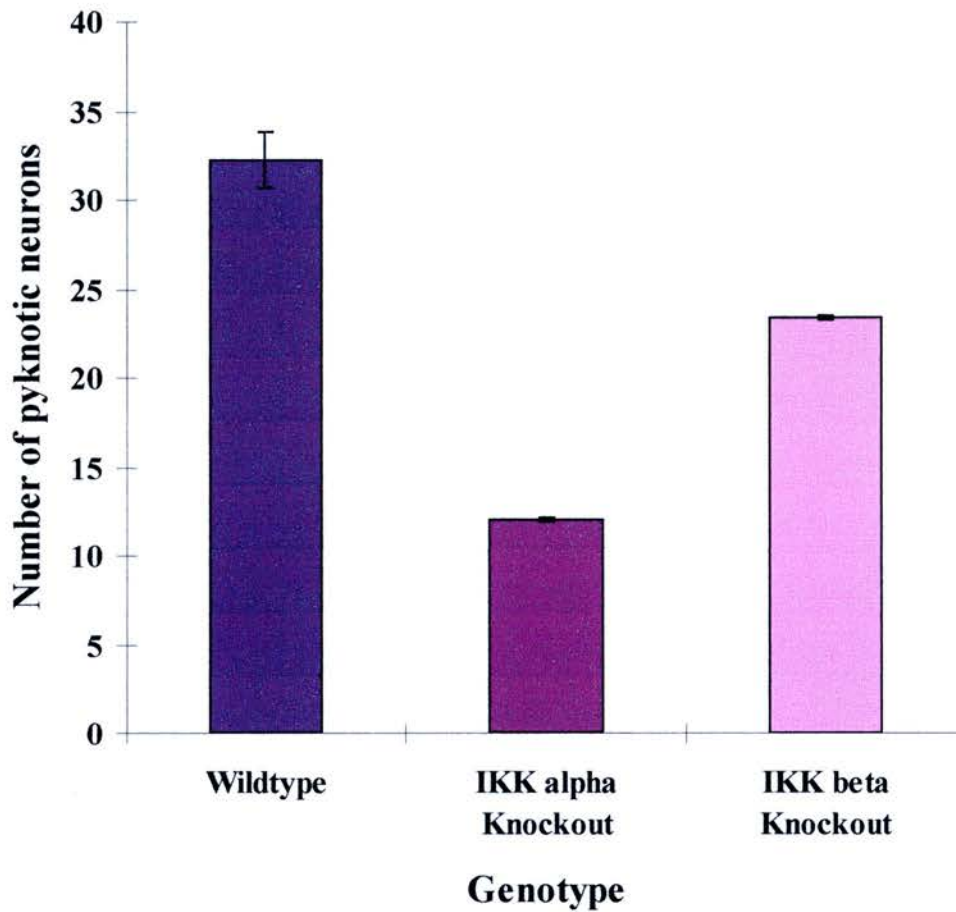
Taken together these data suggest that NF- $\kappa$ B is important in bringing about the death of spinal motoneurons at E12 which represents a time at which NGF binding to p75 is bringing about cell death in this same neuronal population (Frade and Barde, 1999). However, the additive effect observed when both the p65 subunit of NF- $\kappa$ B and the p75 ligand NGF are knocked out suggests that p75 and NF- $\kappa$ B are inducing cell death as part of two different pathways.

### 3.2.2 IKK $\alpha$ and IKK $\beta$ act Upstream of NF- $\kappa$ B in the Induction of Motoneuron Cell Death in the Spinal Cord

Similar experiments to those described above were carried out to discover the role of the IKK complex that is upstream of NF- $\kappa$ B in cell death in the E12 spinal cord. The mean number of pyknotic neurons at the same level of cervical spinal cord as previously studied was determined for mice with specific null mutations in either the IKK $\alpha$  or IKK $\beta$  subunit of IKK and was compared to the number of dying neurons observed in wild type embryos. All histology slides were coded so that observer bias could not influence the results. There was a significant decrease in the magnitude of cell death and a significant increase in the number of Islet-1 positive cells in the spinal cord of IKK $\alpha$ <sup>-/-</sup> (cell death - p<0.0001, unpaired t-test; Islet-1 - p<0.0001, unpaired t-test) and IKK $\beta$ <sup>-/-</sup> (cell death - p<0.0001, unpaired t-test; Islet-1 - p<0.0001, unpaired t-test) mice as compared to wild types (figures 3.5-3.7 and table 3.1). The decrease in cell death observed in the IKK $\beta$ <sup>-/-</sup> spinal cords was less than that found in the IKK $\alpha$ <sup>-/-</sup> spinal cord, which was comparable to that observed in *p65*<sup>-/-</sup>*NGF*<sup>+/+</sup> mice. Consistent with this the increase in Islet-1 positive cells in the IKK $\beta$ <sup>-/-</sup> spinal cords was smaller than in the IKK $\alpha$ <sup>-/-</sup> spinal cord. This suggests that the influence of the IKK $\alpha$  subunit of the IKK complex in bringing about cell death in the developing spinal cord is greater than that of the IKK $\beta$  subunit. These results also indicate that cell death is achieved by the ultimate activation of NF- $\kappa$ B following the targeting of I $\kappa$ B $\alpha$  for degradation by the IKK complex. Some Islet-1 positive cells can be seen to be present in the upper section of the spinal cord these are probably motoneurons in the sympathetic lateral horn (figure 3.7).

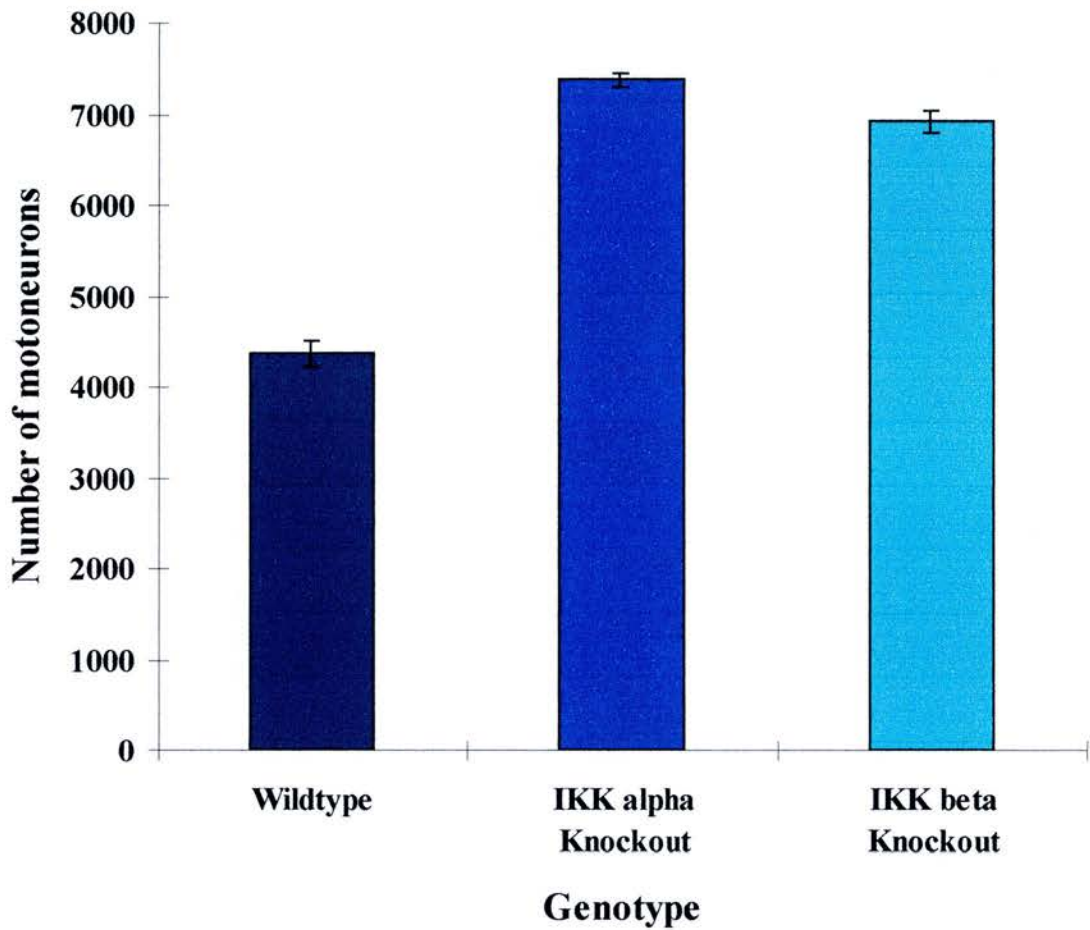


### E12 Spinal Cord: Pyknotic Neurons

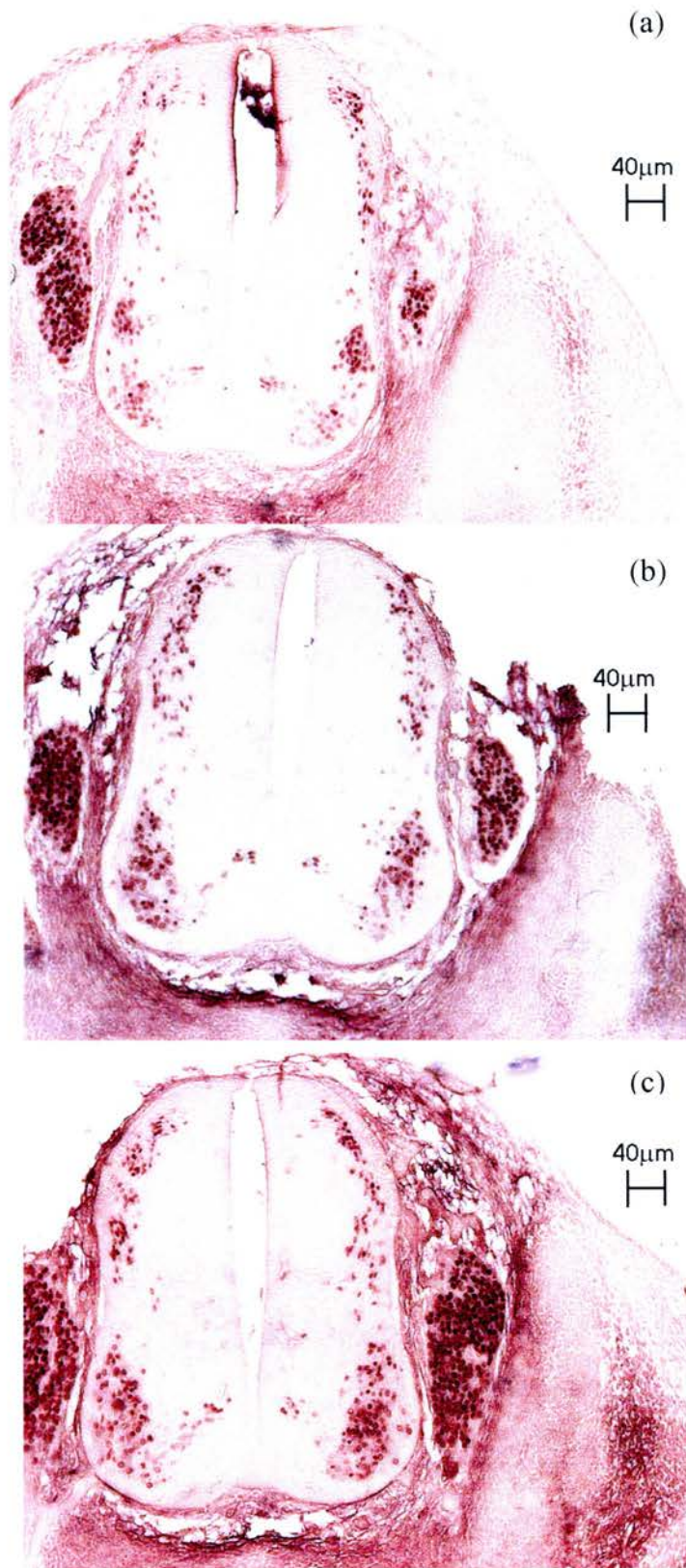


**Figure 3.5:** Bar chart showing the number of pyknotic neurons in cross-sections of cervical spinal cord in E12 wildtype,  $IKK\alpha$  and  $IKK\beta$  knockout mice. The means and standard errors of 5 embryos of each genotype are shown. The number of pyknotic neurons, and therefore cell death, are reduced significantly in the spinal cords of  $IKK\alpha$  and  $IKK\beta$  deficient embryos compared to their wildtype littermates. This apoptosis is decreased to a greater extent in the *ikk $\alpha$*  knockout than in the *ikk $\beta$*  knockout indicating that  $IKK\alpha$  has a greater influence on cell death.

### E12 Spinal Cord: Motoneurons



**Figure 3.6: Bar chart showing the number of motoneurons in cross-sections of cervical spinal cord in E12 wildtype, IKK $\alpha$  or IKK $\beta$  knockout mice.** This is determined by counting the total number of Islet-1 positive cells at the level of C3 to C5. The means and standard errors of 5 embryos of each genotype are shown. The number of Islet-1 positive cells, and therefore motoneurons, are increased significantly in the spinal cords of IKK $\alpha$  and IKK $\beta$  deficient embryos compared to their wildtype littermates. This apoptosis is increased to a lesser extent in the *ikk $\alpha$*  knockout than in the *ikk $\beta$*  knockout indicating that IKK $\alpha$  has a greater influence on cell death



**Figure 3.7: Photomicrograph showing the motoneurons in the E12 spinal cord.** The number of motoneurons in the spinal cord increases from a) wildtype to b)  $IKK\alpha^{-/-}$  and c)  $IKK\beta^{-/-}$ .

	<b>Pyknotic Neurons</b>	<b>Standard Error</b>	<b>Islet-1 positive Neurons</b>	<b>Standard Error</b>
<b>Wildtype</b>	31.7	0.77	5200	35.16
<b>NGF+/+: p65-/-</b>	18.6	0.51	9480	234.65
<b>NGF-/-: p65+/+</b>	16.7	0.68	10173	310.33
<b>NGF-/-: p65-/-</b>	11.37	0.36	15920	551.5
<b>IKK<math>\alpha</math>-/-</b>	12.03	0.11	6912	111.9
<b>IKK<math>\beta</math>-/-</b>	23.4	0.09	7377	70.37

**Table 3.1: Summary of spinal cord results.** The means and standard errors for pyknotic neurons (dying neurons) and Islet-1 positive neurons (motoneurons) are shown for NGF, p65, IKK $\alpha$ , IKK $\beta$  and NGF/p65 deficient and wildtype embryos. Embryos deficient in all these proteins show decreased levels of cell death and increased numbers of motoneurons compared to wildtype embryos.



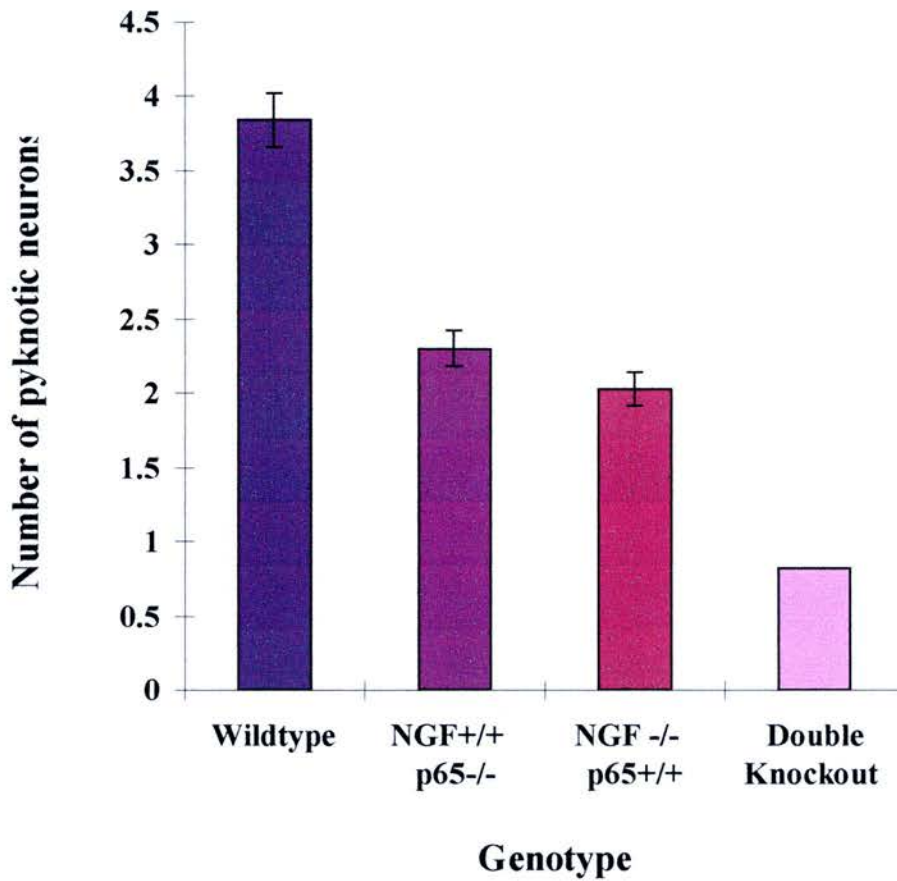
### 3.2.3 p65 and NGF act Independently to Induce Cell Death of Neurons in the Retina

To ascertain the generality of the finding that NF- $\kappa$ B and NGF binding to p75 can both bring about apoptosis in the same neuronal populations but by different mechanisms, I extended my study to the developing retina where neurons also die due to the pro-apoptotic effects of NGF binding to p75 (Frade and Barde, 1999). In the retina of E14.5  $p65^{-/-}NGF^{+/+}$ ,  $p65^{+/+}NGF^{-/-}$ ,  $p65^{-/-}NGF^{-/-}$  and wildtype mice I counted the mean number of pyknotic neurons per 8 $\mu$ m section in serial sections at the level of the optic cup (figures 3.8, 3.9 and table 3.2) and confirmed the findings using TUNEL staining (figure 3.10). Embryos were fixed, embedded and serially sectioned through the eye and CFV and TUNEL stained. All histology slides were coded so that observer bias could not influence the results.

There was a significant reduction in the number of pyknotic neurons in the developing retina of  $p65^{-/-}NGF^{+/+}$  and  $p65^{+/+}NGF^{-/-}$  animals as compared to their wild type littermates ( $p < 0.0001$ , one way ANOVA with Tukey post hoc multiple comparisons test). Furthermore there were significantly less dying neurons in the developing retina of  $p65^{-/-}NGF^{-/-}$  mice as compared to the other three genotypes studied (figure 3.8 and table 3.2) ( $p < 0.0001$ , one way ANOVA with Tukey post hoc multiple comparisons test). This suggests that there is an additive effect of knocking out  $p65$  and  $ngf$  and therefore that these two molecules are not acting exclusively as part of the same signalling pathway, although they may both contribute to a common pathway. These findings were

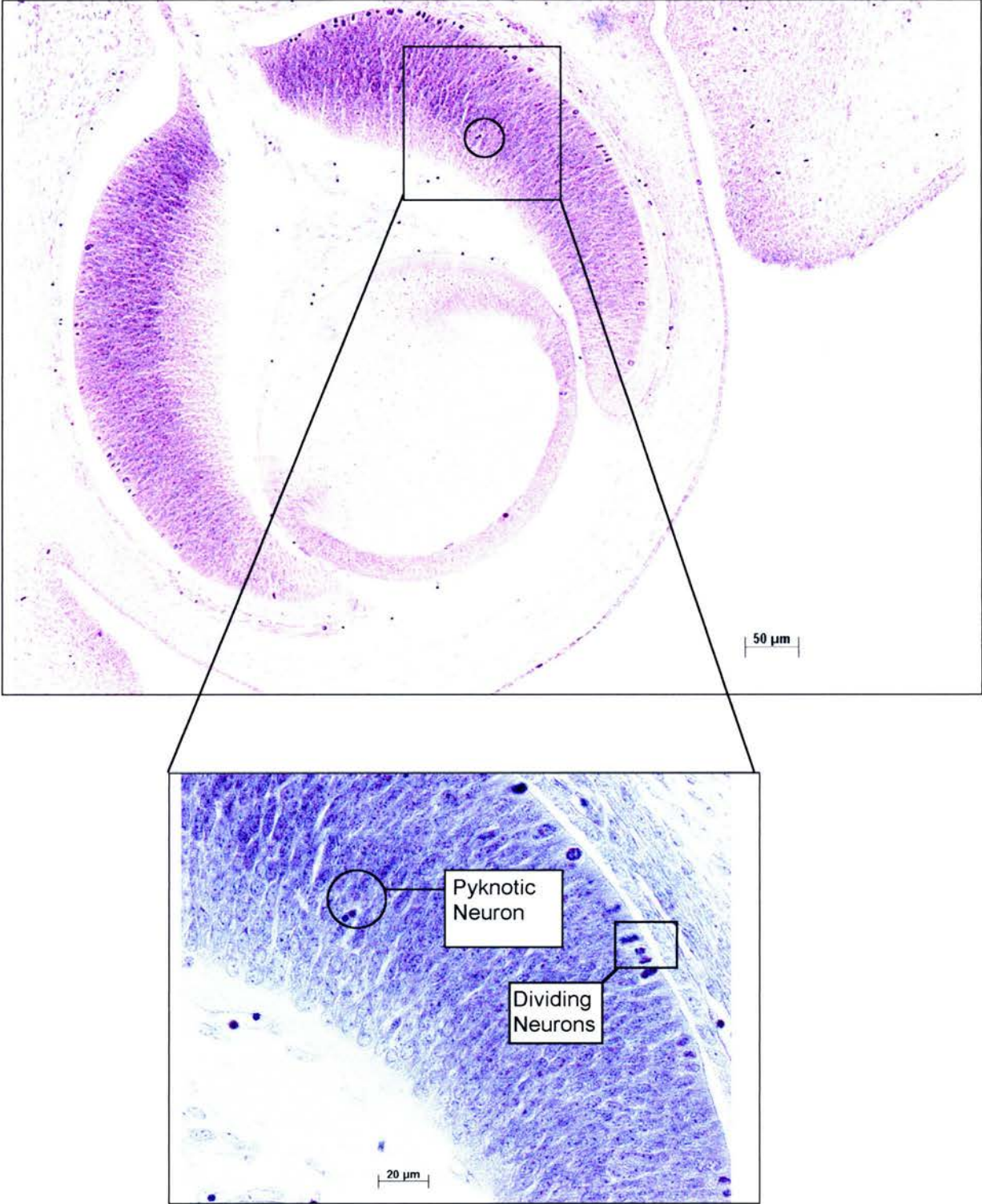
supported by TUNEL staining (figure 3.10) in which fewer TUNEL positive cells were seen in the retina of  $p65^{-/-}NGF^{+/+}$  and  $p65^{+/+}NGF^{-/-}$  animals as compared to their wild type litter-mates and even fewer were seen in  $p65^{-/-}NGF^{-/-}$  mice. The numbers of apoptotic cells determined by TUNEL staining were seen to be similar to the numbers of pyknotic neurons found in the retina. It should be noted that these two methods label cells at different stages of apoptosis and that apoptosis is a very short lived process with high rates of clearance. This means that it is very difficult to quantify total levels of apoptosis and only relative levels at a given stage can be examined.

## E14.5 Retina: Pyknotic Neurons

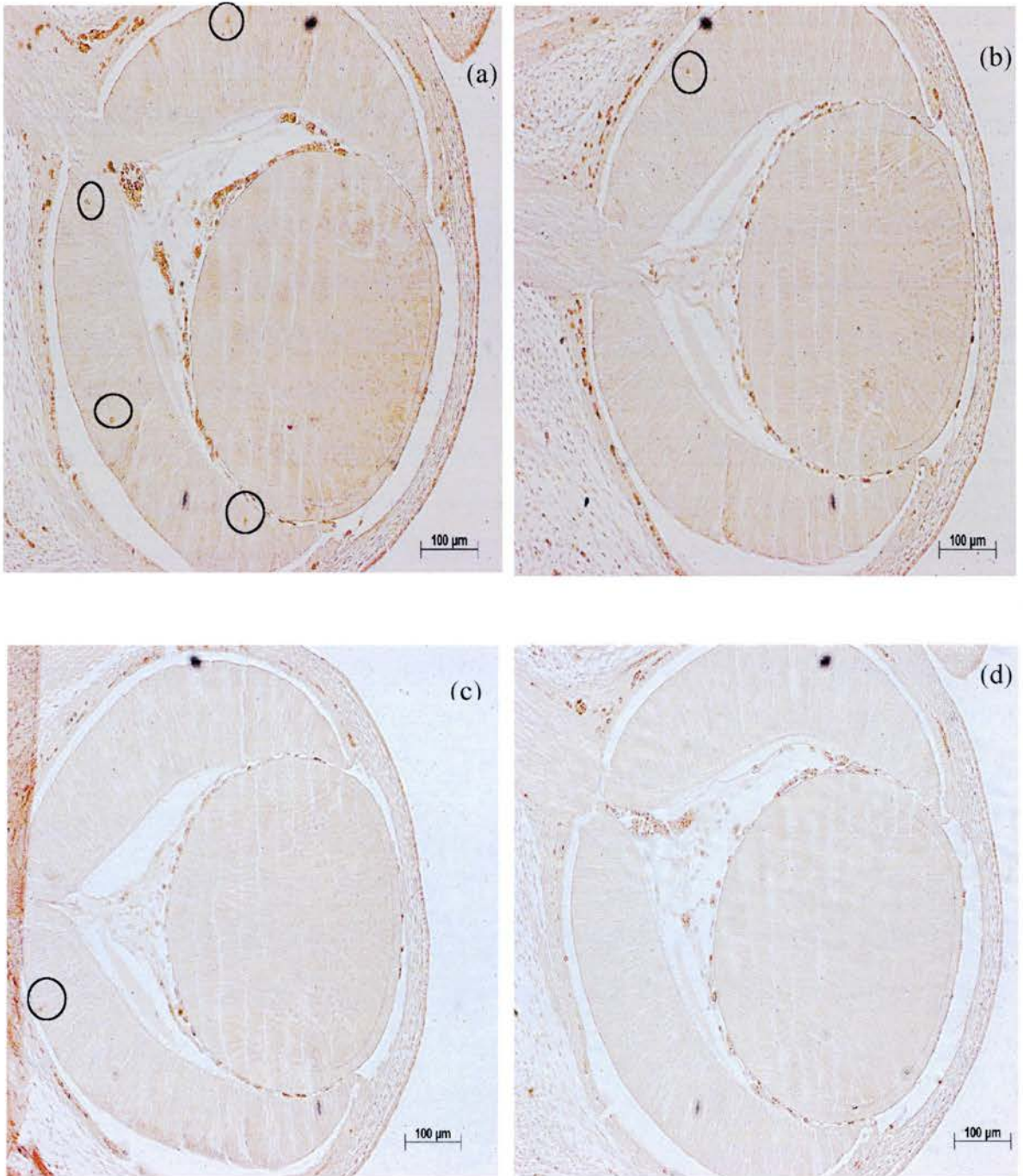


**Figure 3.8:** Bar chart showing the number of pyknotic neurons in cross-sections of developing retina in E14.5 wildtype,  $p65^{-/-}NGF^{+/+}$ ,  $p65^{+/+}NGF^{-/-}$  and double knockout embryos. The means and standard errors of 5 embryos of each genotype are shown. The number of pyknotic neurons, and therefore cell death, is reduced significantly in the retinas of p65 deficient and NGF deficient embryos compared to their wildtype littermates. This apoptosis is further decreased in the double knockout.





**Figure 3.9: Photomicrograph showing an example of a pyknotic neuron at low and high power in the retina. The pyknotic neuron is ringed.**



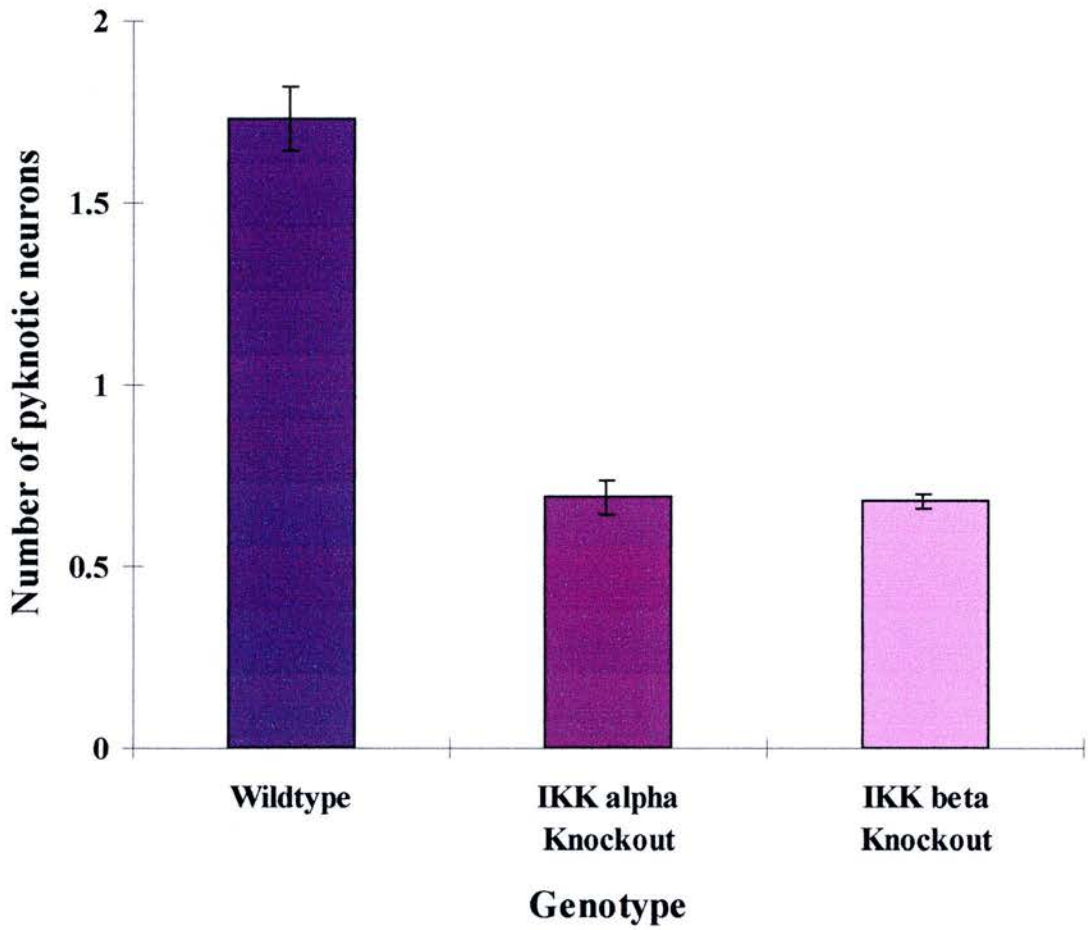
**Figure 3.10: Photomicrograph showing the TUNEL positive cells in the E14.5 retina.** TUNEL positive cells are ringed. The number of TUNEL positive cells in the retina decreases from a) wildtype to b) p65<sup>-/-</sup> and c) NGF<sup>-/-</sup> and then further to d) p65<sup>-/-</sup>:NGF<sup>-/-</sup>.



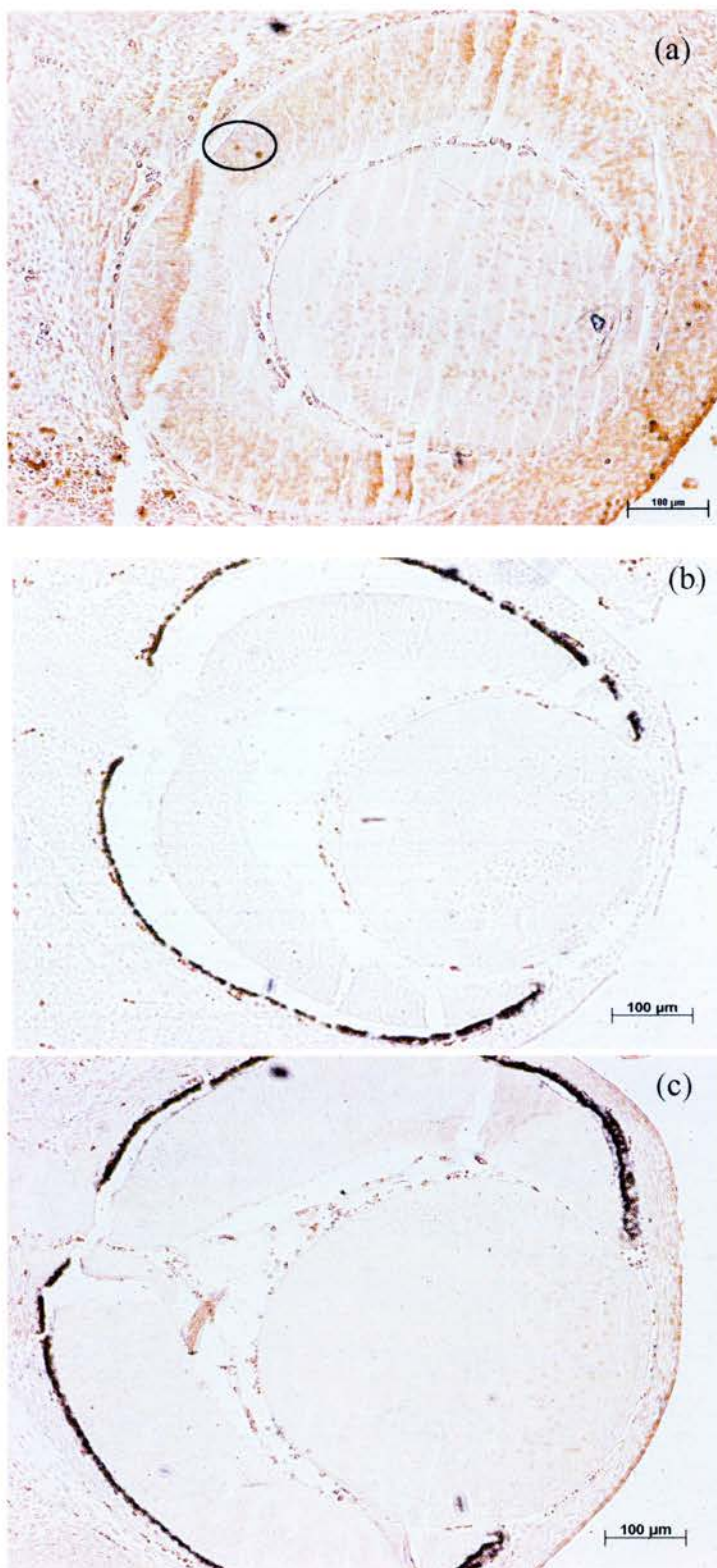
### 3.2.4 IKK $\alpha$ and IKK $\beta$ act Upstream of NF- $\kappa$ B in the Induction of Neuronal Cell Death in the Retina

I tried to elucidate the role of the IKK complex, which acts upstream of NF- $\kappa$ B, in bringing about apoptosis in the developing retina. Deletion of either the IKK $\alpha$  or IKK $\beta$  subunit of IKK significantly reduced the magnitude cell death in the retina by approximately 60% (*IKK $\alpha$* <sup>-/-</sup> -  $p < 0.0001$ , unpaired T-test; *IKK $\beta$* <sup>-/-</sup> -  $p < 0.0001$ , unpaired t-test) and both subunits were equally effective in doing so (figure 3.11 and table 3.2). This was confirmed by TUNEL staining (figures 3.12). These findings suggest that both IKK $\alpha$  and IKK $\beta$  are involved in and even required for cell death in the developing retina. This is probably due to the activation of NF- $\kappa$ B following targeting of the I $\kappa$ B subunit for degradation by the IKK complex.

### E14.5 Retina: Pyknotic Neurons



**Figure 3.11:** Bar chart showing the number of pyknotic neurons in cross-sections of developing retina in E14.5 wildtype,  $IKK\alpha$  and  $IKK\beta$  knockout mice. The means and standard errors of 5 embryos of each genotype are shown. The number of pyknotic neurons, and therefore cell death, is reduced significantly in the retinas of  $IKK\alpha$  and  $IKK\beta$  deficient embryos compared to their wildtype littermates.



**Figure 3.12:**  
**Photomicrograph**  
**showing the TUNEL**  
**positive cells in the E14.5**  
**retina.** TUNEL positive  
cells are ringed. The  
number of TUNEL positive  
cells in the retina decreases  
from a) wildtype to  
b)  $IKK\alpha^{-/-}$  and c)  $IKK\beta^{-/-}$ .

	<b>Pyknotic Neurons</b>	<b>Standard Error</b>
<b>Wildtype</b>	31.7	0.77
<b>NGF+/+: p65-/-</b>	18.6	0.51
<b>NGF-/-: p65+/+</b>	16.7	0.68
<b>NGF-/-: p65-/-</b>	11.37	0.36
<b>IKK<math>\alpha</math>-/-</b>	12.03	0.11
<b>IKK<math>\beta</math>-/-</b>	23.4	0.09

**Table 3.2: Summary of retina results.** The means and standard errors for pyknotic neurons (dying neurons) are shown for NGF, p65, IKK $\alpha$ , IKK $\beta$  and NGF/p65 deficient and wildtype embryos. Embryos deficient in all these proteins show decreased levels of cell death compared to wildtype embryos.

### 3.2.5 p65 acts Downstream of NGF to Induce Cell Survival in Sympathetic Neurons

In contrast to the above findings, NF- $\kappa$ B has recently been shown to play a role in signalling survival in response to certain neurotrophic factors (Bui et al., 2001; Foehr et al., 2000; Hamanoue et al., 1999; Maggirwar et al., 1998; Middleton et al., 2000a). In order to ascertain the potential role of NF- $\kappa$ B in mediating the response of sympathetic neurons of the SCG (which express both TrkA and p75 receptors), to NGF, I quantified the total number of surviving and dying neurons in the SCG of wildtype embryos and *p65*<sup>-/-</sup> or *p65*<sup>+/-</sup> embryos during the period of development when SCG neurons are dependent on NGF. Embryos were fixed, embedded and serially sectioned through the SCG and stained for CFV and  $\beta$ III-tubulin to identify pyknotic and surviving neurons respectively. The total number of neurons and the number of neurons with pyknotic nuclei in the SCG were counted using digital stereology. All histology slides were coded so that observer bias could not influence the results.

In the SCG of E14 *p65*<sup>-/-</sup> embryos there was a three fold increase in the number of pyknotic nuclei (and therefore dying neurons) ( $p < 0.0005$ , unpaired t-test) and a 44% decrease in the total number of neurons compared to the wildtype embryos in the same litter ( $p < 0.005$ , unpaired t-test) (figures 3.13, 3.14 and table 3.3). In the SCG of E16 *p65*<sup>+/-</sup> mice there was a three fold increase in the number of dying neurons ( $p < 0.0005$ , unpaired t-test) and there was a 8% reduction in the total number of neurons (non-significant, unpaired t-test) as compared to the wildtype embryos (figures 3.13, 3.14 and

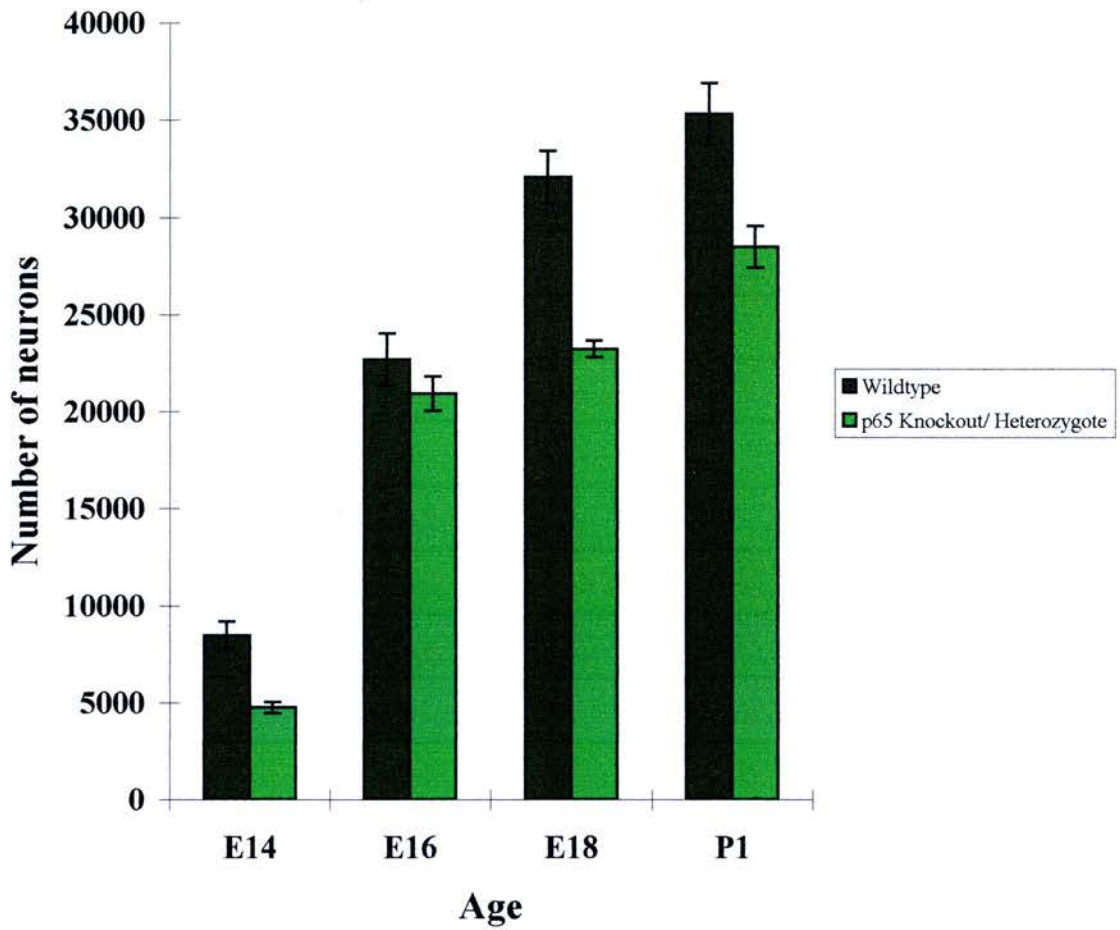


table 3.3). Similarly comparisons of the SCG of  $p65^{+/-}$  and wildtype embryos at E18 revealed an almost five fold increase in the number of dying neurons in the heterozygous embryos ( $p < 0.005$ , unpaired t-test) and a 27% decrease in the total number of surviving neurons ( $p < 0.001$ , unpaired t-test) (figures 3.13, 3.14 and table 3.3). At P1 there was a two fold increase in the number of dying neurons ( $p < 0.0005$ , unpaired t-test) and a 19% reduction in the number of surviving neurons in the  $p65^{+/-}$  as compared to the wildtype ( $p < 0.005$ , unpaired t-test) (figures 3.13, 3.14 and table 3.3). It should be noted that at E16, E18 and P1 heterozygote (+/-) embryos were compared to wildtype embryos whereas homozygote (-/-) embryos were used at E14. This is because  $p65^{-/-}$  embryos do not survive beyond E14.5 *in utero*.

In both neurotrophin treated and neurotrophin deprived low density cultures, there were significant decreases in cell survival in  $p65^{+/-}$  embryos in NGF-dependent sympathetic SCG neurons at E16 [38% increase in neurons grown with a saturating concentration of NGF(2ng/ml) ( $p < 0.0001$ , unpaired t-test) and 24% increase in NGF deprived cultures ( $p < 0.0001$ , unpaired t-test)] (figure 3.15).

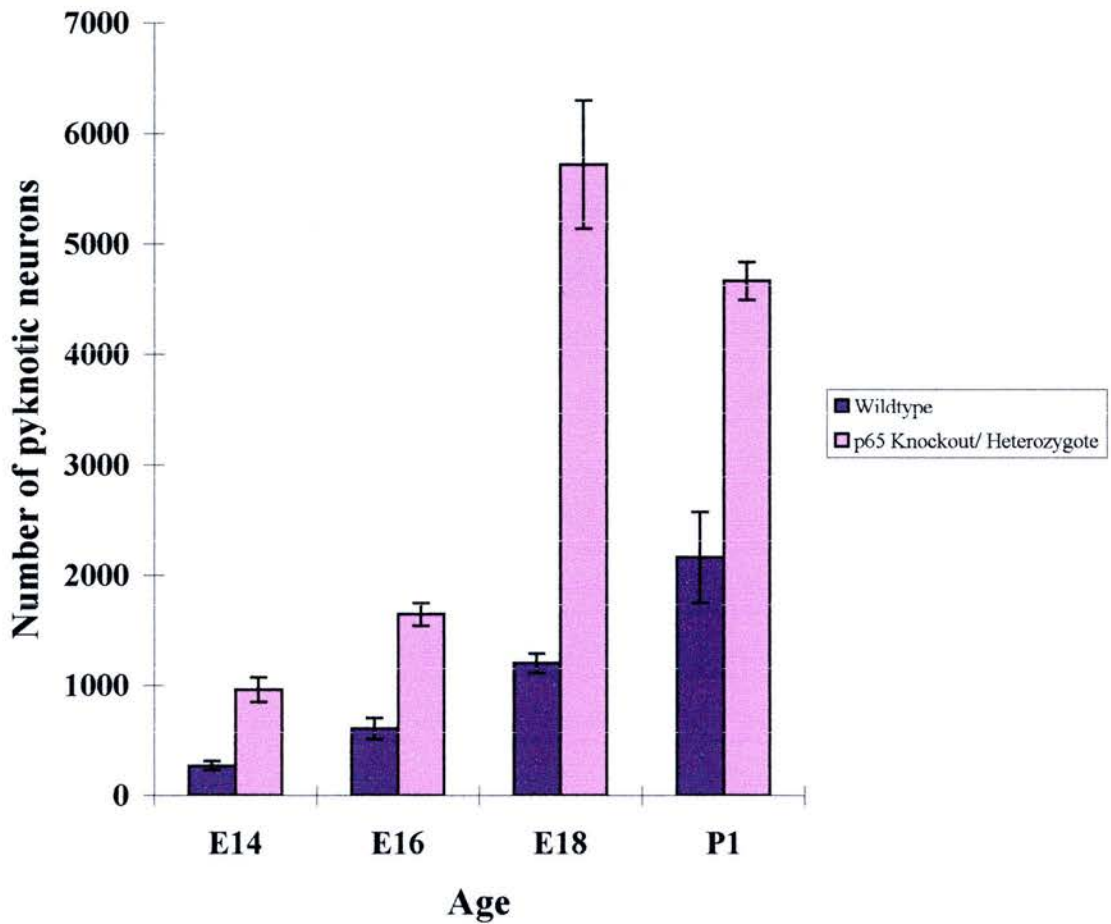
These results demonstrate that the survival of a substantial proportion of SCG neurons is dependent on the expression of p65 during embryonic development.

### E14-P1 SCG: Total Neurons



**Figure 3.13:** Bar chart showing the total number of neurons in the SCG of wildtype and p65 deficient mice at E14, and of wildtype and p65 heterozygote mice at E16, E18 and P1. Means and standard errors from both ganglia in three embryos of each genotype at all ages are shown. The numbers of neurons are decreased at all ages in the p65 deficient embryos compared to their wildtype counterparts.

### E14-P1 SCG: Pyknotic Neurons

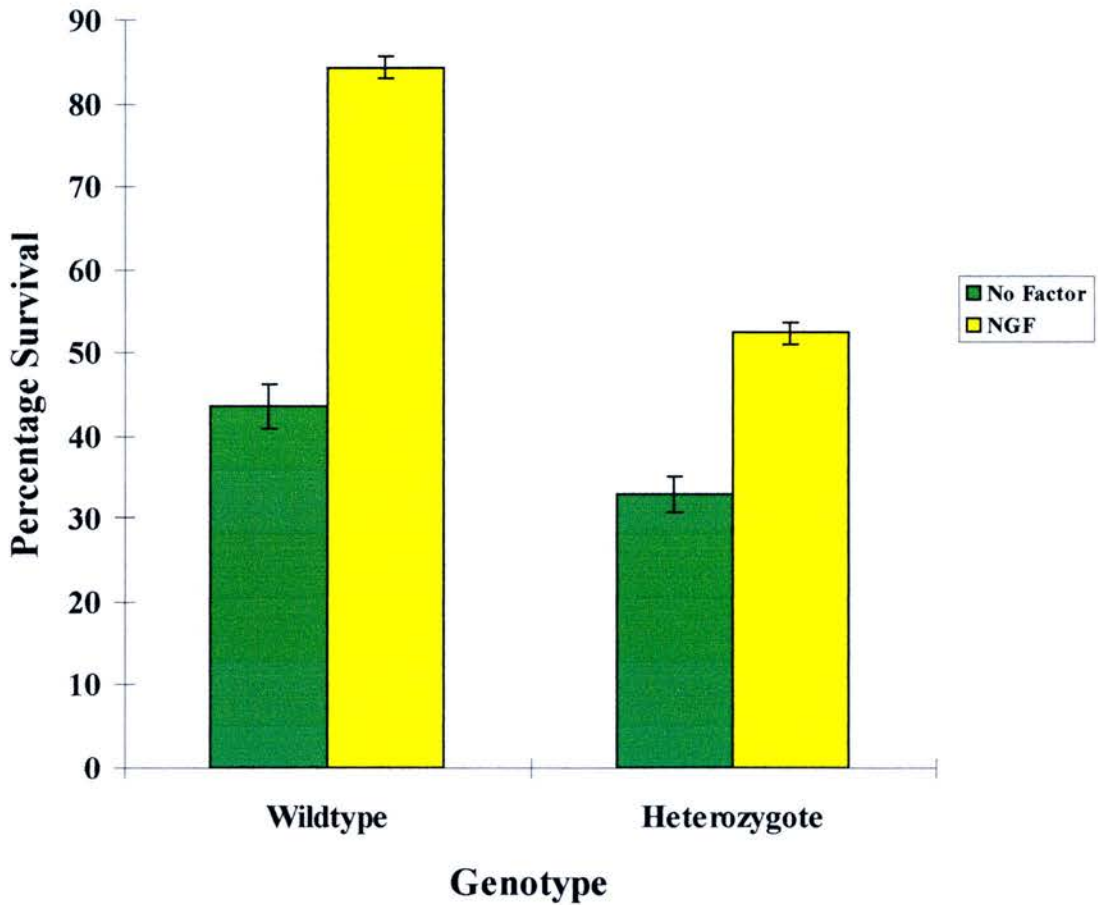


**Figure 3.14:** Bar chart showing the number of pyknotic neurons in the SCG of wildtype and p65 deficient mice at E14, and of wildtype and p65 heterozygote mice at E16, E18 and P1. Means and standard errors from both ganglia in three embryos of each genotype at all ages are shown. The numbers of pyknotic neurons, and therefore apoptosis, are increased at all ages in the p65 deficient embryos compared to their wildtype counterparts.

Age	Neurons				Pyknotic Neurons			
	Wildtype	Std Error	p65-/- or p65+/-	Std Error	Wildtype	Std Error	p65-/- or p65+/-	Std Error
<b>E14</b>	8479	704.8	4767	292.1	272.1	40.7	961.9	111
<b>E16</b>	22678	1347.9	20923	875.6	609.7	94.6	1644.6	102.4
<b>E18</b>	32092	1341.6	23250	429.1	1199.8	89.3	5718.7	579.6
<b>P1</b>	35332	1606.6	28503	1069	2160.8	410.9	4665.9	172.3

**Table 3.3: Summary of SCG results.** The means and standard errors for counts of neurons and pyknotic neurons in the SCG of wildtype and p65 deficient embryos at various ages are shown. The numbers of neurons are decreased and the levels of pyknotic neurons are increased in embryos deficient in p65 as compared to wildtype embryos.

### E16 SCG Neurons



**Figure 3.15:** Bar chart showing the percent survival of SCG neurons from wildtype and *p65* heterozygote E16 mice after 48 hours in culture. The means and standard errors of three separate experiments are shown. Percent survival was decreased significantly in *p65* heterozygous embryos compared with their wildtype littermates both when cultured with 2ng/ml NGF (38%) and in neurotrophin deprived cultures (24%).

### 3.3 Discussion

Taken together these findings demonstrate a clear role for NF- $\kappa$ B and the IKK complex in bringing about cell death in the developing spinal cord and retina. However, it is also clear that NF- $\kappa$ B is not acting in the same pathway as NGF binding to p75 in order to bring about apoptosis as can be seen by the cumulative apoptotic effect in the double knockout of both NGF and NF- $\kappa$ B (Figures 3.1, 3.3 and 3.8). IKK $\alpha$  and IKK $\beta$  were seen to be equally important in inducing cell death in the developing retina (figure 3.11), but in spinal motoneurons IKK $\alpha$  is more influential in bringing about cell death than IKK $\beta$  (figure 35). IKK $\alpha$  in the spinal cord and both IKK $\alpha$  and IKK $\beta$  in the retina showed a similar level of influence as NF- $\kappa$ B in mediating cell death. This reflects the fact that there are sub-populations of cells in both the developing retina and spinal cord that undergo NF- $\kappa$ B-dependent apoptosis and that the signals that lead to NF- $\kappa$ B activation are different in the retina than those in the spinal cord.

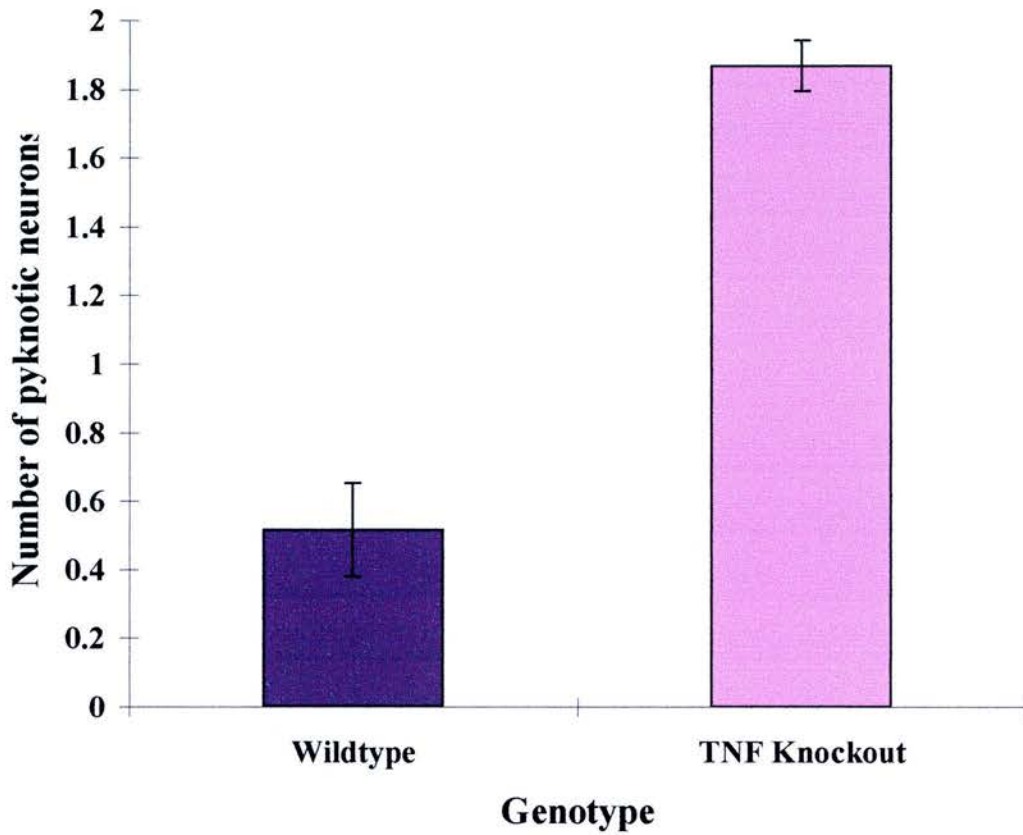
NF- $\kappa$ B may be activated as part of an apoptotic cascade downstream of growth factor deprivation. Alternatively, as there are pathways other than NGF binding to p75 that have been shown to actively trigger neuronal apoptosis in nervous system during development such as death triggered by Fas (Raoul et al., 1999, 2000) and by TNF $\alpha$  (Dawson et al., 1996; New et al., 1998; Barker et al., 2001), it is possible that these or other factors are responsible for the NF- $\kappa$ B-dependent cell death. In order to determine the influence of TNF $\alpha$  in inducing cell death I examined the level of cell death in the

retinas of *tnf $\alpha$*  knockout mice as compared to wildtype littermates. I observed that in TNF $\alpha$  deficient mice the magnitude of cell death in the developing retina does not decrease as compared to wildtype animals (Figures 3.16) and therefore TNF $\alpha$  is not likely to be a contributing factor in the signalling of apoptosis in this region.

Unexpectedly the TNF $\alpha$  deficient mice showed a significant increase in cell death as compared to the wildtype mice ( $p < 0.0001$ , unpaired t-test), suggesting that TNF $\alpha$  is acting in a neurotrophic capacity in influencing the survival of E14 retina neurons.



### E14 Retina: Pyknotic Neurons



**Figure 3.16:** Bar chart showing the number of pyknotic neurons in cross-sections of developing retina in E14 wildtype and TNF $\alpha$  knockout mice. The means and standard errors of 5 embryos of each genotype are shown. There is no significant difference in the number of pyknotic neurons, and therefore cell death, in the retina of TNF $\alpha$  deficient embryos compared to their wildtype littermates.

In contrast I have also found that p65 (and therefore NF- $\kappa$ B) activity plays an important role in the mediation of the cell survival response to NGF in SCG sympathetic neurons. This is supported by the finding that NGF promotes the translocation of NF- $\kappa$ B (p65 and p50) to the nucleus in SCG neurons. 95% of SCG neurons treated with NGF showed nuclear staining for p65 and p50, as compared to 1% of non-treated neurons. This occurred by a TrkA dependent, p75 independent mechanism as demonstrated by the fact that NGF was just as effective in inducing nuclear translocation of NF- $\kappa$ B subunits in p75 deficient neurons as in wildtype neurons (Gayle Middleton, personal communication). This indicates that TrkA is the determinant of cell survival in these neurons.

The expression of a dominant negative IKK $\alpha$  was found to inhibit the survival response of SCG neurons to NGF just as effectively as super-repressor I $\kappa$ B $\alpha$ . This further validates the role of the NF- $\kappa$ B pathway in the activation of cell survival in the SCG. At E14 the NGF dose response of p65-deficient SCG neurons was shifted by over an order of magnitude to a higher concentration compared to that of wildtype neurons. This effect was found to be gene dose dependent, so that although the effect could be seen in the heterozygote, a greater effect was found in the knockout mice. Similar shifts in dose response were seen for SCG neurons from IKK $\alpha$  deficient mice, indicating the importance of the NF- $\kappa$ B pathway in mediating cell survival in the SCG (Gayle Middleton, personal communication).

In summary, these findings indicate that both NF- $\kappa$ B activation and the phosphorylation of I $\kappa$ B $\alpha$  by the IKK $\alpha$  catalytic subunit of the I $\kappa$ B kinase complex are important events in the mediation of the survival response of SCG neurons to NGF. These findings are physiologically relevant as the increased loss of SCG neurons in p65 and IKK $\alpha$  deficient embryos occurs during the period of development when SCG neurons are known to be supported by NGF *in vivo* (Francis et al., 1999; Wyatt and Davies, 1995). Although the activation of NF- $\kappa$ B is a consequence of TrkA activation by NGF, inhibiting NF- $\kappa$ B activation *in vitro* does not completely abolish the survival response of SCG neurons to NGF, suggesting that there is also a NF- $\kappa$ B-independent component of NGF survival signalling in SCG neurons. In future work it will be important to determine the molecular interactions that lead to the selective activation of NF- $\kappa$ B by NGF independently of any interaction with p75. It will also be important to determine whether this NF- $\kappa$ B mediated pathway is responsible for cell survival in response to neurotrophins in other areas of the nervous system during development.

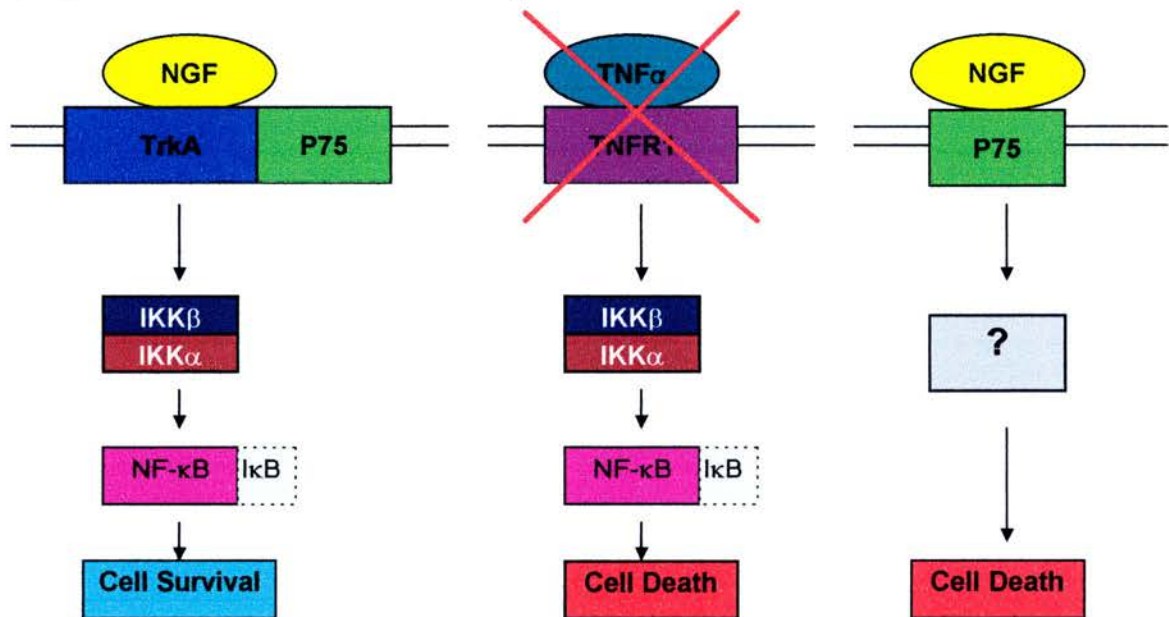
The findings of this study suggest that NF- $\kappa$ B has dual actions during development, the mediation of apoptosis in cells of the spinal cord and retina and the mediation of cell survival in cells of the SCG. Although NF- $\kappa$ B is known to mediate cell survival in response to NGF, I have shown that NF- $\kappa$ B mediated cell death is not induced by NGF. NGF does have an apoptosis-inducing effect in retina and spinal cord neurons but it appears to be mediated by an alternative pathway (figure 3.17). It is possible that the



NGF-induced and NF- $\kappa$ B-mediated pathways act in concert to produce the apoptosis seen during development. That neither are sufficient for developmental cell death is evident from the fact that apoptosis is not completely eliminated in the embryos with null mutations in either NGF or p65 and by the cumulative effect seen in the NGF/p65 double knockout.

**SCG:**

**Spinal Cord and Retina:**



**Figure 3.17: Summary diagram of the findings in chapter 3.** The findings of this study suggest that NF- $\kappa$ B has dual actions during development, the mediation of apoptosis in cells of the spinal cord and retina and the mediation of cell survival in cells of the SCG. Although NF- $\kappa$ B is known to mediate cell survival in response to NGF, I have shown that NF- $\kappa$ B mediated cell death is not induced by NGF. This NF- $\kappa$ B mediated apoptosis is not induced by TNF $\alpha$  binding to TNFR1. NGF does have an apoptosis-inducing effect in retina and spinal cord neurons but it appears to be mediated by an alternative pathway

I have shown that p65 (and therefore NF- $\kappa$ B) is not downstream of NGF binding to p75 in inducing cell death in the spinal cord and retina. It is possible that in the absence of NGF-TrkA pro-survival signalling, NF- $\kappa$ B has a default pro-apoptotic effect. This suggests that the expression of TrkA on cells results in the switch in NF- $\kappa$ B activity

from pro- to anti-apoptotic. p75 has been proposed to mediate cell death in a ligand independent mechanism which is negated by NGF (Rabizadeh et al., 1993; Barret and Georgiou, 1996; Barret and Bartlett, 1994). Marked increases in sensory and sympathetic neuronal apoptosis have been found in mice expressing only the p75 intracellular domain (Majdan et al., 1997). Also in a mutant PC12 cell line that did not express p75, apoptosis was reduced (Rabizadeh and Bresden, 1994). It is possible that this p75 constitutive pathway acts via NF- $\kappa$ B activation and that the binding of NGF to p75 rather than negating cell death, activates an alternative pathway and enhances the cytotoxic response, as has been suggested previously (Frade and Barde, 1996, 1999). This constitutive, NF- $\kappa$ B-mediated cell death response is then presumably inhibited by the expression of TrkA on the cell and the binding of NGF to TrkA stimulates NF- $\kappa$ B-mediated cell survival. Indeed, Trk activation was found to inhibit p75-mediated death of sympathetic (Bamji et al., 1998) and TMN sensory neurons (Davey and Davies, 1998). During programmed sympathetic cell death, deficiencies in p75 significantly reduced apoptosis (Bamji et al., 1998), whereas TrkA deficiency leads to the apoptosis of the majority of sympathetic neurons (Smeyne et al., 1994). This indicates that developmental apoptosis is to some extent due to constitutive p75-mediated and possibly NGF-induced death signals that must be inhibited by the expression of TrkA as a mediator of survival. This hypothesis requires examination in a study of the effect of null mutations in *p75* on NF- $\kappa$ B activity and also the level of cell death in *p65/p75* double knockouts as compared to embryos with null mutations in *p65*, *p75* and wildtype embryos.

# **CHAPTER 4: THE EFFECTS OF TUMOR NECROSIS FACTOR $\alpha$ ON CRANIAL SENSORY AND SYMPATHETIC NEURONS DURING EMBRYONIC DEVELOPMENT I: NEUROTROPHIC ACTIVITY.**

## **4.1 Introduction**

### **4.1.1 TNF and its Receptors**

Cytokines play crucial roles in cell-to-cell communication and cellular activation. Functionally they are classified as being either pro-inflammatory or anti-inflammatory depending on the final balance of their effects on the immune system (Mosmann et al., 1986). Tumour necrosis factor (TNF) is a pleiotropic pro-inflammatory cytokine produced by macrophages, monocytes, lymphocytes, keratinocytes and fibroblasts, in response to inflammation, infection, injury and other environmental challenges (Baud and Karin, 2001). It was first identified as a factor that induced rapid haemorrhagic necrosis of transplantable tumours (Carswell et al., 1975). A number of TNFs exist of which the predominant two are TNF $\alpha$  and TNF $\beta$ . TNF $\alpha$  is a 17kDa protein of 157 amino acids in length. It is produced by the processing of a 27kDa, 233 amino acid precursor. This reaction is catalysed by TNF $\alpha$  converting enzyme (TACE). Once converted, TNF $\alpha$  forms both dimers and trimers (Aggarwal et al., 1985; Fransen et al.,

1985; Davies, J.M. et al., 1987; Jones et al., 1989). TNF $\beta$  holds 36 percent homology with TNF $\alpha$  (Meager, 1991) and the genes for the two proteins lie close to each other on murine chromosome 17 (Marmenout et al., 1985). The tertiary structure of TNF $\alpha$  and TNF $\beta$  are very similar and both bind to TNF receptors 1 and 2 (TNFR1 and TNFR2).

TNFR1 and 2 are expressed on all somatic cell types with the exception of erythrocytes. TNFR1 is a 55kDa protein and TNFR2 is a 75kDa protein (Goodwin et al., 1991). TNFR1 is a glycosylated protein of 455 amino acids that contains an extracellular domain of 171 amino acids and a cytoplasmic domain of 221 amino acids (Loetscher et al., 1990). Sequence homologies in the cysteine-rich domains of the extracellular region show that TNFR1 is related to the p75 receptor of the neurotrophin family (discussed in section 1.7.2) (Marsters et al., 1992). The activation of TNFR1 is sufficient to trigger cytotoxic activity towards transformed cells and transgenic mice with null mutations in TNFR1 that still express TNFR2 are resistant to lethal doses of endotoxins and enterotoxins (Pfeffer et al., 1993). This indicates an important role for TNFR1 but not TNFR2 in the induction of the cytotoxic effect of TNF. Although TNFR2 contributes to a number of cell specific responses (e.g., T lymphocyte proliferation), the TNF-induced effects in most cells are mediated by TNFR1 (Heyninck and Beyaert, 2001). TNFR2 has been found to synergistically enhance TNF $\alpha$ -induced cytotoxicity (Grell et al., 1999). Two further TNF receptors have been identified, one of which is expressed in normal human liver and binds TNF $\alpha$  only and the other which is a truncated soluble form (Nophar et al., 1990; Schwalb et al., 1993). Several soluble TNF binding proteins have



been described. These include T-BP-1 and T-BP-2. They are truncated proteins of approximately 30kDa, derived from the TNF-binding domain of the membrane receptor. They can be isolated from both blood serum and urine and are thought to act as endogenous inhibitors of TNF $\alpha$  by competing with the cell surface receptor for its binding (Engelmann et al., 1990; Schall et al., 1990). TNF $\alpha$  plays a vital and unique role in the control of pro- and anti-apoptotic signalling pathways. The stimulation of TNFR1 can simultaneously activate signalling pathways within cells, one of which ends in apoptosis and another in cell survival. When both pathways are activated by TNF $\alpha$  the cell receiving the anti-apoptotic signal survives and multiplies, but if the death pathway predominates the cell undergoes apoptosis.

#### **4.1.2 TNF in the Nervous System**

Members of the cytokine family have roles in both the immune response and a number of physiological and pathological processes (reviewed by Szelenyi, 2001). There is evidence for the presence of functional TNF $\alpha$  and its receptors in the brain. Genes for TNF $\alpha$  and its receptor have been found to be constitutively expressed in astrocytes and microglia. Genes for the TNF $\alpha$  receptor have also been demonstrated to be constitutively expressed on neurons and oligodendrocytes (reviewed by Szelenyi, 2001). This constitutive expression indicates that TNF $\alpha$  may contribute to the normal functioning of the brain. Certain cytokines may affect the CNS directly by local release

or indirectly through CNS contact with peripherally released cytokines (Sternberg, 1997; Besedovsky, et al., 1991; Breder et al., 1988; Plata-Salaman, et al., 1988). Glial cells are able to produce TNF $\alpha$  (Lieberman et al., 1989). It was found that after systemic admission of endotoxin, TNF $\alpha$  mRNA is induced initially in perivascular cells, meningeal cells, and neurons in different brain regions, including median eminence and medulla, and later in the neurons of the hypothalamus and tractus solitarius (Breder et al., 1994). This suggests that peripheral cytokines may stimulate the synthesis of cytokines in the CNS. They may move into the CNS through leaky areas or by active transport (Banks et al., 1991; Watkins et al., 1995). In the CNS, microglia and astrocytes are believed to be the primary producers of TNF $\alpha$ , although it is also produced by fibroblasts (Arnett et al., 2001; Baud and Karin, 2001). Also TNF $\alpha$  has been observed to be synthesised by neurons of the hypothalamus, amygdala, caudal raphe nuclei and areas of the pontine and medulla (Breder et al., 1993). The production of TNF $\alpha$  in the CNS suggests that it has a functional role in the nervous system. The permanent presence of cytokine receptors causes neural cells to be sensitive to cytokines even at low levels (Wong et al., 1996; Wong and Licinio, 1994). *In vivo* studies using transgenic mice in which TNF $\alpha$  is targeted to the brain, indicate that CNS over-expression of TNF $\alpha$  is an important factor in the pathogenesis of CNS neurotoxic and neurodegenerative disorders (Campbell et al., 1998). It is unclear as of yet whether the altered cytokine expression reflects a primary pathogenic process, or a secondary consequence of the disease.

### 4.1.3 Functions of TNF

TNF's name reflects its activities in tumour cell lines. It causes cytolysis and cytostasis in many tumour cell lines *in vitro* (Larrick and Wright, 1990) and in transplanted tumours it induces haemorrhagic necrosis. Injection of exogenous TNF $\alpha$  results in the destruction of small blood vessels in malignant tumours (Trinchieri, 1992) and TNF is responsible for necrosis of the sarcoma Meth A (Carswell et al., 1975). TNF $\alpha$  also has a number of other functions. Being a cytokine, it is highly involved in the immune response. TNF $\alpha$  is a potent chemoattractant for neutrophils, concurrently enhancing their adherence to endothelial cells. In macrophages it stimulates phagocytosis and superoxide dismutase, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and interleukin-1 (IL1) synthesis. In the presence of IL2, TNF $\alpha$  promotes the proliferation and differentiation of B lymphocytes and in IL2 absence it enhances T lymphocyte proliferation. TNF $\alpha$  is also responsible for some of the severe effects of sepsis (Bonavida, 1991; Larrick and Kunchel, 1988). Further effects of TNF $\alpha$  are the promotion of angiogenesis *in vivo* (Frater-Schroder et al., 1987) and of astroglial and microglial proliferation (Merril, 1991), activation of osteoclasts, inhibition of lipoprotein lipase synthesis and suppression of lipogenic metabolism in adipocytes (Grunfeld and Feingold, 1991). TNF $\alpha$  has been found to induce apoptosis (Liu and Han, 2001) and also act as a growth factor for human fibroblasts, promoting their production of collagenase and PGE<sub>2</sub>. Another response to TNF $\alpha$  is pyrexia (Tracy and Cerami, 1993). IL-1 (Luheshi et al., 1997) and IL-6

(Cartmell et al., 2000) have also been shown to induce the pyrogenic response to inflammation in the rat.

#### **4.1.4 The Mechanisms of TNF $\alpha$ activity**

##### **4.1.4a The Mechanism of TNF $\alpha$ Induced Cell Survival**

The binding of TNF $\alpha$  to its receptors can cause activation of the transcription factors NF- $\kappa$ B and AP-1. These induce genes that are often involved in inflammatory responses (Barnes and Karin, 1997). A number of these genes suppress TNF $\alpha$ -induced apoptosis, in a possible feedback mechanism. The suppression of apoptosis is mostly dependent on NF- $\kappa$ B (reviewed by Baud and Karin, 2001).

In response to TNF $\alpha$  and other agonists, I $\kappa$ B (and other inhibitory subunits of NF- $\kappa$ B) is phosphorylated by the IKK complex, resulting in their ubiquitination, degradation and the nuclear translocation of NF- $\kappa$ B (Karin and Ben-Neriah, 2000). Once in the nucleus, NF- $\kappa$ B transcriptional activity can be modulated further through phosphorylation by various protein kinases that are TNF $\alpha$  responsive, like mitogen activated protein kinases (MAPKs). This provides a point of cross-talk with other signalling pathways (Baud and Karin, 2001). TNF $\alpha$  mediated IKK activation may depend on an interaction between RIP1 and the IKK $\gamma$ /NEMO regulatory subunit of the IKK complex, also TRAF2 is thought to be required for the recruitment of the IKK complex to the activated TNFR1

(Karin and Ben-Neriah, 2000; Devin et al., 2000; Zhang, S.Q. et al., 2000). Activation of IKK is thought to involve the phosphorylation of two serines within the activation loop of IKK $\beta$  (Delhase et al., 1999). Both IKK $\alpha$  and IKK $\beta$  contain an activation loop similar to that found in the MAPK kinase family of proteins. This loop contains two serine residues that become phosphorylated in response to interleukin 1 (IL1) or TNF $\alpha$  (Delhase et al., 1999). IKK $\beta$  knockout cells exhibit almost no NF- $\kappa$ B activity in response to IL1 or TNF $\alpha$ , indicating that IKK $\beta$  is the main kinase involved in NF- $\kappa$ B activation by pro-inflammatory cytokines. NF- $\kappa$ B inducing kinase (NIK) interacts with TRAF2 and is capable of activating NF- $\kappa$ B when over-expressed. The presence of catalytically inactive NIK blocks NF- $\kappa$ B activation by a number of stimuli including TNF $\alpha$  (Malinin et al., 1997). Although studies of NIK knockout mice indicated that it is not essential for TNF $\alpha$  induced NF- $\kappa$ B DNA binding or transcriptional activity (Yin et al., 2001), it has been reported to be a potent inducer of processing of the precursor p100/NF- $\kappa$ B to the mature p52 subunit of NF- $\kappa$ B (Xiao et al., 2001) which is then available for activation by TNF $\alpha$ . When over-expressed in mammalian cells, other MAP3Ks and protein kinases, including MEKK1, can activate IKK or NF- $\kappa$ B (Lee et al., 1998; Lee et al., 1997a). MEKK1 is also involved in TNF $\alpha$  signalling as demonstrated by catalytically inactive mutants (Xia et al., 1998; Liu, ZG., et al., 1996; Minden et al., 1994). Phosphorylation of I $\kappa$ B by such kinases in response to TNFR1 signalling is much less efficient than that produced by the soluble IKK complex (Zheng, S.Q. et al., 2000).

AP-1 is a heterogeneous group of dimeric transcription factors, whose activity is regulated by mechanisms, including not only TNF $\alpha$  binding but also the phosphorylation by MAPKs. Upon stimulation, these protein kinases enter the nucleus and phosphorylate DNA-bound AP-1 transcription factors, activating them (Karin et al., 1997). The activation of AP-1 results in its induction of genes involved in inflammatory responses (Barnes and Karin, 1997; Karin et al., 1997).

However simple linear sequences of events do not lead to NF- $\kappa$ B and AP-1 activation. Their activities can be modulated in a number of different ways. For example, the serine threonine kinase Akt/PKB can contribute to TNF $\alpha$ -induced NF- $\kappa$ B transcriptional activity (Madrid et al., 2001), but not to IKK activation (Delhase et al., 2000). This indicates that Akt/PKB is involved in activating NF- $\kappa$ B downstream from the IKK complex. As discussed previously Akt is activated via the phosphatidyl 3-OH kinase (PI3'K) signalling pathway in response to growth factors and is involved in the inhibition of Bad-mediated apoptosis. Thus, Akt may be involved in the activation of TNF $\alpha$ -induced, NF- $\kappa$ B mediated cell survival while concurrently inhibiting Bad-mediated apoptosis. There may also be interactions between TNFRs and the atypical protein kinase C (aPKC) pathway. p62, an aPKC associated protein, is thought to be involved in TNF $\alpha$ -induced IKK activation, as its down regulation greatly inhibits TNF $\alpha$ -mediated NF- $\kappa$ B activation. p62 appears to interact with RIP1 and therefore can connect aPKC molecules to TNF $\alpha$  signalling complexes. All this indicates that p62 is

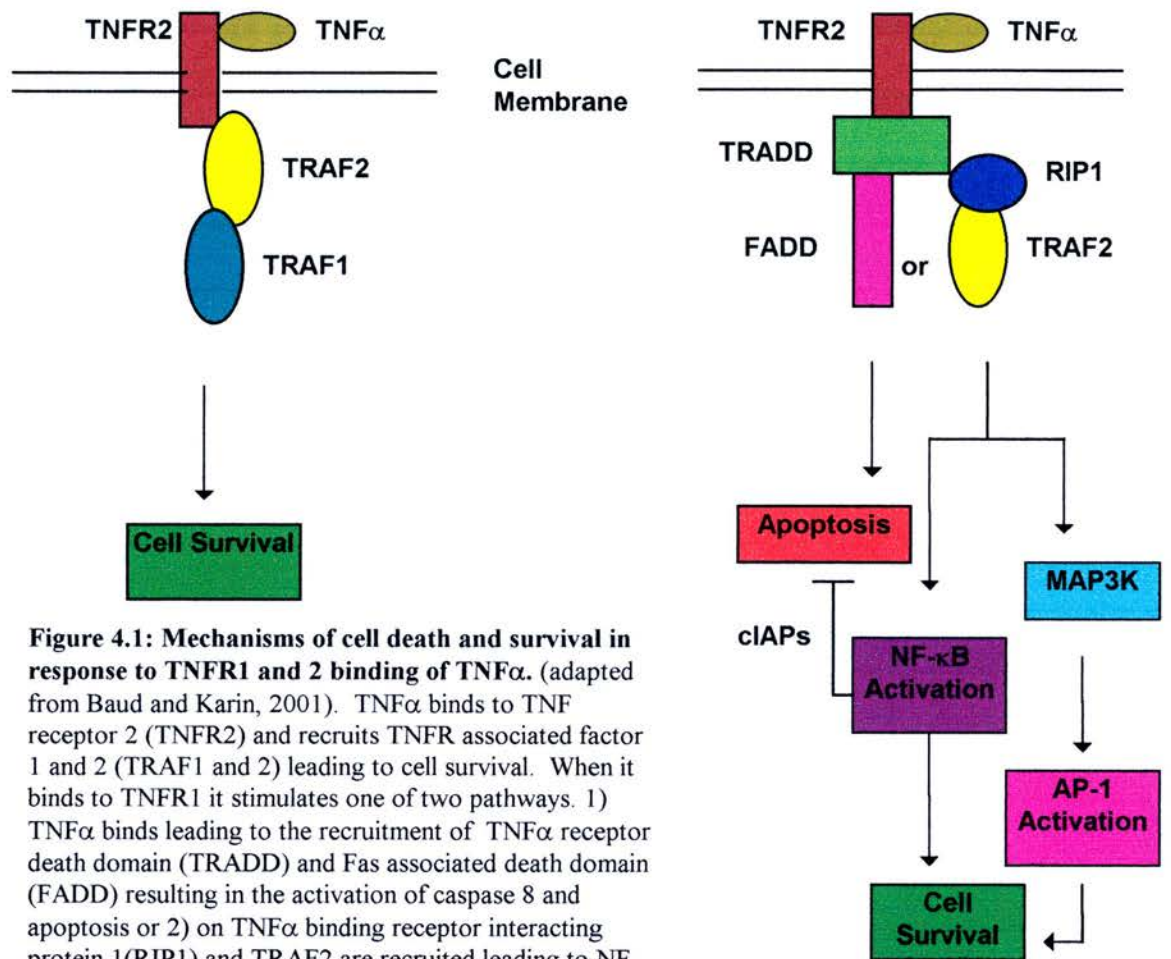
involved in TNF $\alpha$  activation of NF- $\kappa$ B (Sanz et al., 1999). Thus a number of factors may be involved in the modulation of cell survival by TNF $\alpha$ -induced, NF $\kappa$ B activation.

The binding of trimeric TNF $\alpha$  to TNFR1 and 2 induces receptor trimerisation and the recruitment of several signalling proteins to the cytoplasmic domains of the receptors (See Figure 4.1). The initial protein recruited to TNFR1 is TNFR1-associated death domain protein (TRADD), which serves as a platform for the recruitment of other mediators, including the serine threonine kinase, receptor interacting protein 1 (RIP1), FAS associated death domain (FADD) and TNFR associated factor 2 (TRAF2) (Jiang et al., 1999; Kelliher et al., 1998; Natoli et al., 1997; Hsu et al., 1996a; Rothe et al., 1995a; Stanger et al., 1995; Chinnaiyan et al., 1995; Rothe et al., 1994). The intracellular domain of TNFR1 contains an 80 amino acid death domain, which is responsible for the induction of cell death signals and NF- $\kappa$ B generation (Heyninck and Beyaert, 2001). This death domain is similar to that found in TRADD, FADD (Hsu et al., 1995) and RIP1 (Hsu et al., 1996b). It is the polymerisation of this domain that results in protein recruitment (Hsu et al., 1995).

Binding to TNFR2 leads to the direct recruitment of TRAF2, which then recruits TRAF1 (Rothe et al., 1995a; Rothe et al., 1994) (see Figure 4.1). TRAF2 plays a pivotal role in events leading to IKK and MAPK activation. It is an effective activator of several MAPKs (Liu, Z.G. et al., 1996) and over-expression of TRAF2 is adequate for the activation of signalling pathways resulting in NF- $\kappa$ B and AP-1 activation in the absence



of extracellular stimuli (Song et al., 1997; Liu, Z.G. et al., 1996; Rothe et al., 1995a). TRAF proteins have been shown to co-immunoprecipitate with the neurotrophin receptor p75, and thus may regulate its actions (discussed in section 1.7.2) (Khursigara et al., 1999). It has been demonstrated that optimal NF- $\kappa$ B and IKK activation by TNFR1 requires TRAF2 (Devin et al., 2000). TRAF2 has been found to recruit IKK to TNFR1 through its interaction with IKK $\alpha$  and IKK $\beta$  (Devin et al., 2001) and murine embryonic fibroblast cells deficient in TRAF2 show large reductions in the IKK complex - mediated, I $\kappa$ B phosphorylation - induced NF- $\kappa$ B nuclear translocation (Karin and Ben-Nariah, 2000; Devin et al., 2000). These same fibroblasts are very sensitive to TNF $\alpha$ -induced cell death as are those deficient in RIP1, suggesting that these molecules have an important role in TNF $\alpha$ -induced NF- $\kappa$ B activation (Devin et al., 2000; Yeh et al., 1997; Lee et al., 1997b). Thus at least some of TRAF2's anti-apoptotic activity can be explained by its mediation of NF- $\kappa$ B activation leading to the NF- $\kappa$ B dependant expression of anti-apoptotic proteins. These proteins include cellular inhibitor of apoptosis 2 (cIAP2) and TRAF1 (Song et al., 1996; Rothe et al., 1995b; Rothe et al., 1994). Over-expression of cIAP2 prevents TNF $\alpha$  induced caspase-8 activity, leading to the inhibition of TNF $\alpha$  stimulated apoptosis (Wang, et al., 1998). Optimal repression of apoptosis by cIAPs occurs on co-expression of the NF- $\kappa$ B inducible TRAF1 protein. This is due to the enhanced recruitment of cIAPs to the TNFR complex (Rothe et al., 1995b). Thus the NF- $\kappa$ B pathway may intersect the apoptotic pathway through the induction of cIAPs, which inhibit specific caspases (Deveraux and Reed, 1999).



**Figure 4.1: Mechanisms of cell death and survival in response to TNFR1 and 2 binding of TNF $\alpha$ .** (adapted from Baud and Karin, 2001). TNF $\alpha$  binds to TNF receptor 2 (TNFR2) and recruits TNFR associated factor 1 and 2 (TRAF1 and 2) leading to cell survival. When it binds to TNFR1 it stimulates one of two pathways. 1) TNF $\alpha$  binds leading to the recruitment of TNF $\alpha$  receptor death domain (TRADD) and Fas associated death domain (FADD) resulting in the activation of caspase 8 and apoptosis or 2) on TNF $\alpha$  binding receptor interacting protein 1(RIP1) and TRAF2 are recruited leading to NF- $\kappa$ B or AP-1 activation and cell survival. NF- $\kappa$ B also induces the expression of proteins that inhibit the cell death pathway (e.g., cIAPs).

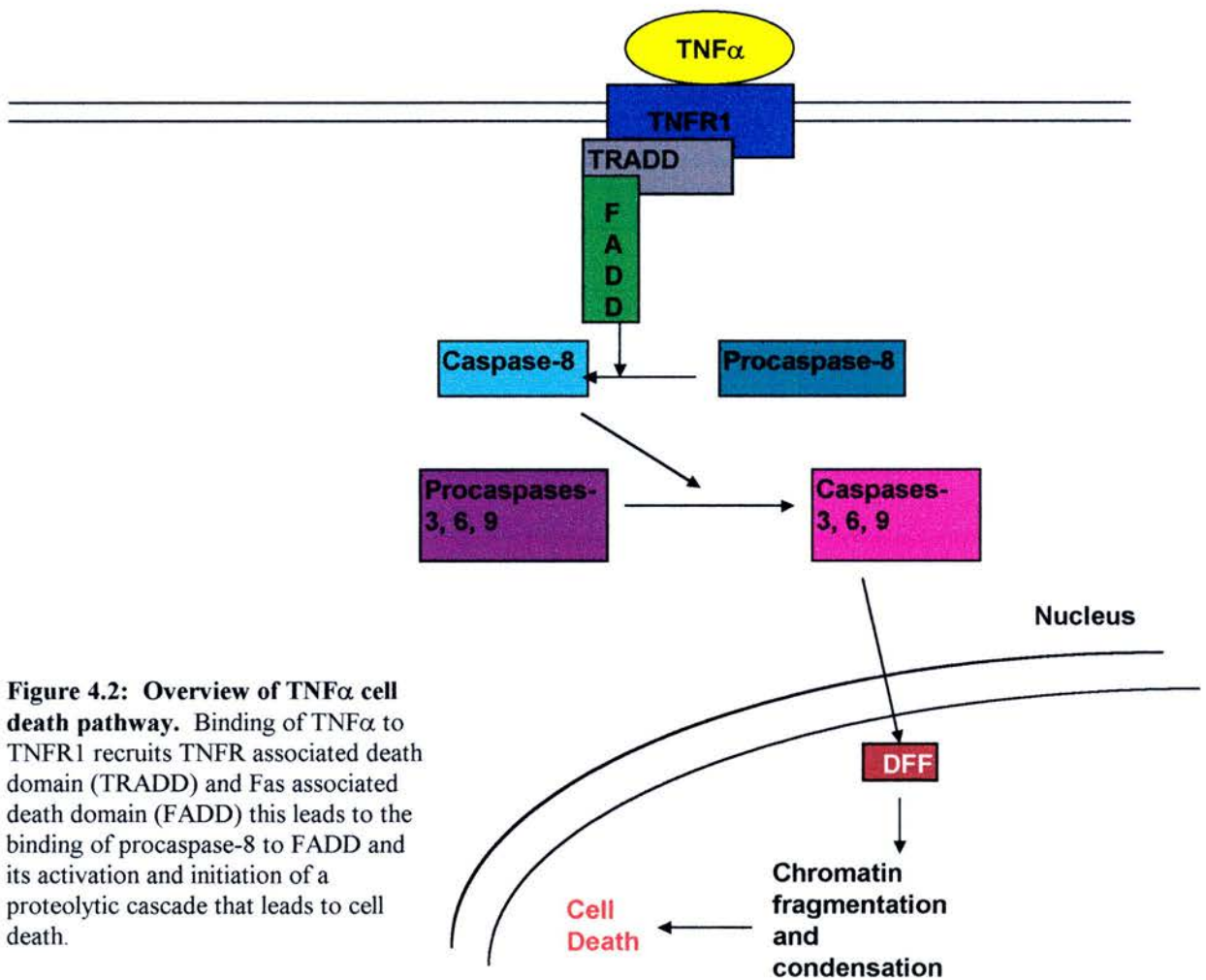
RIP1 was found, through over-expression studies, to be a key effector in the activation of NF- $\kappa$ B by TNFR1 (Liu, Z.G., et al., 1996; Ting et al., 1996). Knockout experiments revealed RIP1 to be vital for TNF $\alpha$ -mediated NF- $\kappa$ B but not MAPK activation (Kelliher et al., 1998) and in cells from RIP1 knockout mice TNF $\alpha$  failed to initiate NF- $\kappa$ B

nuclear translocation and NF- $\kappa$ B mediated gene transcription (Kelliher et al., 1998; Ting et al., 1996). Also, the activation of IKK by TNF $\alpha$  is thought to be dependant on its dimerisation which is mediated by RIP1 (Inohara et al., 2000). RIP1 contains a C-terminal death domain, by which it is recruited to the TNFR complex (Hsu et al., 1996b), it also contains an intermediate domain which directly interacts with the IKK $\gamma$  subunit of the IKK complex (Zhang S.Q., et al., 2000) resulting in its dimerisation and activation. This intermediate domain can be proteolytically cleaved by caspase 8 leading to the interruption of TNF $\alpha$ -induced NF- $\kappa$ B activation (Lin et al., 1999; Martinon et al., 2000; Kim et al., 1999). This enhances the activity of alternative pathways resulting in an increase in TNF $\alpha$ -mediated apoptotic events. Other pathways have been proposed to be activated by TNF $\alpha$  including the JNK pathway, the sphingomyelinase pathway, the p38 pathway and the ERK pathway (reviewed by Liu and Han, 2001). Although as of yet it is unclear what physiological role the activation of these pathways by TNF $\alpha$  has, it is likely that the variety of different signalling cascades activated by TNF $\alpha$  reflects the many and diverse responses to its binding in different cells.

#### **4.1.4b The Mechanism of TNF $\alpha$ Induced Cell Death**

FADD is the major but not the only TNFR1 pro-apoptotic mediator and the inability of TNFR2 to recruit FADD may explain its reduced effectiveness as an inducer of apoptosis (Yeh et al., 1998) (figure 4.1). The death domain of FADD recruits it to the

TNFR1 complex and a death effector domain (DED) promotes the activation of a downstream proteolytic cascade through binding of homologous DEDs of procaspase - 8 (Boldin et al., 1996; Muzio et al., 1996). Caspases are aspartate directed cysteine proteases and are essential effectors in pro-apoptotic pathways induced in response to cytotoxic stimuli, including TNF $\alpha$  (reviewed in Budihardjo et al., 1999). The binding of procaspase-8 to FADD results in its autoproteolytic activation, it can then initiate a proteolytic cascade that leads to cell death (Muzio et al., 1998) (see figure 4.2).



**Figure 4.2: Overview of TNF $\alpha$  cell death pathway.** Binding of TNF $\alpha$  to TNFR1 recruits TNFR associated death domain (TRADD) and Fas associated death domain (FADD) this leads to the binding of procaspase-8 to FADD and its activation and initiation of a proteolytic cascade that leads to cell death.

This pathway involving TRADD, FADD and caspase 8 is the most widely accepted pathway by which TNF $\alpha$  triggers apoptosis although it is not the only one (Sanz et al., 1999). But, FADD and caspase 8 deficient fibroblasts are resistant to TNFR1-induced apoptosis (Yeh et al., 1998; Vadlamudi et al., 1996), supporting its role as the primary route of TNF $\alpha$ -mediated cell death. TNF $\alpha$ -activated caspase 8 subsequently activates caspase 3, which leads to apoptosis by the cleavage of a number of cellular proteins (Rath and Aggarwal, 1999). A residual apoptotic response is observed in FADD deficient cells on TNF $\alpha$  treatment, indicating that there must be another pathway involved in TNF $\alpha$ -induced cell death. The identity of this pathway has yet to be elucidated. An alternative mechanism of TNF $\alpha$ -mediated apoptosis involves intracellular cross-talk between heterologous receptors on a single cell and can result in either the promotion or inhibition of receptor activity. An example of this is TNF $\alpha$  receptor interaction with IGF1, a receptor for the survival signal, in cerebellar granule cells which results in the “silencing of survival signals” (SOSS) thus inhibiting IGF1-mediated neuroprotection (Loddick and Rothwell, 1999; Venters et al., 1999, 2000). It has been shown that TNFR1 has an inherent tendency towards basal signalling which is usually inhibited by an intracellular protein called “silencer of death domains” (SODD). TNFR1 binds SODD in the absence of TNF $\alpha$  and releases it when it binds TNF $\alpha$ , as shown in Jurkat and U937 cells (Jiang et al., 1999). SODD over-expression inhibited TNF $\alpha$  activation of a NF- $\kappa$ B-dependent reporter gene, together with NF- $\kappa$ B activation triggered by TNFR1 over-expression and it also inhibited TNF $\alpha$ -induced apoptosis in HeLa and 293 cells (Jiang et al., 1999). Thus, it is possible that pro-inflammatory

cytokines like TNF $\alpha$  may also influence neuronal survival not only by intracellular interactions but by interactions between cellular receptors as well and that TNF $\alpha$  cytotoxic activity may result from the removal of an inhibitory signal rather than the induction of a stimulatory one.

Thus it can be seen that TNF $\alpha$  has an important and unique involvement in the control of both pro- and anti-apoptotic signalling pathways. The stimulation of TNFR1 can simultaneously activate at least two signalling pathways within cells, one leading to apoptosis, the other to cell survival. When both pathways are activated by TNF $\alpha$  the cell receiving the anti-apoptotic signal survives and multiplies, but if the death pathway predominates the cell undergoes apoptosis.

#### **4.1.5 The Dual Roles of TNF $\alpha$**

Although there is considerable evidence that pro-inflammatory cytokines such as TNF $\alpha$  are neurotoxic this finding is still controversial. TNF $\alpha$  has not been found to result in cell death if infused into a normal rodent brain, but there is evidence that it mediates death after focal ischaemic injury of neurons, whereas the blockage of TNF $\alpha$  activity by antibodies is neuroprotective in this situation (Barone et al., 1997). TNF $\alpha$  induces apoptosis in oligodendrocytes, and in mice with null mutations in TNF, greater numbers of oligodendrocytes were observed and apoptosis in this region was delayed (Arnett et



al., 2001). TNF $\alpha$  in the CNS may directly promote neuronal apoptosis, for example in injured cortical neurons increased TNF $\alpha$  synthesis was found to occur, leading to decreased recovery and survival. Also the inhibition of TNF $\alpha$  by sTNFR:Fc, which neutralises TNF $\alpha$  *in vitro* improved neurological recovery in the affected areas (Knobloch et al., 1999). Another possibility is that TNF $\alpha$  not only exerts direct effects on neurons but it also enhances neurodegeneration through the interruption of survival by growth factors and other cytokines responsible for inhibition of apoptosis (Venters et al., 2001). In ischaemic rat cerebellar cells TNF $\alpha$  expression is increased and NF- $\kappa$ B activity has been shown to be inhibited, as shown by decreased p65 immunoreactivity leading to apoptosis, suggesting that in some situations TNF $\alpha$  acts as a neurotoxic factor through the inhibition of survival signals (Botchkina et al., 1999). *In vitro* experiments have shown that TNF $\alpha$  exacerbates neuronal death on mixed neuronal and glial cell cultures. This neurodegenerative effect may be due to the effect of neurotoxins released from the glial cells in response to TNF $\alpha$  (Jeohn et al., 1998). TNF $\alpha$  is a potent activator of microglial secretion of further TNF $\alpha$ , indicating an auto-regulatory positive feedback mechanism (Nakamura et al., 1999).

TNF $\alpha$  has been implicated as having a role in a number of neurodegenerative conditions, including multiple sclerosis, in which increased levels of TNF $\alpha$  mRNA have been observed in lesions (Bitsch et al., 2000). Also, TNF $\alpha$  protein has been found in the sera of multiple sclerosis patients (Martino et al., 1997). Alzheimer's disease is



associated with dysregulated proinflammatory cytokines, including TNF $\alpha$ , in the CNS (Lanzrein et al., 1998). TNF $\alpha$  is also raised in Alzheimer's patients (Fillit et al., 1991) and a haplotype for TNF $\alpha$  has been associated with late-onset Alzheimer's disease (Collins et al., 2000). TNF $\alpha$  is also thought to be involved in neuronal death following traumatic cranial injury where it has been found to be elevated in both serum (Goodman et al., 1990) and cerebrospinal fluid (Ross et al., 1994). All this supports the idea that TNF $\alpha$  has a neurotoxic effect. The involvement of TNF $\alpha$  in neurological conditions is discussed on greater detail in section 7.2.

Following ischaemic and excitotoxic brain injury TNF $\alpha$  has been seen to have a neuroprotective action, mediated by TNFR1 (Gary et al., 1998). In TNF $\alpha$  knockout mice cerebral ischaemic damage was increased (Bruce et al., 1996) and TNF $\alpha$  was seen to protect cultured embryonic rat hippocampal, septal and cortical neurons against glucose deprivation-induced and excitotoxic injury (Cheng et al., 1994; Houzen et al., 1997). This concurs with the dual role of NF- $\kappa$ B in TNF $\alpha$  neurodestruction and neuroprotection (Lipton, 1997). A possible route for TNF $\alpha$  mediated neuroprotection is the induction of phosphatidyl inositol-3 kinase (PI3'K) activity (Venters et al., 2001). TNF $\alpha$  transiently induces PI3'K activity in myeloid cells and when PI3'K activity is inhibited by the use of antibodies, TNF $\alpha$  induces cell death. This indicates that TNF $\alpha$  may initiate neuronal survival by the induction of PI3'K activity.

The primary determinant of the dual roles of TNF $\alpha$  may well be the NF- $\kappa$ B pathway. It has been suggested that NF- $\kappa$ B is induced by TNF $\alpha$  under certain circumstances leading to cell survival and inhibited under others with concurrent promotion of alternative pro-apoptotic signals (Kaltschmidt et al., 1999). TNF $\alpha$  has also been found to have a biphasic effect in neurons recovering from injury. It exerts a damaging effect in the early post-traumatic (ischaemic) period, where deficits in memory retention were shown to be lower in *TNF $\alpha$ <sup>-/-</sup>* mice as compared to their wildtype counterparts. In contrast to this, the absence of TNF $\alpha$  during the post-injury period may be deleterious as TNF $\alpha$  - deficient mice were seen to have increased levels of cortical tissue loss at this stage, when compared to wildtype mice (Scherbel et al., 1999). Differences in NF- $\kappa$ B activity may partially account for regional differences observed in response to TNF $\alpha$  under identical conditions (Galasso et al., 2000).

Thus there is much evidence that TNF $\alpha$  can have both pro- and anti-apoptotic effects within the nervous system. It has also been shown to induce neuronal death associated with HIV-infection (New et al., 1998), ischaemia (Dawson et al., 1996) and axotomy (Terrado et al., 2000). In contrast to these findings it has been demonstrated that TNF $\alpha$  exerts a neuroprotective effect against glutamate excitotoxicity (Houzen et al., 1997; Carlson et al., 1998) suggesting that although there is growing knowledge of the effects of TNF $\alpha$  in cytotoxic states, little is known about the effects of TNF $\alpha$  on neuronal survival during the development of the nervous system. Because of this together with

the unusual results found in the retina of TNF $\alpha$  deficient mice, which indicated that TNF $\alpha$  is enhancing cell survival in this area during development (Figure 3.15), I extended my study to include the *in vitro* and *in vivo* examination of the effects of TNF $\alpha$  on sensory and sympathetic neurons during development. Extensive tissue culture, dose response, histochemical and histological analysis were carried out to examine how TNF $\alpha$  affects neuronal survival of SCG, trigeminal and nodose ganglia.

## 4.2 Results

### 4.2.1 TNF $\alpha$ Enhances the Responsiveness of Neurons to Neurotrophins *in vitro*

In order to determine whether or not TNF $\alpha$  was acting to enhance the survival response of SCG, trigeminal and nodose neurons to neurotrophins the neurons were grown for 12 hours with saturating concentrations (2ng/ml) of either NGF (trigeminal and SCG neurons) or BDNF (nodose neurons) and then washed extensively to remove the neurotrophins and grown with and without neurotrophic support, with TNF $\alpha$  alone, with saturating concentrations of neurotrophin plus TNF $\alpha$  or with function blocking anti-TNF $\alpha$  or anti-TNFR1 antibodies.

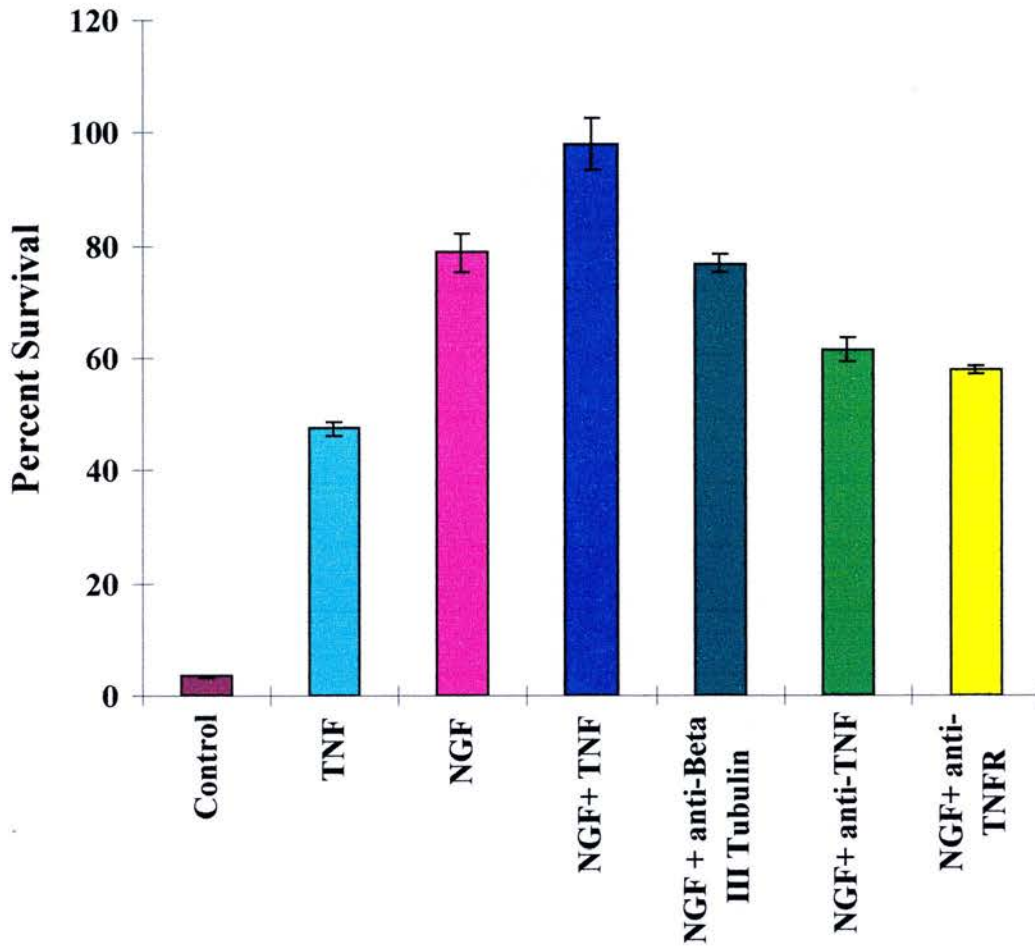
At E14 TNF $\alpha$  is able to promote the survival of developing sensory and sympathetic neurons. Over 50% of the NGF-dependent trigeminal and SCG neurons survived for 48 hours in culture with TNF $\alpha$  (2ng/ml) as compared to less than 10% survival in control untreated cultures (figure 4.3, 4.4 and table 4.1). Similarly over 40% of BDNF-dependent nodose sensory neurons survived when grown with TNF $\alpha$  for 48 hours as compared to less than 10% survival in control cultures (figure 4.5 and table 4.1).

Neurons treated with either NGF or BDNF in addition to TNF $\alpha$  showed enhanced survival as compared to survival with neurotrophin or TNF $\alpha$  alone ([NGF/BDNF + TNF $\alpha$ ] vs TNF $\alpha$  - SCG -  $p < 0.0001$ , trigeminal -  $p < 0.0001$ , nodose -  $p < 0.001$ , unpaired

T-test - [NGF/BDNF+ TNF $\alpha$ ] vs NGF/BDNF - SCG -  $p < 0.005$ , trigeminal -  $p < 0.0001$ , nodose -  $p < 0.01$ , unpaired t-test)(figure 4.3-4.5 and table 4.1).

Under these conditions, addition of either anti-TNF $\alpha$  or anti-TNFR1 antibody markedly reduced the survival response of trigeminal and SCG neurons to NGF and of nodose neurons to BDNF (figures 4.3-4.5 and table 4.1) as presumably it prevented the cell survival enhancing affect of TNF $\alpha$ . In all cases there are around 20% less neurons surviving in response to NGF or BDNF when grown in combination with either of the function blocking antibodies as compared to NGF or BDNF alone (SCG -both cases-  $p < 0.0001$ , one way ANOVA with post hoc tukey multiple comparisons test; nodose - BDNF V's [BDNF + anti-TNFR] -  $p < 0.0001$ ; BDNF V's [BDNF + anti-TNF] -  $p < 0.0005$ , one way ANOVA with post hoc tukey multiple comparisons test; trigeminal - both cases -  $p < 0.0001$ , one way ANOVA with post hoc tukey multiple comparisons test). To ascertain whether this effect was due to non-specific toxicity resulting from the addition of the antibodies to the culture medium, control cultures supplemented with neurotrophin together with antibodies to the intracellular neuron-specific antigen  $\beta$ III tubulin were set up. Under these conditions the neurons survived as well as in control cultures grown with neurotrophin alone (There was no significant difference between these conditions and SCG, nodose or trigeminal control cultures grown with neurotrophin alone, one way ANOVA with post hoc Tukey multiple comparisons test) (figures 4.3-4.5 and table 4.1).

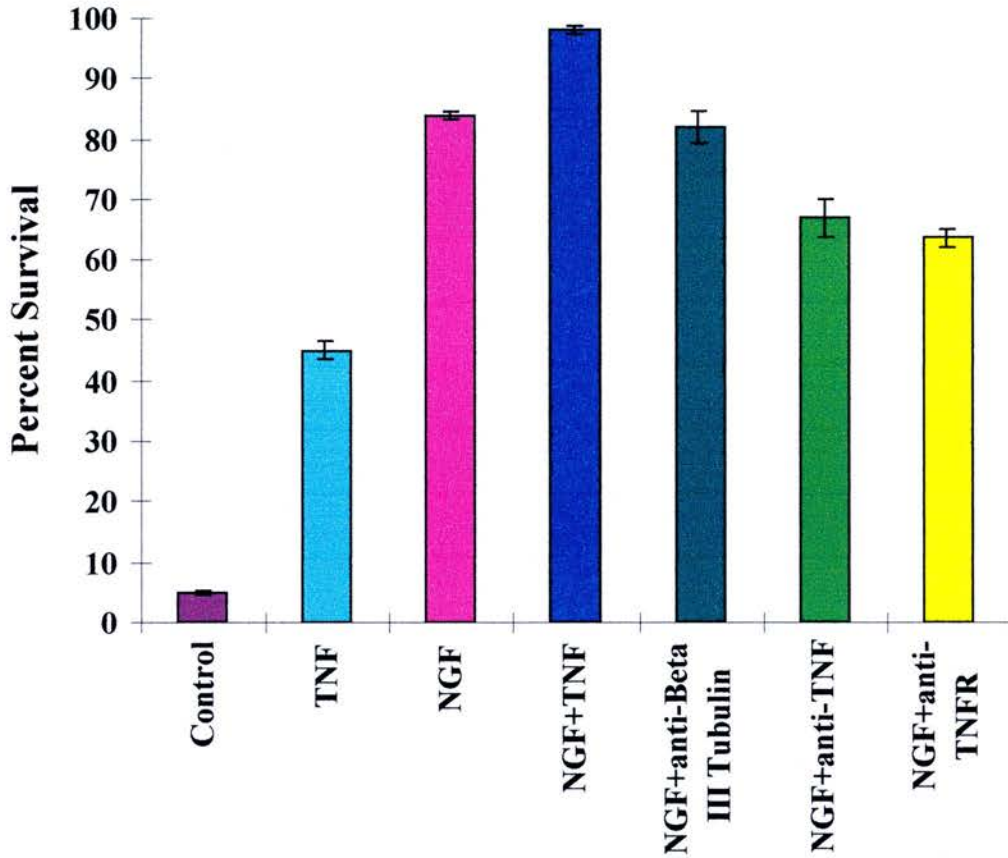
## E14 SCG Neurons



**Figure 4.3:** Bar chart showing the percent survival of cultured SCG neurons grown with 2ng/ml of NGF, TNF $\alpha$ , NGF plus TNF $\alpha$  or antibodies to either TNF $\alpha$ , TNFR or the intracellular neuron-specific antigen  $\beta$ III-tubulin. The means and standard errors of four separate experiments for each population are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. TNF $\alpha$  can be seen to increase the percent survival of SCG neurons and also the neurotrophin induced survival response. Anti-bodies to TNF $\alpha$  and TNFR1 reduced the neurotrophin induced survival response, indicating that if TNF $\alpha$  is blocked cell survival is inhibited. This effect was not due to non-specific actions of anti-bodies on neuronal survival as there was no significant difference between cultures treated with NGF and those treated with NGF and anti- $\beta$ III tubulin anti-bodies.



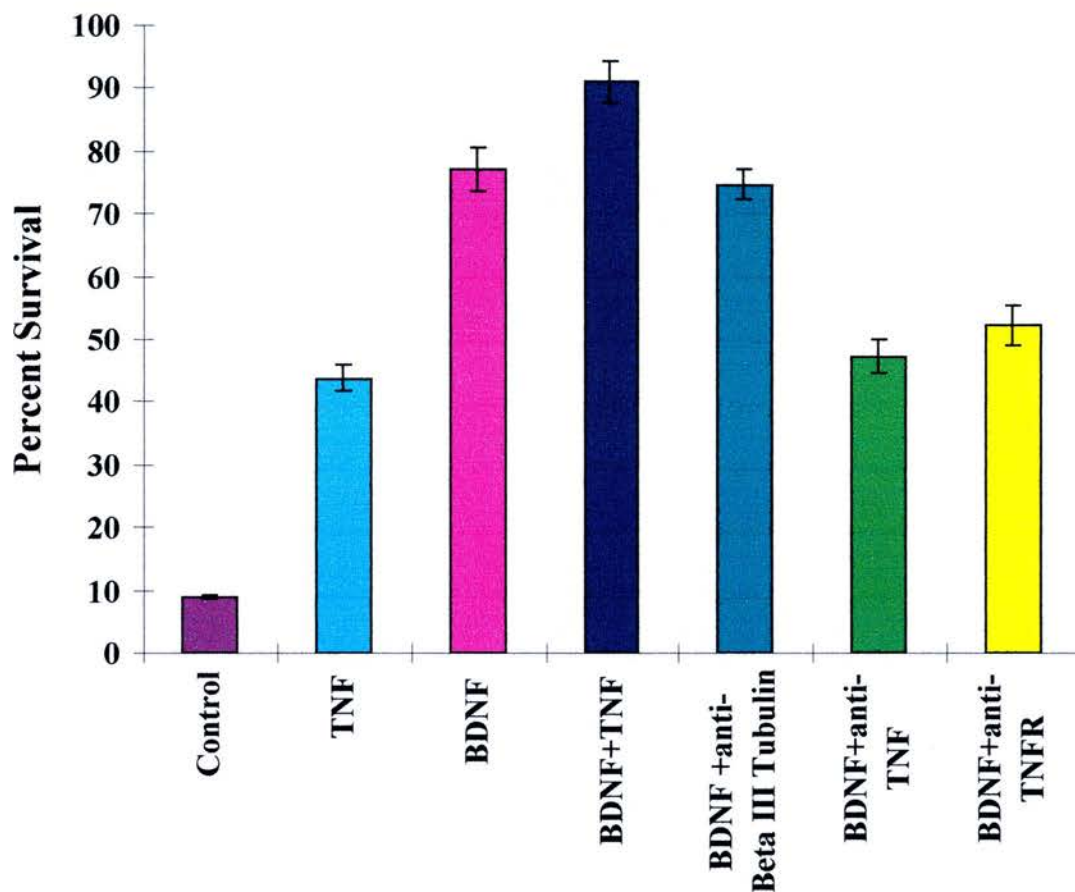
## E14 Trigeminal Neurons



**Figure 4.4:** Bar chart showing the percent survival of cultured trigeminal neurons grown with 2ng/ml NGF, TNF $\alpha$ , NGF plus TNF $\alpha$  or antibodies to either TNF $\alpha$ , TNFR or the intracellular neuron-specific antigen  $\beta$ III-tubulin. The means and standard errors of four separate experiments for each population are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. TNF $\alpha$  can be seen to increase the percent survival of trigeminal neurons and also the neurotrophin induced survival response. Anti-bodies to TNF $\alpha$  and TNFR1 reduced the neurotrophin induced survival response, indicating that if TNF $\alpha$  is blocked cell survival is inhibited. This effect was not due to non-specific actions of anti-bodies on neuronal survival as there was no significant difference between cultures treated with NGF and those treated with NGF and anti- $\beta$ III tubulin anti-bodies.



## E14 Nodose Neurons



**Figure 4.5:** Bar chart showing the percent survival of cultured nodose neurons grown with 2ng/ml BDNF,  $\text{TNF}\alpha$ , BDNF plus  $\text{TNF}\alpha$  or antibodies to either  $\text{TNF}\alpha$ , TNFR or the intracellular neuron-specific antigen  $\beta\text{III-tubulin}$ . The means and standard errors of four separate experiments for each population are shown. Survival is expressed as a percentage of a count taken 6 hours after plating.  $\text{TNF}\alpha$  can be seen to increase the percent survival of nodose neurons and also the neurotrophin induced survival response. Anti-bodies to  $\text{TNF}\alpha$  and TNFR1 reduced the neurotrophin induced survival response, indicating that if  $\text{TNF}\alpha$  is blocked cell survival is inhibited. This effect was not due to non-specific actions of anti-bodies on neuronal survival as there was no significant difference between cultures treated with BDNF and those treated with BDNF and anti- $\beta\text{III tubulin}$  anti-bodies.

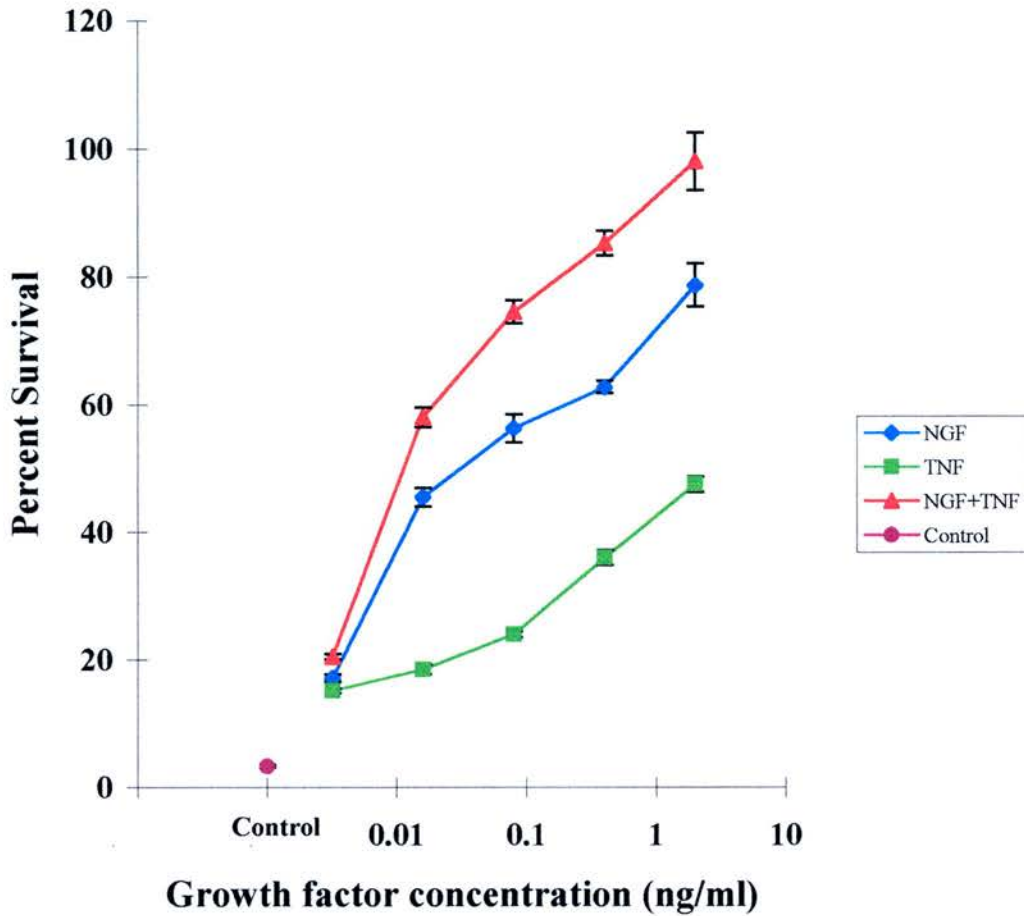
Reagent	SCG		Trigeminal		Nodose	
	% Survival	Std Error	% Survival	Std Error	% Survival	Std Error
Control	3.37	0.2	4.39	0.3	8.84	0.3
TNF $\alpha$	47.5	1.2	45	1.6	43.78	1.9
NGF/BDNF	78.7	3.3	83.85	0.8	77.12	3.5
NGF/BDNF + TNF $\alpha$	98	4.5	98.02	0.8	91.02	3.3
NGF+ anti-TNF $\alpha$	61.33	2.2	66.9	3.2	47.23	2.7
NGF/BDNF + anti-TNFR1	57.75	0.7	63.57	1.6	52.23	3.3
NGF/BDNF + anti- $\beta$ III Tubulin	76.75	1.6	81.77	2.6	74.62	2.4

**Table 4.1: Summary of SCG, trigeminal and nodose neurons cultured with 2ng/ml neurotrophin, TNF $\alpha$ , neurotrophin plus TNF $\alpha$  or antibodies to either TNF $\alpha$ , TNFR or the intracellular neuron-specific antigen  $\beta$ III-tubulin.** The mean percent survival and standard errors for SCG, trigeminal and nodose neurons cultured with 2ng/ml NGF/BDNF, TNF $\alpha$ , NGF/BDNF plus antibodies to either TNF $\alpha$ , TNFR or the intracellular neuron-specific antigen  $\beta$ III-tubulin. TNF $\alpha$  can be seen to increase the percent survival of SCG, trigeminal and nodose neurons and also the neurotrophin induced survival response. Anti-bodies to TNF $\alpha$  and TNFR1 reduced the neurotrophin induced survival response, indicating that if TNF $\alpha$  is blocked cell survival is inhibited. This effect was not due to non-specific actions of anti-bodies on neuronal survival as there was no significant difference between cultures treated with neurotrophin and those treated with anti- $\beta$ III tubulin anti-bodies.

#### **4.2.2 TNF $\alpha$ Promotes the Survival of Neurons *in vitro***

Detailed dose response analysis revealed that for SCG, trigeminal and nodose neurons, the addition of TNF $\alpha$  at a concentration of 3.2pg/ml or above was sufficient to bring about a significant enhancement of the normal neurotrophin survival response (SCG - p<0.01, unpaired t-test; nodose - p<0.0005, unpaired t-test; trigeminal- p<0.0001, unpaired t-test) and significantly enhance survival above the control level (SCG - p<0.001, unpaired t-test; nodose - p<0.05, unpaired t-test; trigeminal- p<0.005, unpaired t-test)(figures 4.6- 4.8).

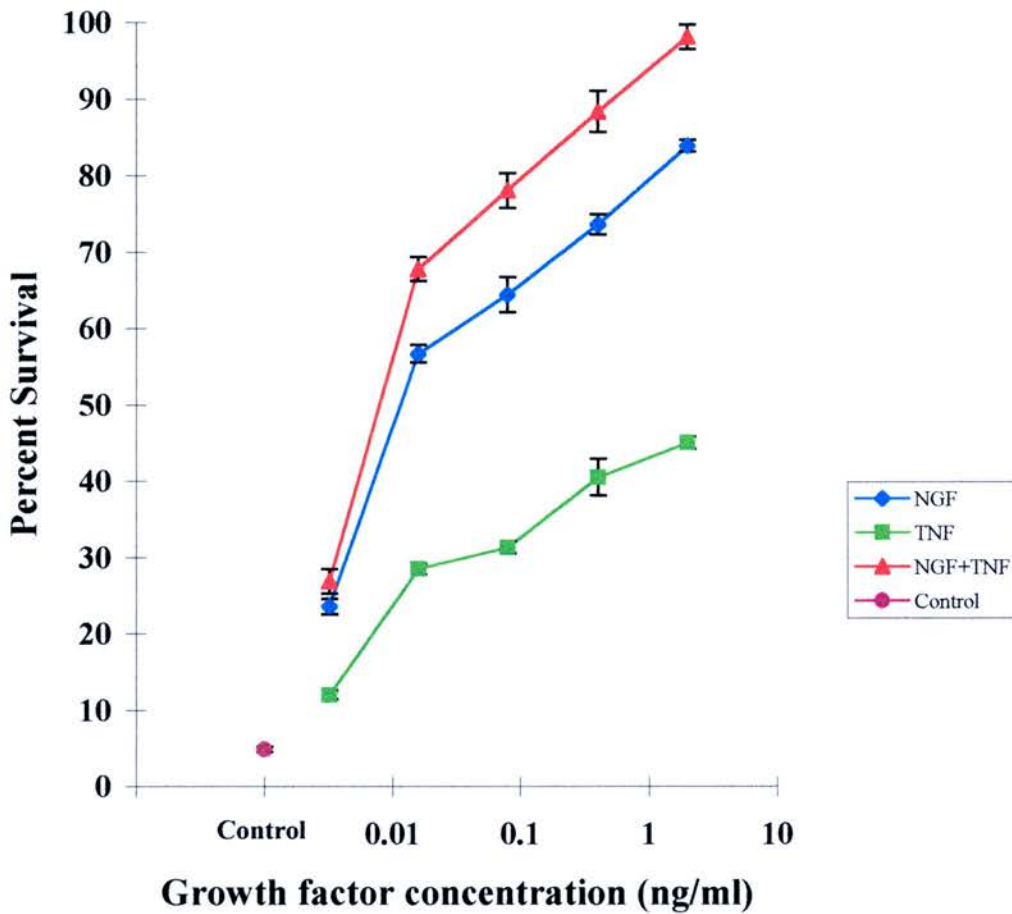
## E16 SCG Neurons



**Figure 4.6: Graph showing the dose response of E14 SCG neurons after 48 hours incubation in culture with neurotrophin or  $TNF\alpha$  alone or in combination or grown without trophic support.** The percent survival was quantified following treatment with increasing concentrations of reagent, in order to determine whether any effects seen occurred in a dose dependent manner. Control cultures received no reagents following NGF deprivation. The means and standard errors of three separate experiments for are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. Survival is expressed as a percentage of a count taken 6 hours after plating.  $TNF\alpha$  can be seen to increase the percent survival of SCG neurons above the control level and also enhance the neurotrophin induced survival response.  $TNF\alpha$  produced these effects in a dose dependent manner.

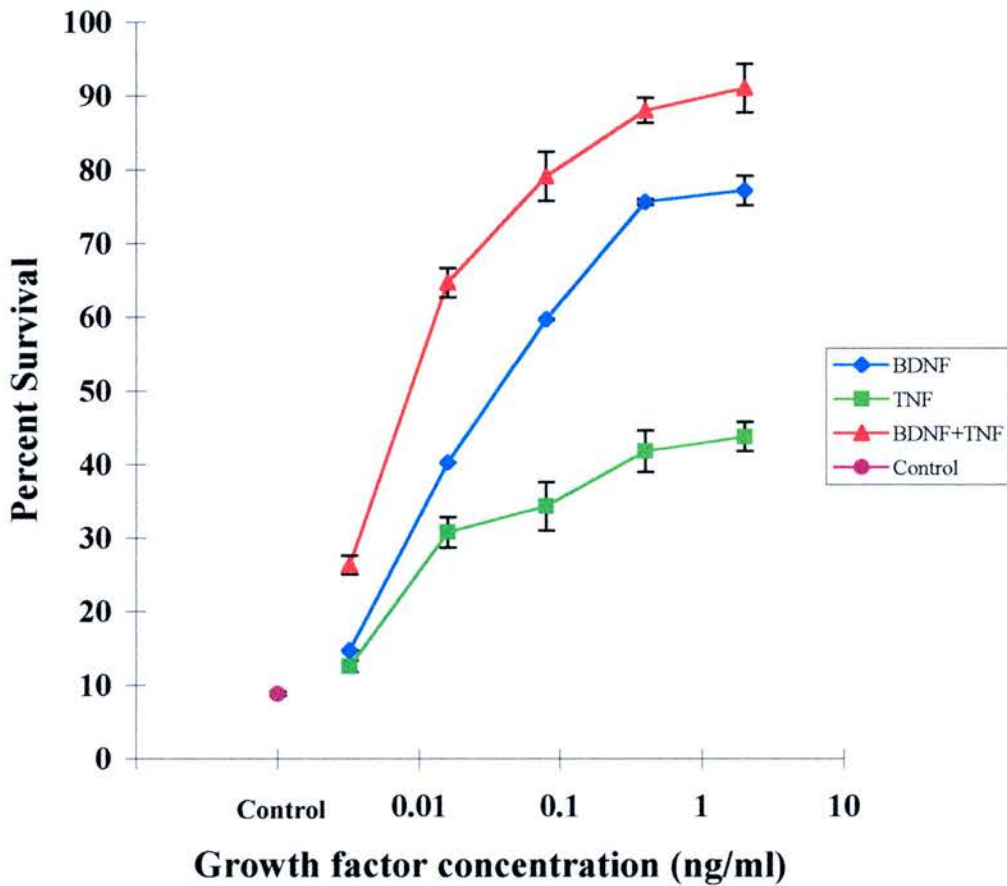


## E14 Trigeminal Neurons



**Figure 4.7: Graph showing the dose response of E14 trigeminal neurons after 48 hours incubation in culture with neurotrophin or  $TNF\alpha$  alone or in combination or grown without trophic support.** The percent survival was quantified following treatment with increasing concentrations of reagent, in order to determine whether any effects seen occurred in a dose dependent manner. Control cultures received no reagents following NGF deprivation. The means and standard errors of three separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating.  $TNF\alpha$  can be seen to increase the percent survival of trigeminal neurons above the control level and also enhance the neurotrophin induced survival response.  $TNF\alpha$  produced these effects in a dose dependent manner.

## E14 Nodose Neurons



**Figure 4.8:** Graph showing the dose response of E14 nodose neurons after 48 hours incubation in culture with neurotrophin or  $\text{TNF}\alpha$  alone or in combination or grown without trophic support. The percent survival was quantified following treatment with increasing concentrations of reagent, in order to determine whether any effects seen occurred in a dose dependent manner. Control cultures received no reagents following BDNF deprivation. The means and standard errors of three separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating.  $\text{TNF}\alpha$  can be seen to increase the percent survival of nodose neurons above the control level and also enhance the neurotrophin induced survival response.  $\text{TNF}\alpha$  produced these effects in a dose dependent manner.

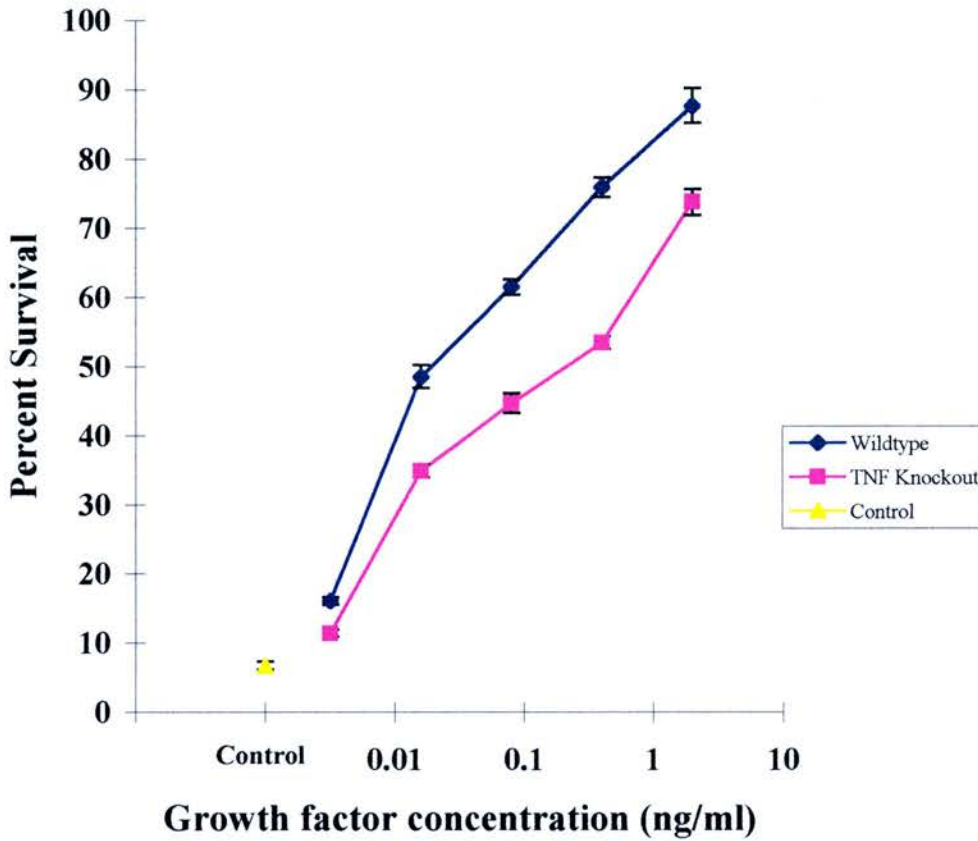
This suggests that at E14 TNF $\alpha$  acts as a neurotrophic factor for developing sensory and sympathetic neurons. The additive effect seen when neurotrophins and TNF $\alpha$  are added to the culture medium in combination suggests that these factors may act on different subsets of developing neurons although there is clearly some overlap between the two populations.



### 4.2.3 Reduced Survival TNF $\alpha$ -Deficient Neurons *in vitro*

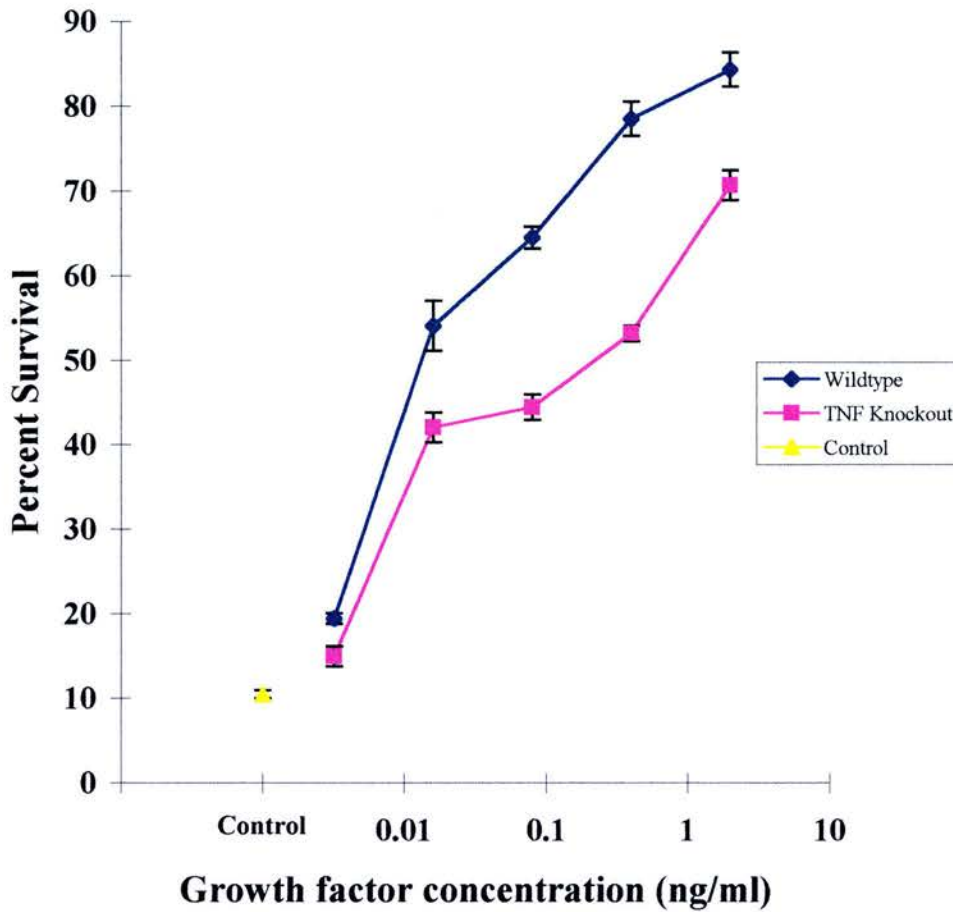
To provide additional evidence that TNF $\alpha$  is involved in maintaining the survival of E14 sensory and sympathetic neurons in culture, I compared the *in vitro* survival of neurons derived from the nodose, trigeminal and SCG of wild type mice and mice that are homozygous for a null mutation in the *tnf $\alpha$*  gene. Detailed dose response analyses were carried out on these neurons to measure their responsiveness to their appropriate neurotrophin. There was a significant reduction in the sensitivity of the TNF $\alpha$  - deficient neurons from all three populations to either NGF or BDNF. Although there was no significant difference in the survival of wild type and TNF $\alpha$ -deficient neurons at very low concentrations of neurotrophin (Figure 4.9-4.11 and table 4.2), there was a marked decrease in the sensitivity of TNF $\alpha$ -deficient neurons to NGF at concentrations of 3.2pg/ml and above (SCG -  $p < 0.005$ , unpaired t-test; trigeminal -  $p < 0.01$ , unpaired t-test) and to BDNF at concentrations of 16pg/ml and above (nodose -  $p < 0.005$ , unpaired t-test). These results lend further weight to the idea that endogenously produced TNF $\alpha$  contributes to the survival of E14 sensory and sympathetic neurons as when TNF expression is eliminated there is a reduction in cell survival.

## E14 SCG Neurons



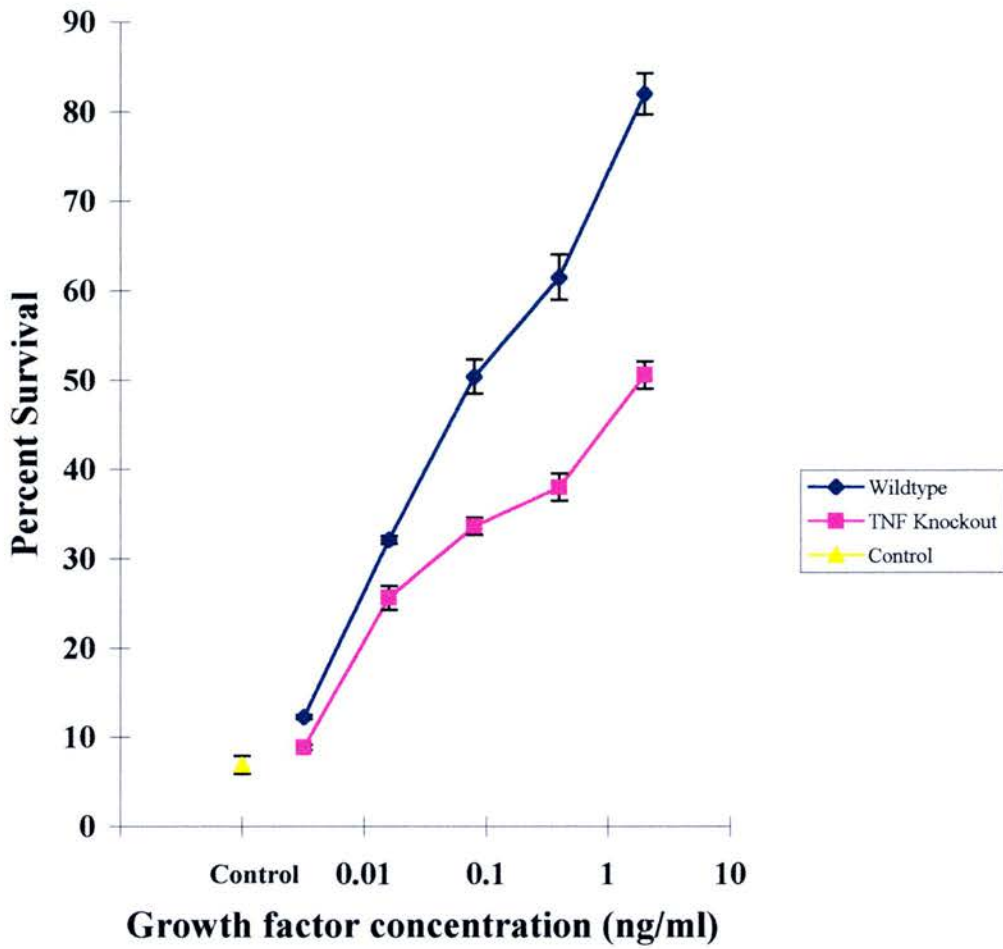
**Figure 4.9:** Graph showing the percent survival of E14 wild type and TNF $\alpha$ -deficient SCG neurons grown in culture for 48 hours with a range of concentrations of NGF. The percent survival was quantified following treatment with increasing concentrations of reagent, in order to determine whether any effects seen occurred in a dose dependent manner. Control cultures received no reagents following NGF deprivation. The means and standard errors of four separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. TNF $\alpha$  deficient neurons show decreased sensitivity to NGF at concentrations of 3.2pg/ml and above, indicating that TNF $\alpha$  enhances cell survival in SCG neurons.

## E14 Trigeminal Neurons



**Figure 4.10:** Graph showing the percent survival of E14 wild type and TNF $\alpha$ -deficient trigeminal neurons grown in culture for 48 hours with a range of concentrations of NGF. The percent survival was quantified following treatment with increasing concentrations of reagent, in order to determine whether any effects seen occurred in a dose dependent manner. Control cultures received no reagents following NGF deprivation. The means and standard errors of four separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. TNF $\alpha$  deficient neurons show decreased sensitivity to NGF at concentrations of 3.2pg/ml and above, indicating that TNF $\alpha$  enhances cell survival in trigeminal neurons.

## E14 Nodose Neurons



**Figure 4.11: Graph showing the percent survival of E14 wild type and TNF $\alpha$ -deficient nodose neurons grown in culture for 48 hours with a range of concentrations of BDNF.** The percent survival was quantified following treatment with increasing concentrations of reagent, in order to determine whether any effects seen occurred in a dose dependent manner. Control cultures received no reagents following BDNF deprivation. The means and standard errors of four separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. TNF $\alpha$  deficient neurons show decreased sensitivity to BDNF at concentrations of 16pg/ml and above, indicating that TNF $\alpha$  enhances cell survival in nodose neurons.



<b>SCG</b>				
<b>NGF Conc. (ng)</b>	<b>Wildtype</b>	<b>Std Error</b>	<b>TNF<math>\alpha</math>-/-</b>	<b>Std Error</b>
<b>0.0032</b>	16.10	0.53	11.43	0.48
<b>0.016</b>	48.55	1.66	34.92	0.96
<b>0.08</b>	61.53	1.11	44.71	1.41
<b>0.4</b>	75.94	1.42	53.44	0.91
<b>2</b>	87.69	2.50	73.78	1.87
<b>Trigeminal Ganglia</b>				
<b>NGF Conc. (ng)</b>	<b>Wildtype</b>	<b>Std Error</b>	<b>TNF<math>\alpha</math>-/-</b>	<b>Std Error</b>
<b>0.0032</b>	19.46	0.47	14.98	0.69
<b>0.016</b>	54.06	0.64	42.04	1.74
<b>0.08</b>	64.5	1.28	44.42	1.49
<b>0.4</b>	78.47	2.03	53.17	0.92
<b>2</b>	84.29	2.01	70.6	1.77
<b>Nodose Ganglia</b>				
<b>BDNF Conc. (ng)</b>	<b>Wildtype</b>	<b>Std Error</b>	<b>TNF<math>\alpha</math>-/-</b>	<b>Std Error</b>
<b>0.0032</b>	12.29	0.26	8.9	1.54
<b>0.016</b>	32.12	0.45	25.63	0.93
<b>0.08</b>	50.38	1.89	33.63	1.35
<b>0.4</b>	61.5	2.53	38.02	0.29
<b>2</b>	82	2.29	50.55	0.51

**Table 4.2: Summary of *in vitro* transgenic dose response results.** The mean percent survival and standard errors for tissue cultures of SCG, trigeminal and nodose neurons from wildtype and TNF $\alpha$  deficient embryos grown with increasing concentrations of neurotrophin are shown. TNF $\alpha$  deficient SCG and trigeminal neurons show decreased sensitivity to NGF at concentrations of 3.2pg/ml and above and TNF $\alpha$  deficient nodose neurons show decreased sensitivity to BDNF at concentrations of 16pg/ml and above, indicating that TNF $\alpha$  enhances cell survival in all these neurons.

#### 4.2.4 Enhanced Neuronal Death in TNF $\alpha$ -Deficient Embryos *in vivo*

To determine whether or not TNF $\alpha$  is able to promote the survival of developing sensory and sympathetic neurons *in vivo*, I estimated the number of surviving neurons and the extent of cell death in the SCG, nodose and trigeminal ganglia of wild type mice and their TNF $\alpha$  deficient litter-mates at E14. This was done by counting the total number of neurons that are positive for a neuron-specific marker (anti- $\beta$ III tubulin) and the number of pyknotic neurons in serial sections using digital stereology.

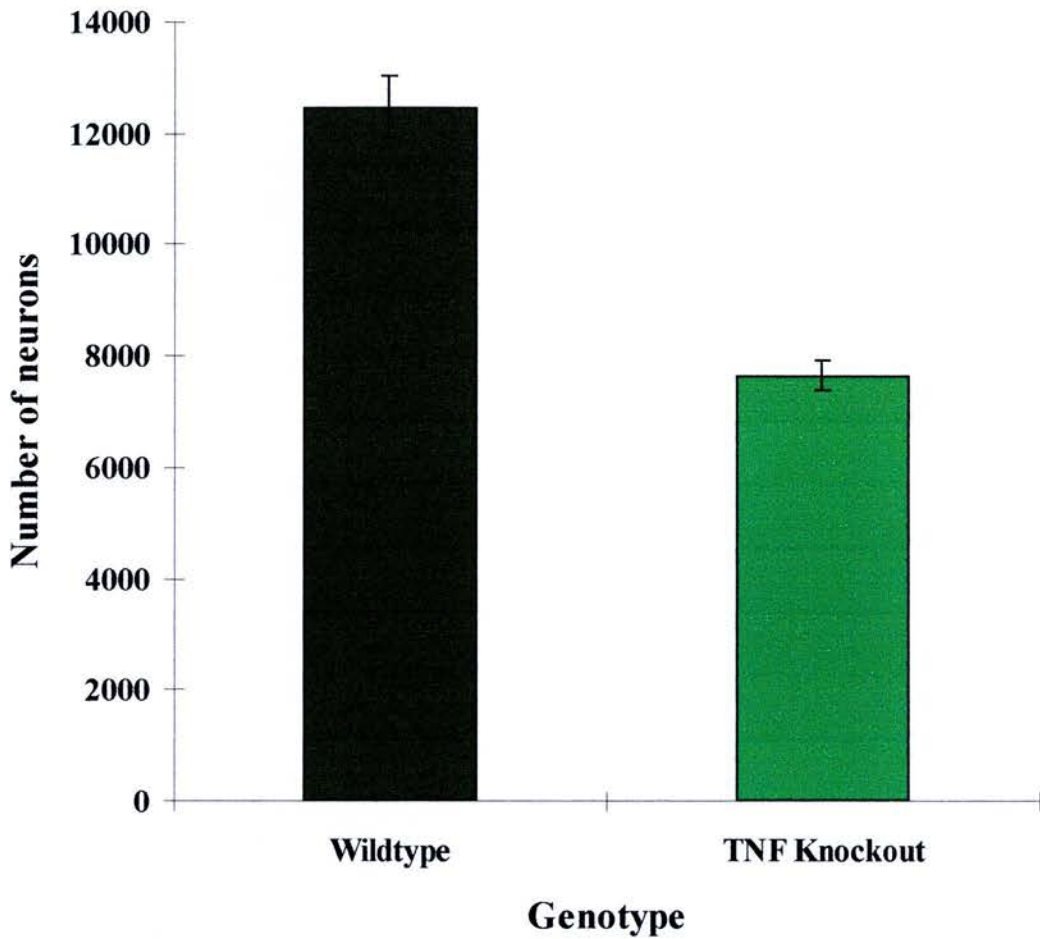
At E14 there is a statistically significant reduction in the number of neurons in all three ganglia derived from *tnf $\alpha$ <sup>-/-</sup>* embryos as compared to those from their wild type litter-mates (39% for SCG neurons [p<0.0001, unpaired t-test], 41% for nodose neurons [p<0.0005, unpaired t-test] and 24% for trigeminal neurons [p<0.05, unpaired t-test]) (figures 4.12, 4.14, 4.16 and table 4.3). Accordingly, there was a marked enhancement in the magnitude of neuronal death in all three populations (figures 4.13, 4.15, 4.17 and table 4.3). In the SCG there were 158% more dying neurons (p<0.0001, unpaired t-test), 123% more dying neurons in the nodose ganglion (p<0.005, unpaired t-test) and in the trigeminal ganglion there were 85% more dying neurons (p<0.005, unpaired t-test).

It should be noted that there are some differences in the numbers of neurons and pyknotic in the SCG of E14 embryos in this set of experiments and those in chapter 3 (figure 3.13 and 3.14). This did not affect the results as comparisons in these experiments were made independently of each other between wildtype and knockout

embryos. It should also be noted that the relationship between the numbers of pyknotic neurons and final neuronal number were relatively consistent between the three ganglia (ratio of pyknotic neurons/ total neurons: SCG = 3.6, trigeminal ganglia = 2.4, nodose ganglia = 5.5).

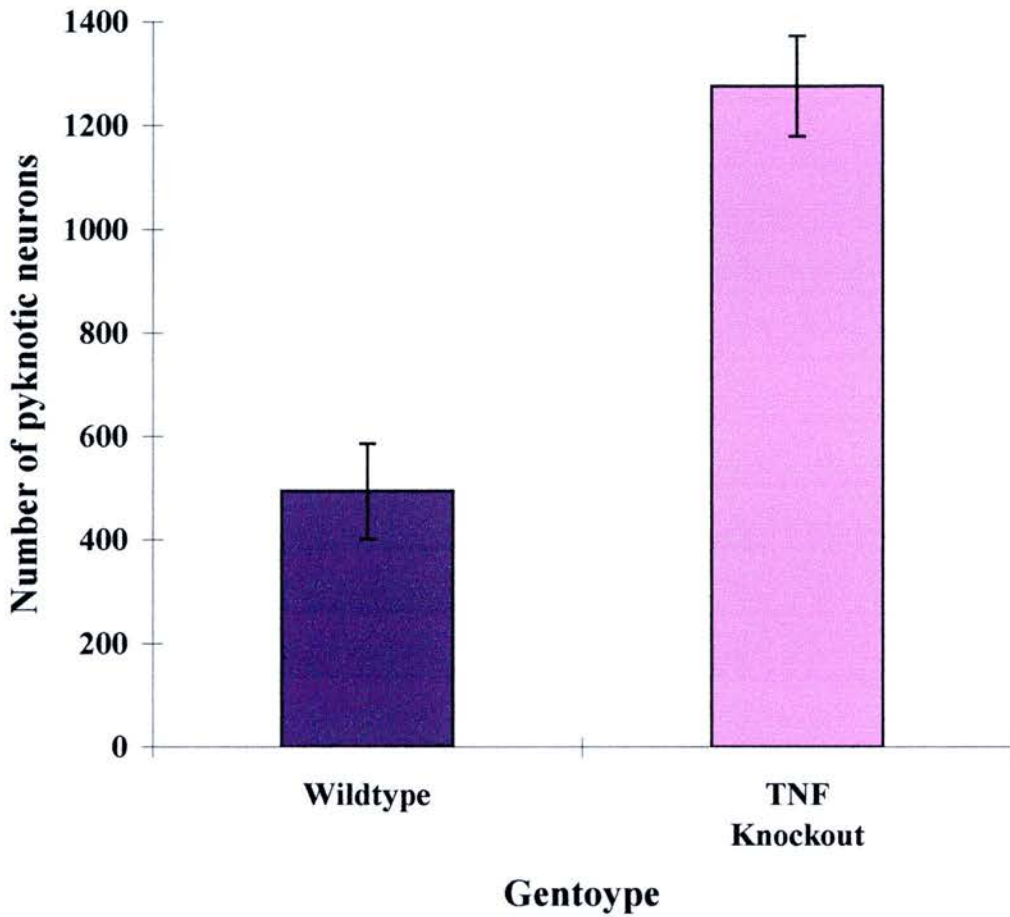


### E14 SCG: Total Neurons



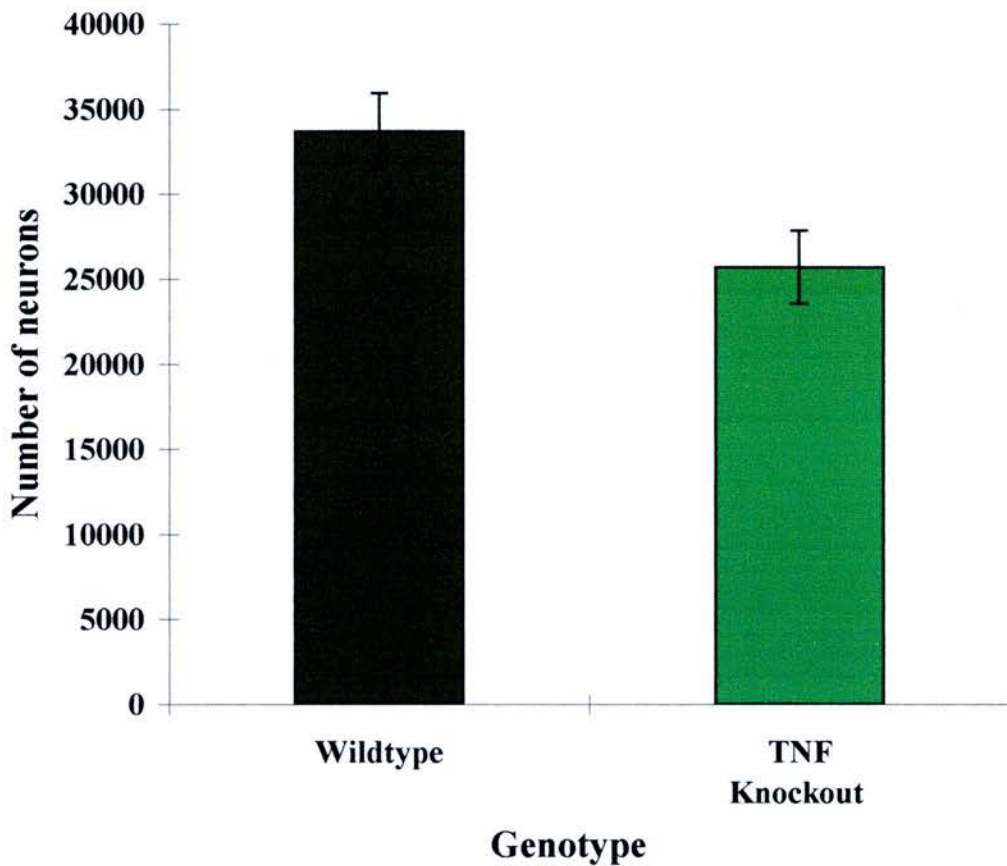
**Figure 4.12: Bar chart showing the total number of neurons in the SCG of E14 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors shown are taken from counts of both right and left ganglia in 5 wild type and 5 TNF $\alpha$  deficient mice. The numbers of neurons in the SCG were decreased by 39% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.

### E14 SCG: Pyknotic Neurons



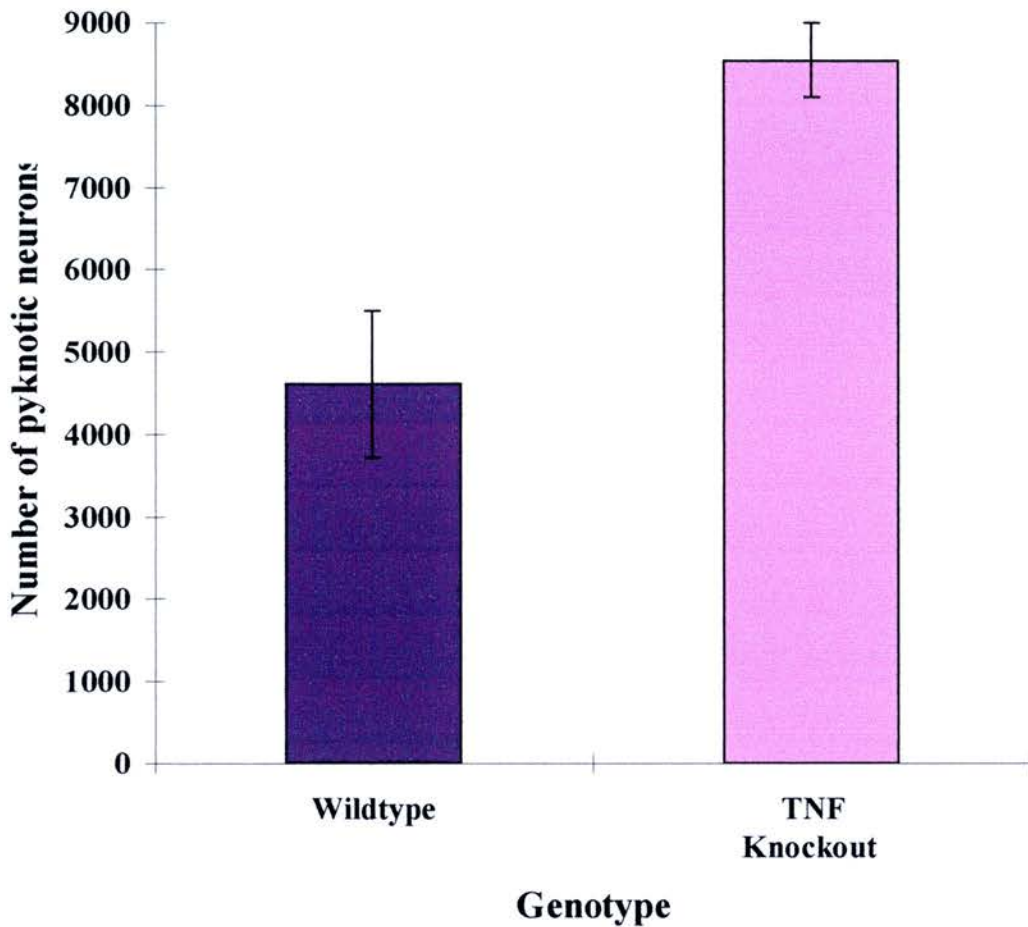
**Figure 4.13: Bar chart showing the number of pyknotic neurons in the SCG of E14 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors shown are taken from counts of both right and left ganglia in 5 wild type and 5 TNF $\alpha$  deficient mice. The numbers of pyknotic neurons, and therefore cell death, in the SCG were increased by approximately 158% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.

### E14 Trigeminal: Total Neurons



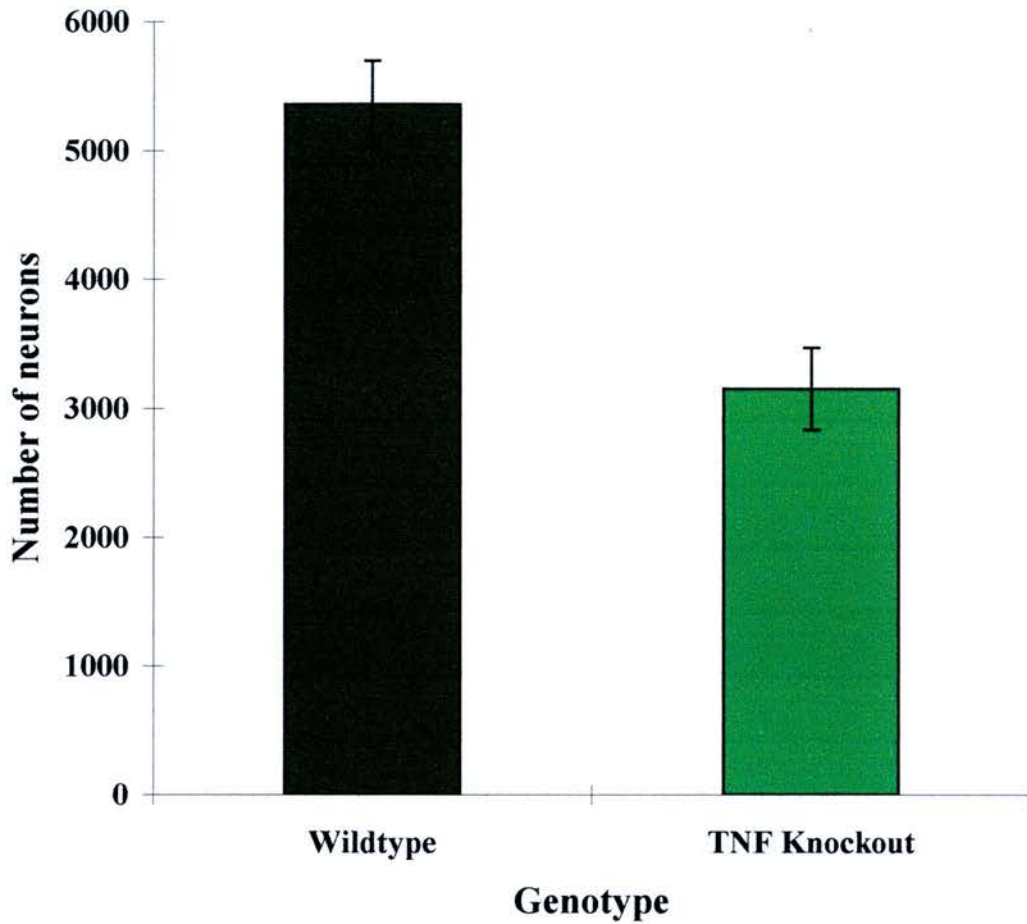
**Figure 4.14:** Bar chart showing the total number of neurons in the trigeminal ganglia of E14 wildtype and TNF $\alpha$  knockout mice. The means and standard errors shown are taken from counts of both right and left ganglia in 5 wild type and 5 TNF $\alpha$  deficient mice. The numbers of neurons in the trigeminal ganglia were decreased by 24% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.

### E14 Trigeminal: Pyknotic Neurons



**Figure 4.15: Bar chart showing the number of neurons in the trigeminal ganglia at E14 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors shown are taken from counts of both right and left ganglia in 5 wild type and 5 TNF $\alpha$  deficient mice. The numbers of pyknotic neurons, and therefore cell death, in the trigeminal ganglia were increased by approximately 85% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.

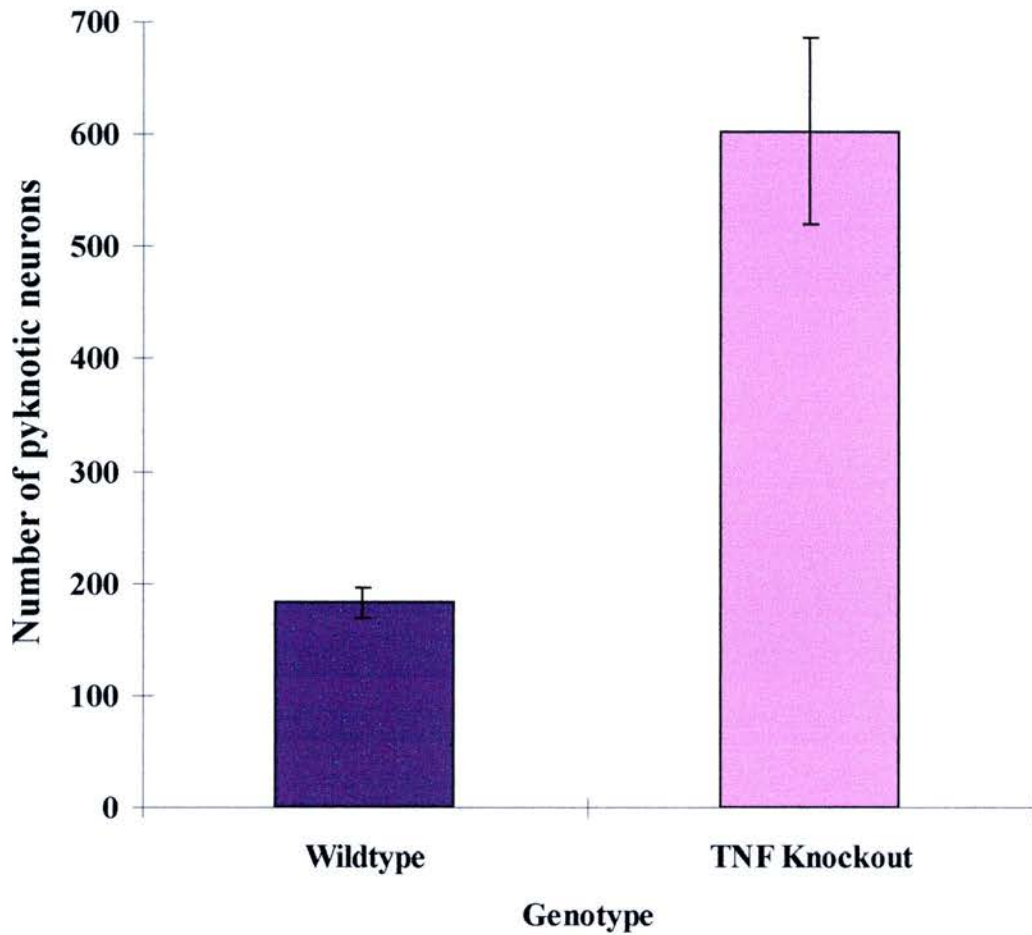
### E14 Nodose: Total Neurons



**Figure 4.16: Bar chart showing the total number of neurons in the nodose ganglia of E14 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors shown are taken from counts of both right and left ganglia in 5 wild type and 5 TNF $\alpha$  deficient mice. The numbers of neurons in the nodose ganglia were decreased by 41% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.



## E14 Nodose: Pyknotic Neurons



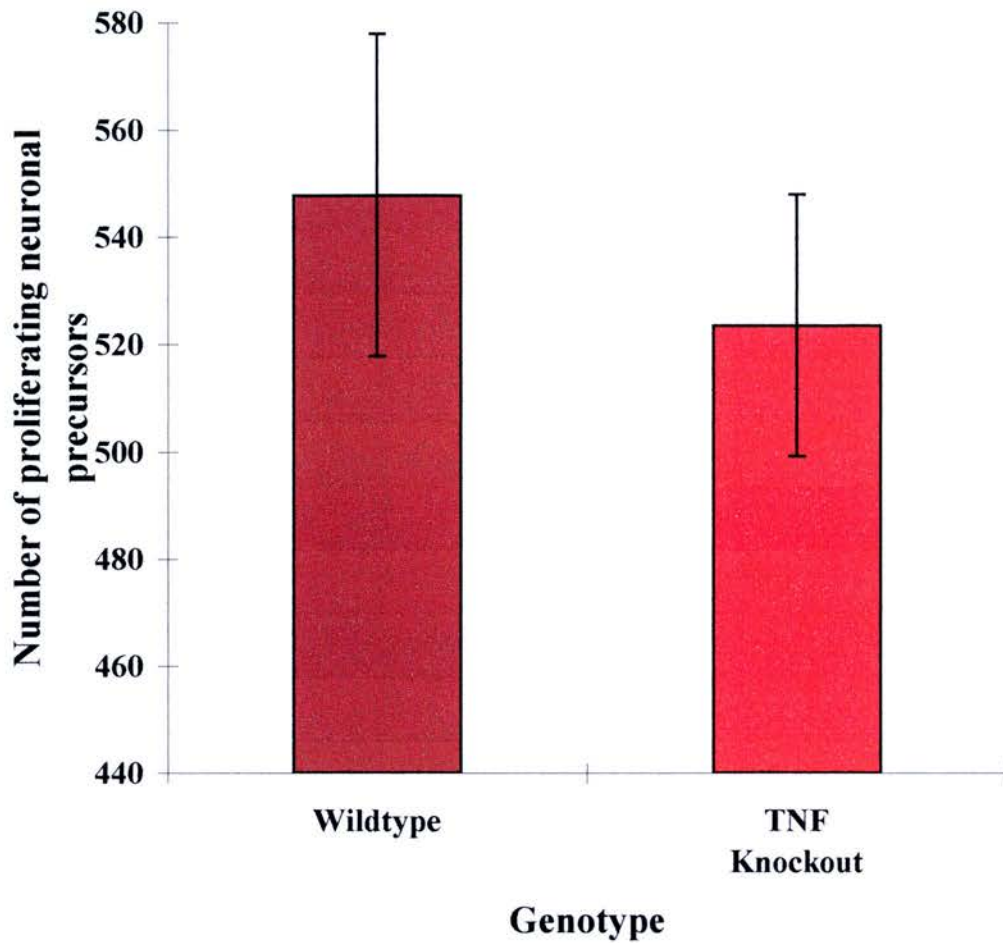
**Figure 4.17: Bar chart showing the number of pyknotic neurons in the nodose ganglia of E14 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors shown are taken from counts of both right and left ganglia in 5 wild type and 5 TNF $\alpha$  deficient mice. The numbers of pyknotic neurons, and therefore cell death, in the nodose ganglia were decreased by approximately 123% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.

#### **4.2.5 TNF $\alpha$ does not Enhance Neuronal Number by Modulating Cell Proliferation**

In order to test the hypothesis that TNF $\alpha$  could be enhancing neuronal number by modulating cell proliferation, I counted serial sections through SCG, trigeminal and nodose ganglia derived from TNF $\alpha$  deficient and wild type mice that were stained for proliferating cell nuclear antigen and  $\beta$ III-tubulin to ascertain the number of dividing cells. In all cases there was no significant difference between the number of dividing cells in TNF $\alpha$  deficient ganglia as compared to wild type (non-significant, unpaired t-test) (figures 4.18-4.20 and table 4.3).

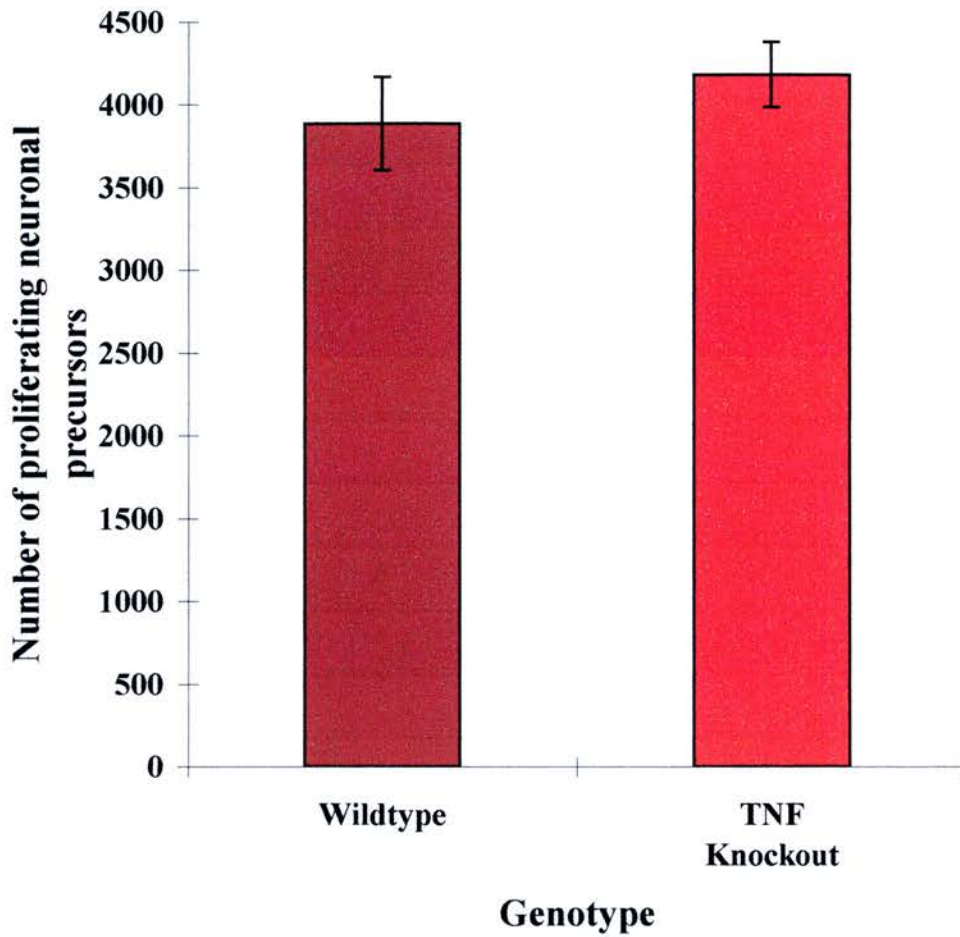


### E14 SCG: Proliferating Neuronal Precursors

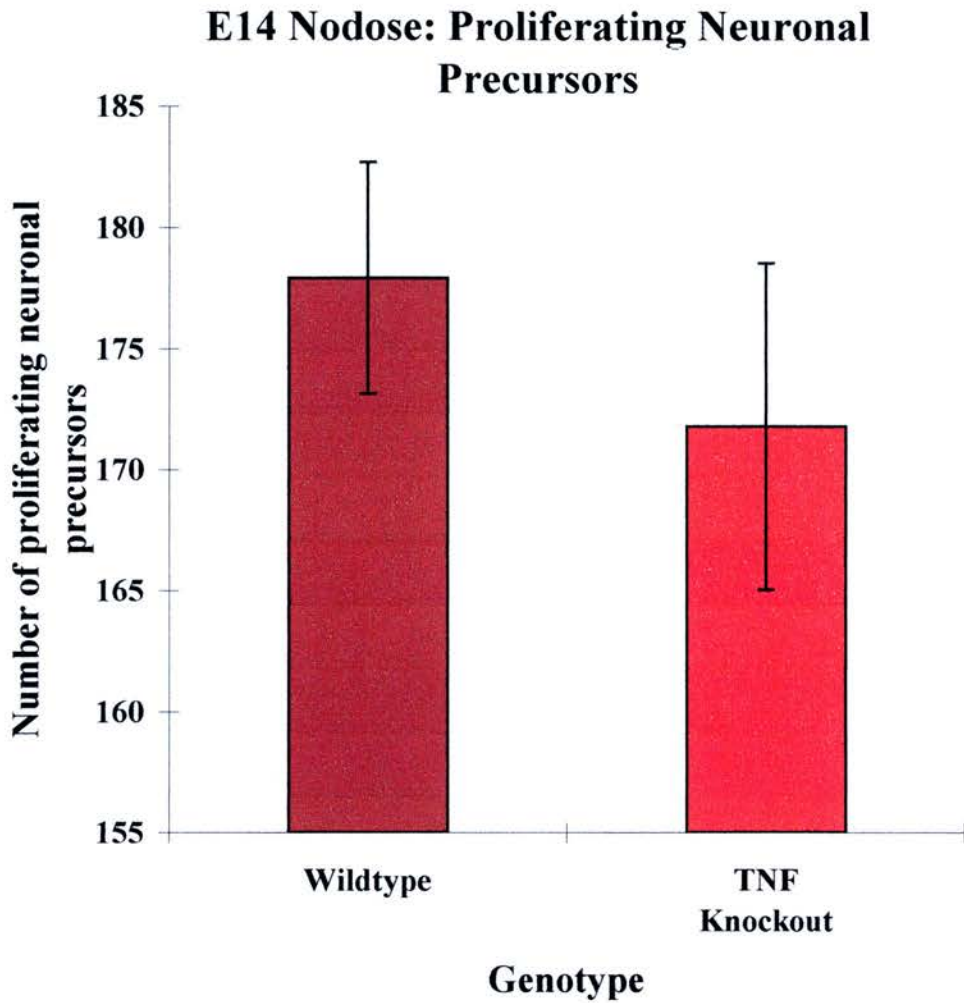


**Figure 4.18: Bar chart showing the number of proliferating neurons in the SCG of E14 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors shown are taken from counts of both right and left ganglia in 5 wild type and 5 TNF $\alpha$  deficient mice. There was no significant difference between the numbers of PCNA staining, and therefore proliferating neuroblasts, in the SCG of wildtype and TNF $\alpha$  deficient embryos.

### E14 Trigeminal: Proliferating Neuronal Precursors



**Figure 4.19:** Bar chart showing the number proliferating neurons in the trigeminal ganglia of E14 wildtype and TNF $\alpha$  knockout mice. The means and standard errors shown are taken from counts of both right and left ganglia in 5 wildtype and 5 TNF $\alpha$  deficient mice. There was no significant difference between the numbers of PCNA staining, and therefore proliferating neuroblasts, in the trigeminal ganglia of wildtype and TNF $\alpha$  deficient embryos.



**Figure 4.20: Bar chart showing the number proliferating neurons in the nodose ganglia of E14 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors shown are taken from counts of both right and left ganglia in 5 wild type and 5 TNF $\alpha$  deficient mice. There was no significant difference between the numbers of PCNA staining, and therefore proliferating neuroblasts, in the nodose ganglia of wildtype and TNF $\alpha$  deficient embryos.

Taken together these findings indicate that TNF $\alpha$  is required *in vivo* to support a proportion of developing sensory and sympathetic neurons during development. However it does not influence the proliferation of these neurons.

	<b>Neurons</b>			
<b>Ganglia</b>	<b>Wildtype</b>	<b>Std Error</b>	<b>TNF<math>\alpha</math>-/-</b>	<b>Std Error</b>
<b>SCG</b>	12473	549.99	7659.12	272.85
<b>Trigeminal</b>	33701.12	2227.24	25726.57	2153.16
<b>Nodose</b>	5364.2	331.68	5364.2	3155.92
	<b>Pyknotic Neurons</b>			
<b>Ganglia</b>	<b>Wildtype</b>	<b>Std Error</b>	<b>TNF<math>\alpha</math>-/-</b>	<b>Std Error</b>
<b>SCG</b>	494.86	92.31	1276.02	96.75
<b>Trigeminal</b>	4611.82	888.44	8535.31	449.12
<b>Nodose</b>	183.34	13.42	602.72	83.2
	<b>Proliferating Neuroblasts</b>			
<b>Ganglia</b>	<b>Wildtype</b>	<b>Std Error</b>	<b>TNF<math>\alpha</math>-/-</b>	<b>Std Error</b>
<b>SCG</b>	547.93	29.97	523.67	24.43
<b>Trigeminal</b>	3888.52	279.06	4184.92	196.5
<b>Nodose</b>	177.93	4.76	547.93	29.97

**Table 4.3: Summary of *in vivo* results from wildtype and TNF $\alpha$  deficient embryos.** The means and standard errors for counts of neurons and pyknotic neurons in the SCG, trigeminal and nodose ganglia of wildtype and TNF $\alpha$  deficient embryos at E14 are shown. The means and standard errors for counts of PCNA stained cells (proliferating neuroblasts) in the SCG, trigeminal and nodose ganglia of wildtype and TNF $\alpha$  deficient embryos at E14 are shown. The numbers of neurons are decreased and the levels of pyknotic neurons are increased in embryos deficient in TNF $\alpha$  as compared to wildtype embryos. There was no significant difference between the numbers of PCNA staining, and therefore proliferating neuroblasts, in the SCG, trigeminal and nodose ganglia of wildtype and TNF $\alpha$  deficient embryos

#### 4.2.6 p65 is Downstream of TNF $\alpha$ in Mediating Cell Survival

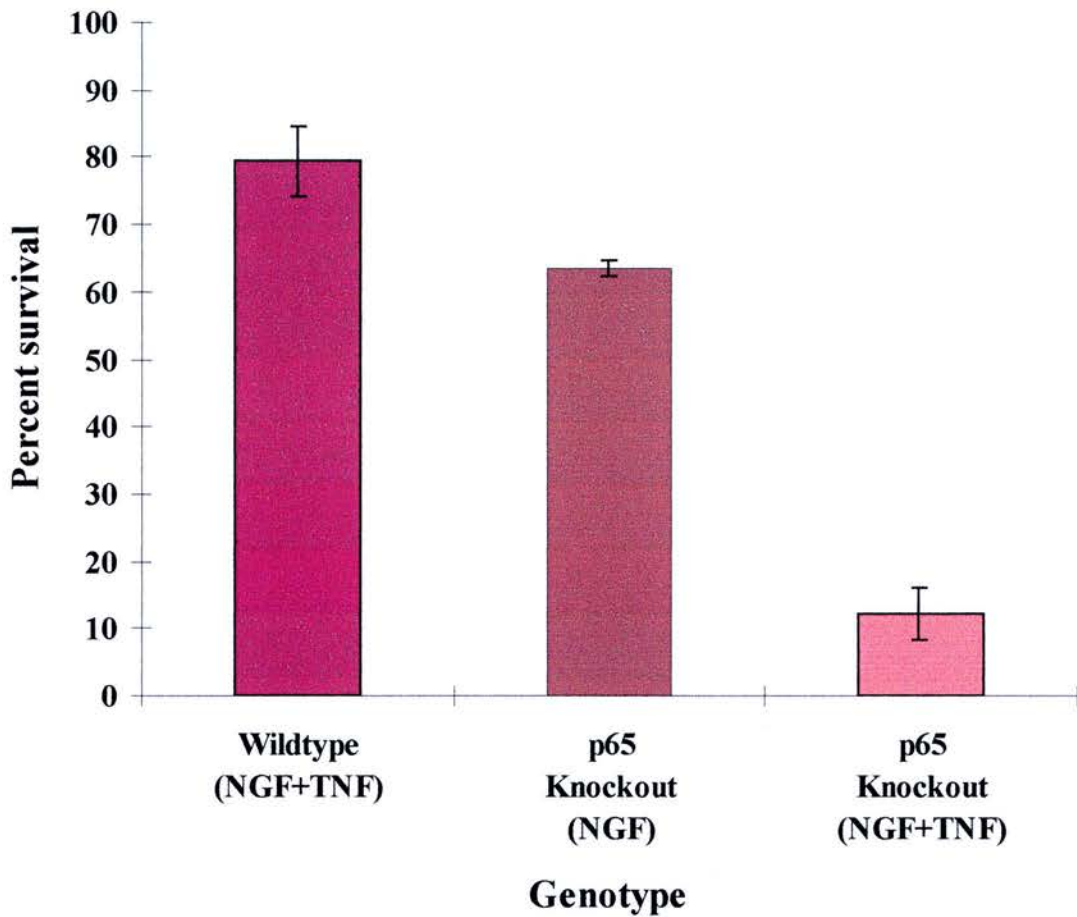
In order to determine whether p65 is involved in mediating cell survival in response to TNF $\alpha$  signalling I examined the effect of saturating concentrations of TNF $\alpha$  on the neurotrophin response of wildtype and p65 deficient litter-mates at E14. Low density dissociated cultures of SCG, trigeminal and nodose ganglion neurons were set up. Neurons were grown in chemically defined serum-free medium with neurotrophic support plus TNF $\alpha$ . The survival of the neurons was assessed after 48 hours incubation and expressed relative to the number of neurons attached 6 hours after plating.

If TNF $\alpha$  were acting via p65 to induce cell survival it would be expected that cell survival in response to TNF $\alpha$  would be abrogated in neuronal cultures from p65 deficient mice. This was found to be the case. There were significant reductions in percent survival of p65 deficient neurons of the SCG, trigeminal or nodose ganglia as compared to wildtype neurons when cultured with saturating levels of TNF $\alpha$  (SCG, nodose and trigeminal -  $p < 0.01$ , unpaired t-test) (figures 4.21-4.23). Neurotrophin support maintained survival of these neurons at high levels so that the effects of TNF $\alpha$  could be assessed. There was a 67% decrease in survival of SCG neurons in the p65 deficient culture as compared to the wildtype. The survival of trigeminal ganglion neurons was decreased by 70% and that of nodose neurons was decreased by 62% in the p65 deficient cultures as compared to the wildtype cultures. This indicates that p65 is involved in mediating the cell survival response to TNF $\alpha$  in E14 neurons. It should be noted that NGF acts through NF- $\kappa$ B to produce its neurotrophic effects. Because of this

the decrease in neuronal survival may be due to the lack of neurotrophic activity rather than lack of TNF $\alpha$  activity when p65 is deficient. This effect can be seen when p65 deficient neurons are cultured with NGF/BDNF (figures 4.21- 4.23) but when the same neurons were cultured with both NGF/BDNF and TNF $\alpha$  the percent survival is not enhanced by TNF $\alpha$  as would be expected indicating that NF- $\kappa$ B is mediating the TNF $\alpha$  survival pathway in these neurons. Instead survival is further decreased indicating that TNF $\alpha$  activates a cytotoxic signal when the NF- $\kappa$ B survival pathway is removed . Thus TNF $\alpha$  may act via NF- $\kappa$ B, inhibiting an apoptotic signal and leading to cell survival.

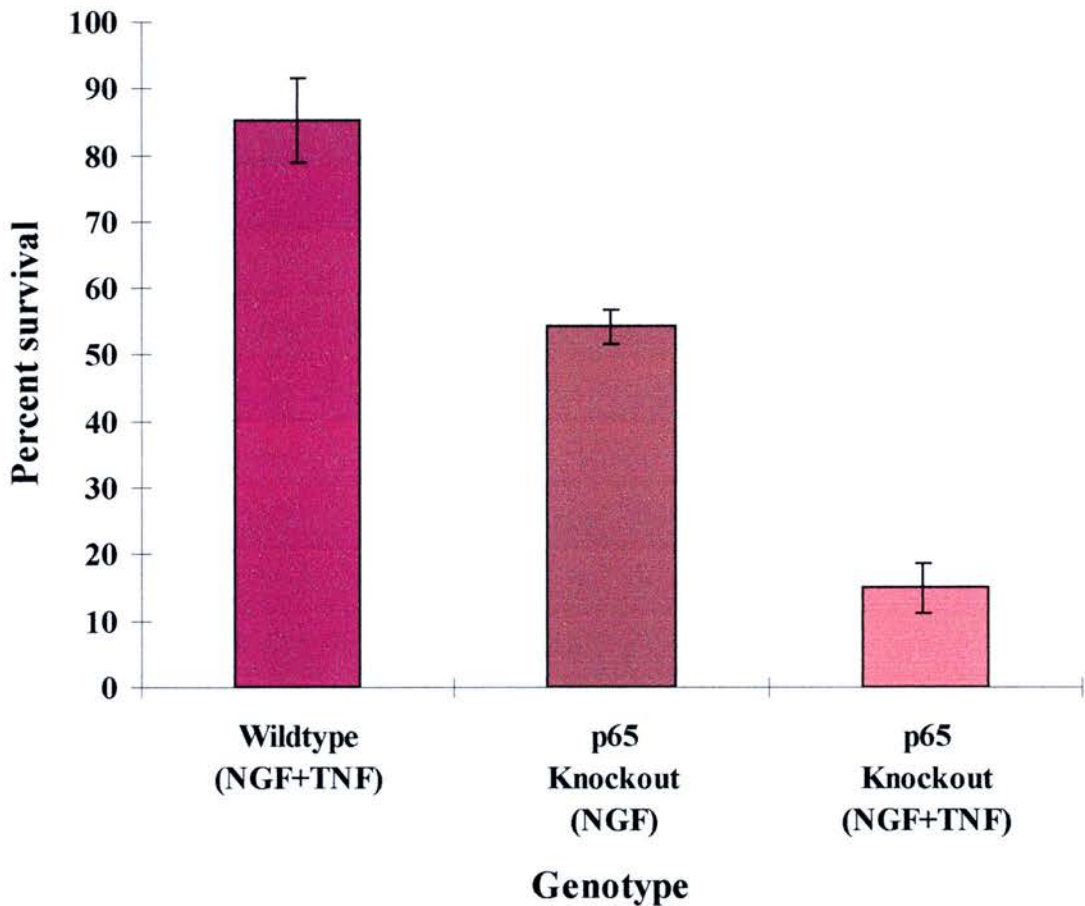


## E14 SCG Neurons



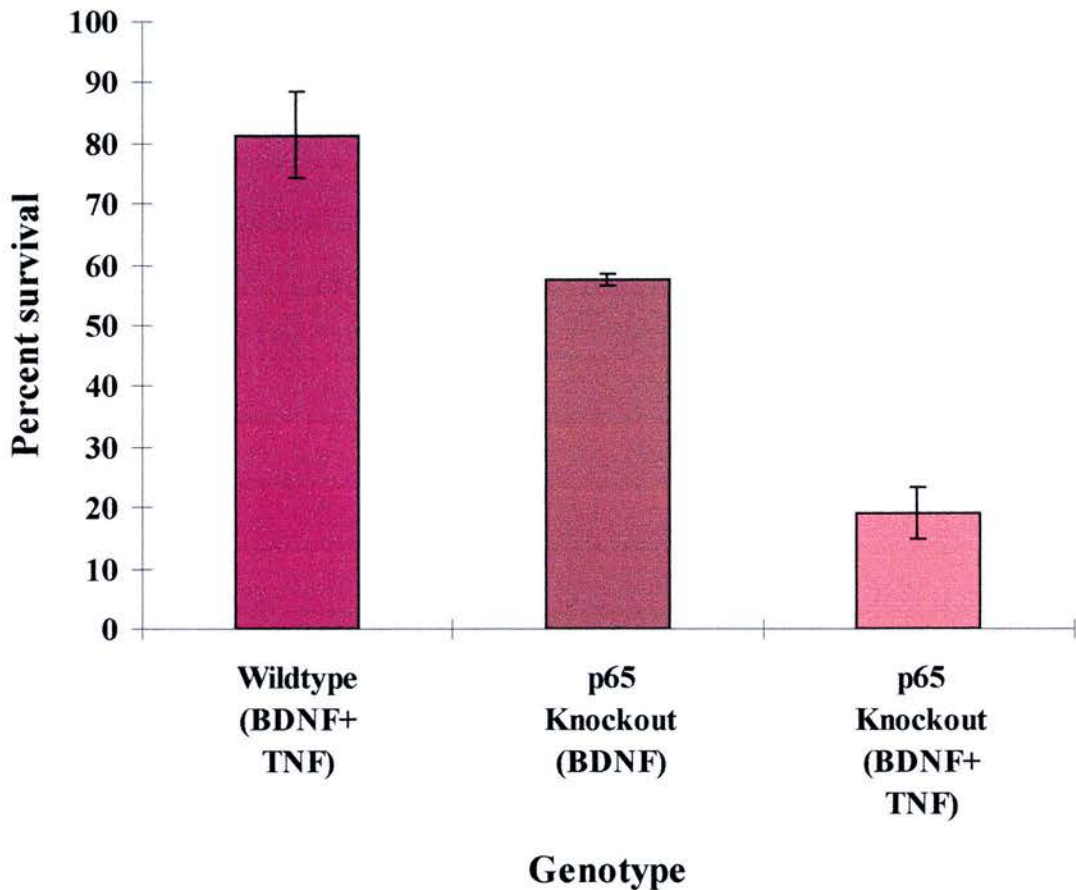
**Figure 4.21: Bar chart showing the percent survival of E14 wildtype and p65 deficient SCG neurons.** Neurons were grown in vitro for 48 hours with neurotrophic support plus  $\text{TNF}\alpha$ . Neurons from *p65* knockout mice were also grown with NGF alone. The means and standard errors of 3 separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. Cell survival is seen to be decreased in *p65* knockout SCG neurons when they are cultured with NGF alone and NGF together with  $\text{TNF}\alpha$ . The percent survival of the neurons is reduced more extensively in cultures with  $\text{TNF}\alpha$  than cultures with neurotrophin alone. This indicates that  $\text{TNF}\alpha$  induces cell survival that is mediated by NF- $\kappa$ B and that the reduction in cell survival is not due to the removal of NF- $\kappa$ B mediated neurotrophin response.

## E14 Trigeminal Neurons



**Figure 4.22: Bar chart showing the percent survival of E14 wildtype and p65 deficient trigeminal ganglion neurons.** Neurons were grown in vitro for 48 hours with neurotrophic support plus  $TNF\alpha$ . Neurons from *p65* knockout mice were also grown with NGF alone. The means and standard errors of 3 separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. Cell survival is seen to be decreased in *p65* knockout trigeminal neurons when they are cultured with NGF alone and NGF together with  $TNF\alpha$ . The percent survival of the neurons is reduced more extensively in cultures with  $TNF\alpha$  than cultures with neurotrophin alone. This indicates that  $TNF\alpha$  induces cell survival that is mediated by NF- $\kappa$ B and that the reduction in cell survival is not due to the removal of NF- $\kappa$ B mediated neurotrophin response.

## E14 Nodose Neurons



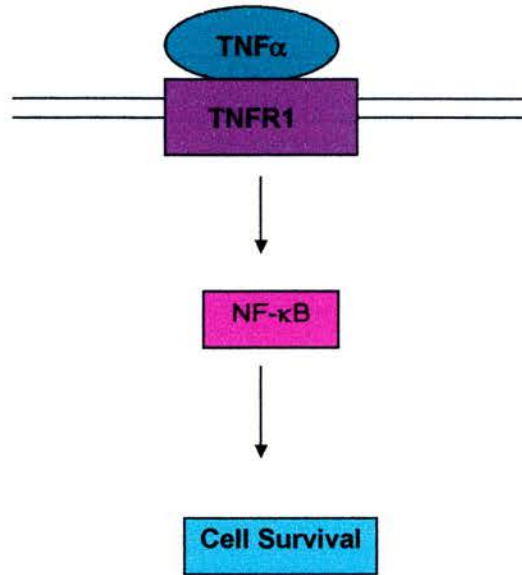
**Figure 4.23: Bar chart showing the percent survival of E14 wildtype and p65 deficient nodose ganglion neurons.** Neurons were grown in vitro for 48 hours with neurotrophic support plus  $\text{TNF}\alpha$ . Neurons from *p65* knockout mice were also grown with BDNF alone. The means and standard errors of 3 separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. Cell survival is seen to be decreased in *p65* knockout nodose neurons when they are cultured with BDNF alone and BDNF together with  $\text{TNF}\alpha$ . The percent survival of the neurons is reduced more extensively in cultures with  $\text{TNF}\alpha$  than cultures with neurotrophin alone. This indicates that  $\text{TNF}\alpha$  induces cell survival that is mediated by  $\text{NF-}\kappa\text{B}$  and that the reduction in cell survival is not due to the removal of  $\text{NF-}\kappa\text{B}$  mediated neurotrophin response.



### 4.3 Discussion

In this study I have used several approaches to demonstrate that TNF $\alpha$  can support the survival of developing sensory and sympathetic neurons. These neurons are able to survive well in cultures supplemented with TNF $\alpha$  and show a dose dependent response to it (figures 4.6-4.8). In addition, function blocking antibodies to either TNF $\alpha$  or TNFR1 reduce the survival response of sensory and sympathetic neurons to the neurotrophins NGF and BDNF (figures 4.3-4.5) indicating that TNF $\alpha$  contributes to the *in vitro* survival of neurons grown with these factors. Accordingly, the sympathetic and sensory ganglia of TNF $\alpha$  deficient mice have a decreased number of neurons and increased number of dying cells *in vivo* as compared to wildtype animals (4.12-4.17) and these neurons survive less well than wildtype neurons *in vitro* (figures 4.9-4.11). It will be important to ascertain whether TNF $\alpha$  acts in a neurotrophic manner in other areas of the nervous system during development.

The finding that p65 acts downstream from TNF $\alpha$  induced cell survival indicates that NF- $\kappa$ B activation is involved in the stimulation of cell survival in response to TNF $\alpha$  (see figure 4.24). This is consistent with the widely postulated theory that TNF $\alpha$  signals by a pathway leading to NF- $\kappa$ B activation to bring about cell survival and via receptor interacting proteins FADD and TRADD to activate caspases and bring about neuronal death (Baud and Karin, 2001).



**Figure 4.24: Summary diagram of the findings in chapter 4.** The findings of this study suggest that TNF $\alpha$  binding to TNFR1 induces cell survival in E14 developing SCG, nodose and trigeminal neurons. This cell survival is mediated by the activation of the transcription factor NF- $\kappa$ B.

It was found that in p65 deficient neurons that were cultured with saturating levels of NGF and TNF $\alpha$  the level of cell survival was reduced compared to the wildtype neurons and to p65 deficient neurons that were cultured with saturating levels of NGF alone. This suggests that in the absence of NF- $\kappa$ B TNF $\alpha$  acts to signal cell death possibly via receptor interacting proteins FADD and TRADD to activate caspases as described previously (Baud and Karin, 2001). Under normal circumstances the presence of NF- $\kappa$ B signalling would inhibit this cytotoxic TNF $\alpha$  pathway by inducing the expression of proteins that inhibit apoptosis such as cIAP2 (Rothe et al., 1994) and A20 (He and Ting, 2002; Song et al., 1996). It may also be due to the fact that NGF and TNF $\alpha$  activate the NF- $\kappa$ B pathway independently of each other. A p65/TNFR1 double knockout has been produced that survives beyond birth (Rosenfield et al., 2000), which could be used in future work to further investigate the role of p65 downstream of TNF $\alpha$  in mediating cell

survival. This could further be aided by the use of IKK $\alpha$ /TNF and IKK $\beta$ /TNF double knockout mice. Unfortunately p65/TNF knockout mice are not viable and the majority of heterozygotes do not survive beyond birth therefore they cannot be used to elucidate this pathway.

Although TNF $\alpha$  has previously been demonstrated to have anti-apoptotic effects in neurotoxic situations such as glutamate excitotoxicity (Houzen et al., 1997; Carlson et al., 1998), and cerebral ischaemic damage (Bruce et al., 1996), these results indicate for the first time that it also has survival promoting capabilities during normal neurodevelopment. This is consistent with the fact that TNF $\alpha$  genes are constitutively expressed in neurons (Szelenyi, 2001) and why TNF $\alpha$  is synthesised by neurons (Breder et al., 1993). Future work to determine the role and influence of TNF $\alpha$ -mediated cell survival during the development of the nervous system and to examine whether TNF $\alpha$  induces a survival response in other areas during neurodevelopment will be important.

# CHAPTER 5: THE EFFECTS OF TUMOUR NECROSIS FACTOR $\alpha$ ON CRANIAL SENSORY AND SYMPATHETIC NEURONS DURING EMBRYONIC DEVELOPMENT II: CYTOTOXIC ACTIVITY.

## 5.1 Introduction

During the development of the nervous system, neurons are generated in excess and subsequently eliminated in a wave of programmed cell death that occurs shortly after they innervate their target tissues. The survival of neurons at this time is thought to be dependent upon a supply of one or more neurotrophic factors from the target field (Davies and Lumsden, 1984). Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are members of the neurotrophin family of structurally related secreted proteins that promote and regulate the survival of neurons of the peripheral nervous system. These proteins are synthesised in limiting amounts by neuronal target fields and bind to specific receptors on the surface of the neurons to initiate a signalling cascade that leads to cell survival by inhibition of caspases and therefore inhibition of apoptosis (Kaplan and Miller, 2000).

Because the binding of NGF to TrkA generates survival signals within the cell that prevent caspase activation and subsequent apoptosis (Kaplan and Miller, 2000), it is



assumed that neurons die following NGF deprivation because of the withdrawal of these survival signals. However, I show here that this death is due in part to the action of TNF $\alpha$ .

TNF $\alpha$  has been shown to induce neuronal death associated with a number of cytotoxic circumstances, such as HIV (New et al., 1998), ischaemia (Dawson et al., 1996) and axotomy (Terrado et al., 2000). It binds to the cell surface receptors, TNFR1 in order to bring about cell death. Following binding the cytoplasmic death domain of TNFR1 interacts with an adaptor protein TRADD which in turn interacts with a further adaptor protein FADD. FADD then recruits and activates pro-caspase 8 which triggers activation of the cell death machinery (Ashkenzai and Dixit, 1998). TNFR2 lacks a death domain but synergistically enhances TNFR1-induced cytotoxicity (Grell et al., 1999). (TNF $\alpha$  has been discussed in more detail in section 4.1). In spite of its demonstration as a cytotoxic factor in the above circumstances, the role of TNF $\alpha$  in developmental cell death has to date not been elucidated. Following the discovery that in the early stages of development TNF $\alpha$  is involved in stimulating sensory and sympathetic survival, I extended my examination to the later stages of neurodevelopment. I carried out extensive tissue culture, dose response, histochemical and histological analysis on SCG, trigeminal and nodose ganglia from E16 murine embryos. (The results detailed in this chapter were published in the paper: Barker, V., Middleton, G., Davey, F., and Davies, A.M. (2001). TNF $\alpha$  contributes to the

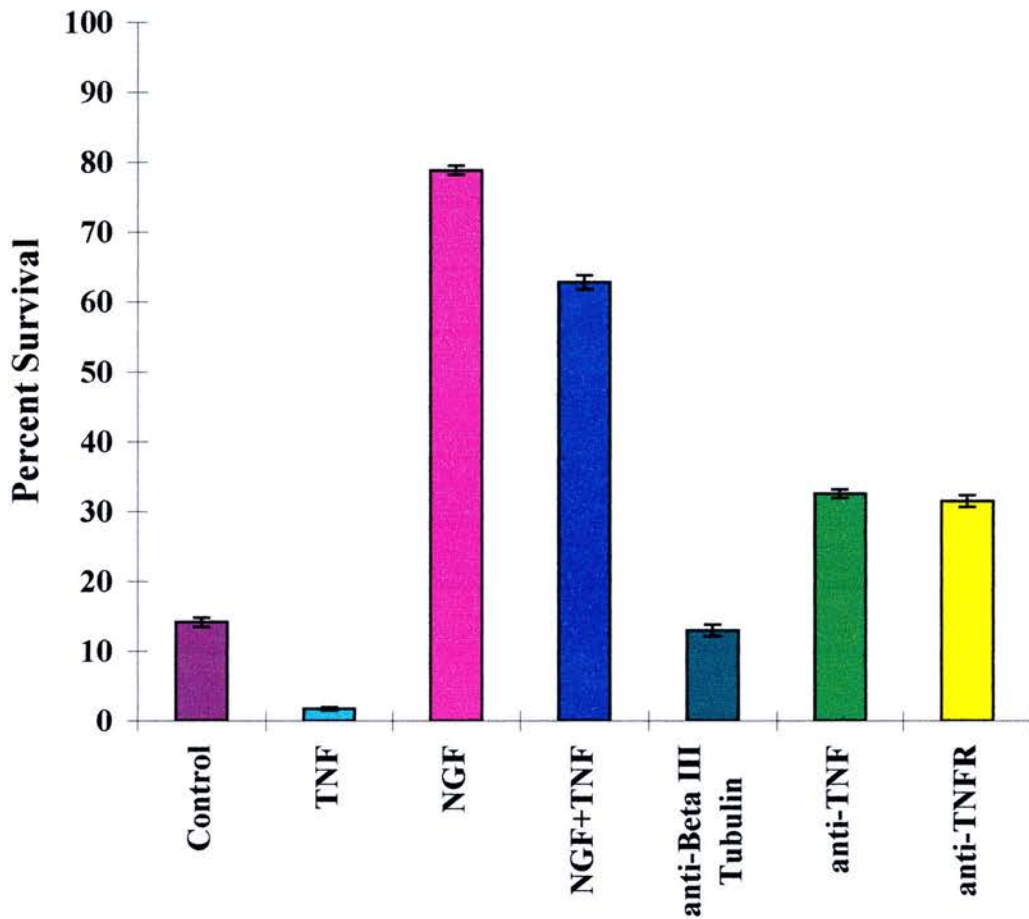
death of NGF-dependent neurons during development. *Nature Neurosci.* **4**, 1194 -1198 -  
see appendix 8).

## 5.2 Results

### 5.2.1 TNF $\alpha$ Promotes Neuronal Apoptosis *in vitro*, an Effect Inhibited by anti-TNF $\alpha$ and anti-TNFR1 Antibodies

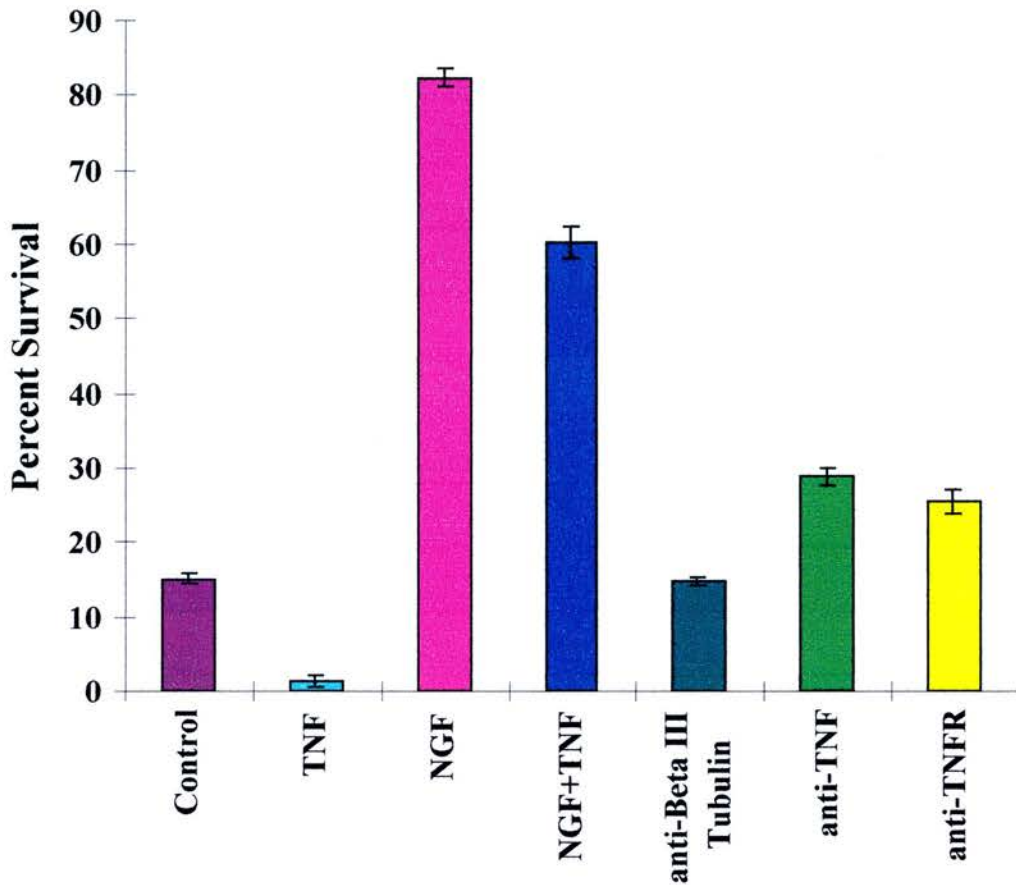
To investigate if TNF $\alpha$  is involved in promoting apoptosis of embryonic neurons deprived of trophic support, I studied the effects of function-blocking anti-TNF $\alpha$  and anti-TNFR1 antibodies on the survival of NGF-dependent neurons following NGF deprivation. I established low-density, dissociated cultures of sympathetic neurons from the SCG and sensory neurons from the trigeminal and nodose ganglia of mouse embryos at E16, when most of these neurons have become dependent on NGF for survival *in vitro* and naturally occurring neuronal death is occurring *in vivo* (Wyatt and Davies, 1995; Davies and Lumsden, 1984; Francis et al., 1999). After 12 hours incubation with NGF, the cultures were washed extensively to remove this neurotrophin and were either resupplemented with NGF, or grown without NGF and treated with anti-TNF $\alpha$  or anti-TNFR1 antibodies or TNF $\alpha$ . Whereas about 80% of the sympathetic and sensory neurons survived for at least another 48 hours after resupplementation with NGF, over 80% died within 48 hours of NGF deprivation (figures 5.1, 5.2 and 5.3). Treatment with TNF $\alpha$  increased the percentage dying by at least a further 15% (figures 5.1, 5.2 and 5.3). Treatment of NGF deprived neurons with either anti-TNF $\alpha$  or anti-TNFR1 antibodies rescued a quarter to a third of the neurons that would have otherwise died (figure 5.1, 5.2 and 5.3).

## E16 SCG Neurons



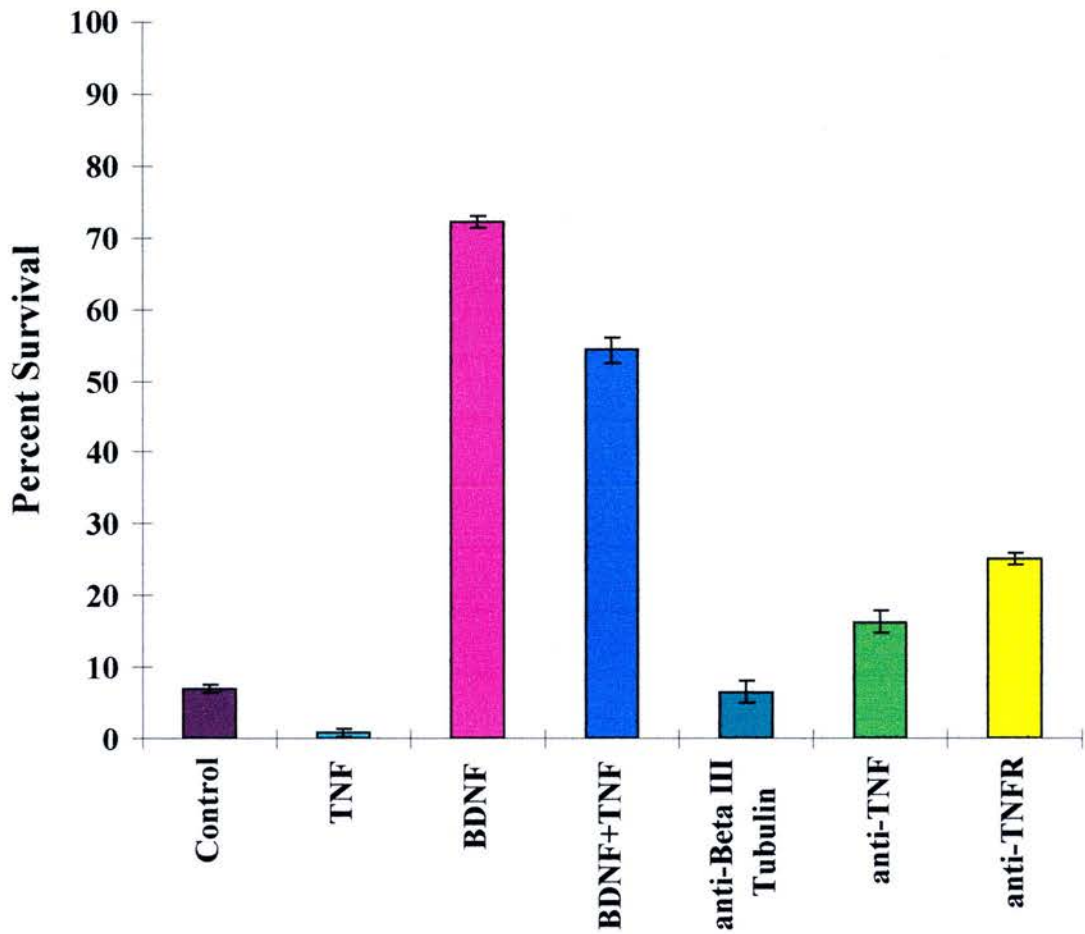
**Figure 5.1:** Bar chart showing the percent survival of E16 SCG neurons cultured for 48 hours with 2ng/ml of NGF, TNF $\alpha$ , both NGF and TNF $\alpha$  and function blocking TNF $\alpha$ , TNFR1 or  $\beta$ III tubulin antibodies after NGF deprivation. Control cultures received no reagents following NGF deprivation. The means and standard errors of six separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. TNF $\alpha$  can be seen to reduce the percent survival of SCG neurons and also reduce the neurotrophin induced survival response. Anti-bodies to TNF $\alpha$  and TNFR1 rescued approximately a third of neurons that die in control cultures, indicating that if TNF $\alpha$  is blocked cell death is inhibited. This effect was not due to non-specific actions of anti-bodies on neuronal survival as there was no significant difference between control cultures and those treated with anti- $\beta$ III tubulin anti-bodies.

## E16 Trigeminal Neurons



**Figure 5.2:** Bar chart showing the percent survival of E16 trigeminal neurons cultured for 48 hours with 2ng/ml of NGF, TNF $\alpha$ , both NGF and TNF $\alpha$  and function blocking TNF $\alpha$ , TNFR1 or  $\beta$ III tubulin antibodies after NGF deprivation. Control cultures received no reagents following NGF deprivation. The means and standard errors of six separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. TNF $\alpha$  can be seen to reduce the percent survival of trigeminal neurons and also reduce the neurotrophin induced survival response. Anti-bodies to TNF $\alpha$  and TNFR1 rescued approximately a quarter of neurons that die in control cultures, indicating that if TNF $\alpha$  is blocked cell death is inhibited. This effect was not due to non-specific actions of anti-bodies on neuronal survival as there was no significant difference between control cultures and those treated with anti- $\beta$ III tubulin anti-bodies.

## E16 Nodose Neurons



**Figure 5.3: Bar chart showing the percent survival of E16 nodose neurons cultured for 48 hours with 2ng/ml of BDNF, TNF $\alpha$ , both BDNF and TNF $\alpha$  and function blocking TNF $\alpha$ , TNFR1 or  $\beta$ III tubulin antibodies after BDNF deprivation. Control cultures received no reagents following BDNF deprivation. The means and standard errors of six separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. TNF $\alpha$  can be seen to reduce the percent survival of nodose neurons and also reduce the neurotrophin induced survival response. Antibodies to TNF $\alpha$  and TNFR1 rescued approximately a quarter of neurons that die in control cultures, indicating that if TNF $\alpha$  is blocked cell death is inhibited. This effect was not due to non-specific actions of anti-bodies on neuronal survival as there was no significant difference between control cultures and those treated with anti- $\beta$ III tubulin anti-bodies.**



Reagent	SCG		Trigeminal		Nodose	
	% Survival	Std Error	% Survival	Std Error	% Survival	Std Error
Control	14.15	0.66	15.03	0.64	6.91	0.6
TNF $\alpha$	1.76	0.22	1.25	0.83	0.9	0.6
NGF/BDNF	78.9	0.66	82.29	1.17	72.2	0.9
NGF/BDNF + TNF $\alpha$	62.85	0.97	60.3	2.24	54.3	1.8
anti-TNF $\alpha$	32.57	0.59	28.8	1.15	16.21	1.5
anti-TNFR1	31.5	0.87	25.57	1.6	25.1	0.9
anti- $\beta$ III Tubulin	12.97	0.87	14.73	0.66	6.5	1.5

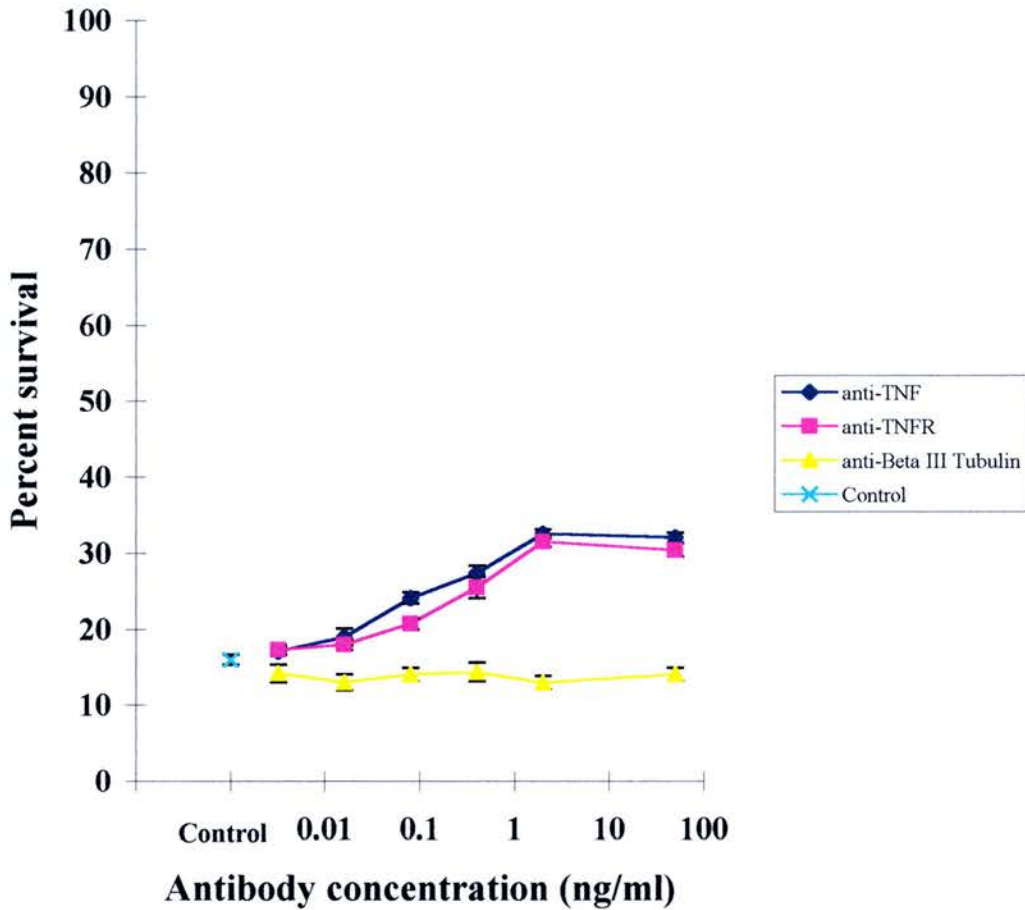
**Table 5.1: Summary of SCG, trigeminal and nodose neurons cultured with 2ng/ml neurotrophin, TNF $\alpha$ , both neurotrophin and TNF $\alpha$  and function blocking TNF $\alpha$ , TNFR1 or  $\beta$ III tubulin antibodies after neurotrophin deprivation.** The mean percent survival and standard errors for SCG, trigeminal and nodose neurons cultured with 2ng/ml NGF/BDNF, TNF $\alpha$  and antibodies to either TNF $\alpha$ , TNFR or the intracellular neuron-specific antigen  $\beta$ III-tubulin. TNF $\alpha$  can be seen to reduce the percent survival of SCG, trigeminal and nodose neurons and also reduce the neurotrophin induced survival response of these neurons. Anti-bodies to TNF $\alpha$  and TNFR1 rescued approximately a quarter of neurons that die in control cultures, indicating that if TNF $\alpha$  is blocked cell death is inhibited. This effect was not due to non-specific actions of anti-bodies on neuronal survival as there was no significant difference between control cultures and those treated with anti- $\beta$ III tubulin anti-bodies.



Dose–response analysis revealed that the effect of the antibodies reached a plateau at a concentration of 2 ng/ml, and higher concentrations of antibodies singularly and in combination did not rescue additional neurons (figures 5.4, 5.5 and 5.6).

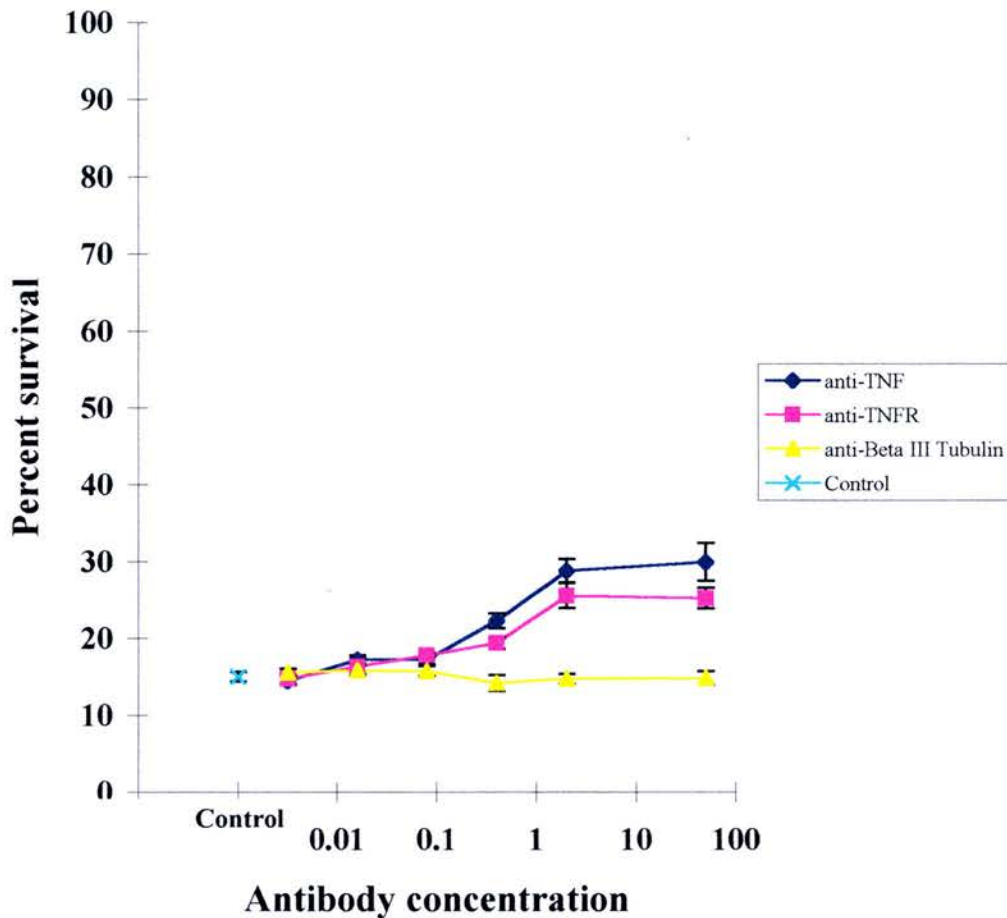
These results were not due to non-specific actions of antibodies on neuronal survival because there was no significant difference between the number of neurons surviving in control cultures and cultures treated with antibodies against an intracellular antigen ( $\beta$ III tubulin) even at concentrations as high as 50 ng/ml (SCG, nodose and trigeminal  $\beta$ III tubulin vs control cultures non-significant, one way ANOVA with post hoc Tukey multiple comparisons test).

## E16 SCG Neurons



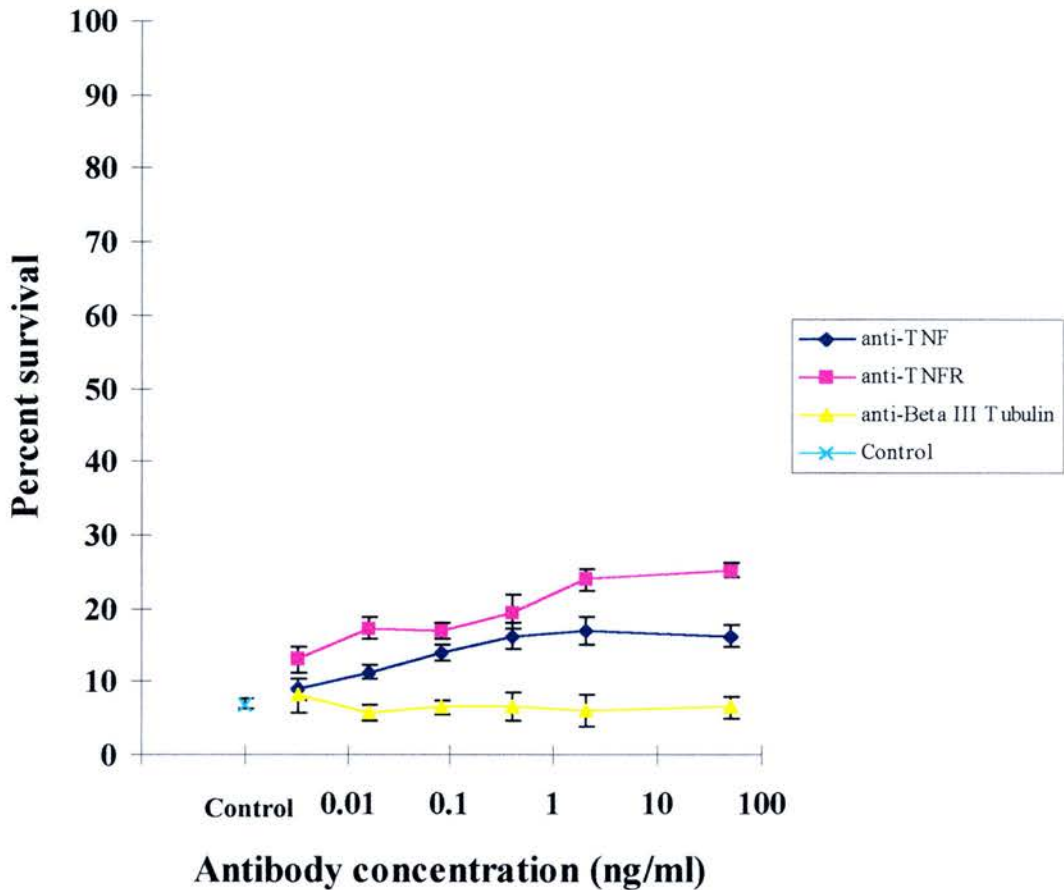
**Figure 5.4: Graph showing the dose response of E16 SCG neurons cultured for 48 hours with function blocking  $TNF\alpha$ , TNFR1 or  $\beta$ III tubulin antibodies after NGF deprivation.** The percent survival was quantified following treatment with increasing concentrations of reagent, in order to determine whether any effects seen occurred in a dose dependent manner. Control cultures received no reagents following NGF deprivation. The means and standard errors of six separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. Anti-bodies to  $TNF\alpha$  and TNFR1 increased the survival of SCG neurons in a dose dependent manner that reached a plateau at 2ng/ml. This effect was not due to non-specific actions of anti-bodies on neuronal survival as there was no significant difference between control cultures and those treated with increasing concentrations of anti- $\beta$ III tubulin anti-bodies.

## E16 Trigeminal Neurons



**Figure 5.5: Dose response of E16 trigeminal neurons cultured for 48 hours with function blocking  $TNF\alpha$ , TNFR1 or  $\beta$ III tubulin antibodies after NGF deprivation.** The percent survival was quantified following treatment with increasing concentrations of reagent, in order to determine whether any effects seen occurred in a dose dependent manner. Control cultures received no reagents following NGF deprivation. The means and standard errors of six separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. Anti-bodies to  $TNF\alpha$  and TNFR1 increased the survival of trigeminal neurons in a dose dependent manner that reached a plateau at 2ng/ml. This effect was not due to non-specific actions of anti-bodies on neuronal survival as there was no significant difference between control cultures and those treated with increasing concentrations of anti- $\beta$ III tubulin anti-bodies.

## E16 Nodose Neurons



**Figure 5.6: Dose response of E16 nodose neurons cultured for 48 hours with function blocking  $TNF\alpha$ ,  $TNFR1$  or  $\beta III$  tubulin antibodies after BDNF deprivation.** The percent survival was quantified following treatment with increasing concentrations of reagent, in order to determine whether any effects seen occurred in a dose dependent manner. Control cultures received no reagents following BDNF deprivation. The means and standard errors of six separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. Anti-bodies to  $TNF\alpha$  and  $TNFR1$  increased the survival of nodose neurons in a dose dependent manner that reached a plateau at 2ng/ml. This effect was not due to non-specific actions of anti-bodies on neuronal survival as there was no significant difference between control cultures and those treated with increasing concentrations of anti- $\beta III$  tubulin anti-bodies.

These results suggest that TNF $\alpha$  synthesised by cells in dissociated cultures of embryonic sympathetic and sensory ganglia is involved in bringing about neuronal death following NGF deprivation, and that this action of TNF $\alpha$  is mediated at least in part by TNFR1 as the addition of both anti-TNF $\alpha$  and anti-TNFR1 antibodies to cultures increased the survival of SCG, trigeminal and nodose neurons in a dose dependent manner.



### 5.2.2 Developing Neurons Express TNF $\alpha$ and TNFR1

To further substantiate the role of TNF $\alpha$  in embryonic sensory and sympathetic neurons and investigate its mode of action, I used immunocytochemistry to identify which cells express TNF $\alpha$  and TNFR1 in dissociated cultures. Because the serum-free medium used in our cultures is not conducive to the survival of non-neuronal cells, most cells remaining in E16 cultures after 48 hours of incubation were neurons (>90%). Virtually all of these neurons were immunoreactive for TNF $\alpha$  protein and for TNFR1 (figure 5.7). The small number of non-neuronal cells in these cultures displayed only a low level of immunoreactivity for TNF $\alpha$  and TNFR1. Neurons in cultures established from *tnf $\alpha$ <sup>-/-</sup>* embryos showed no immunoreactivity for TNF $\alpha$  and no staining was observed in cultures of wild-type neurons if the primary antibodies were omitted, demonstrating specific staining for TNF $\alpha$  and its receptor. These findings indicate that most if not all neurons synthesise TNF $\alpha$  and express the TNFR1 receptor during the stage of NGF dependence, and suggest that TNF $\alpha$  exerts its actions by an autocrine mechanism.

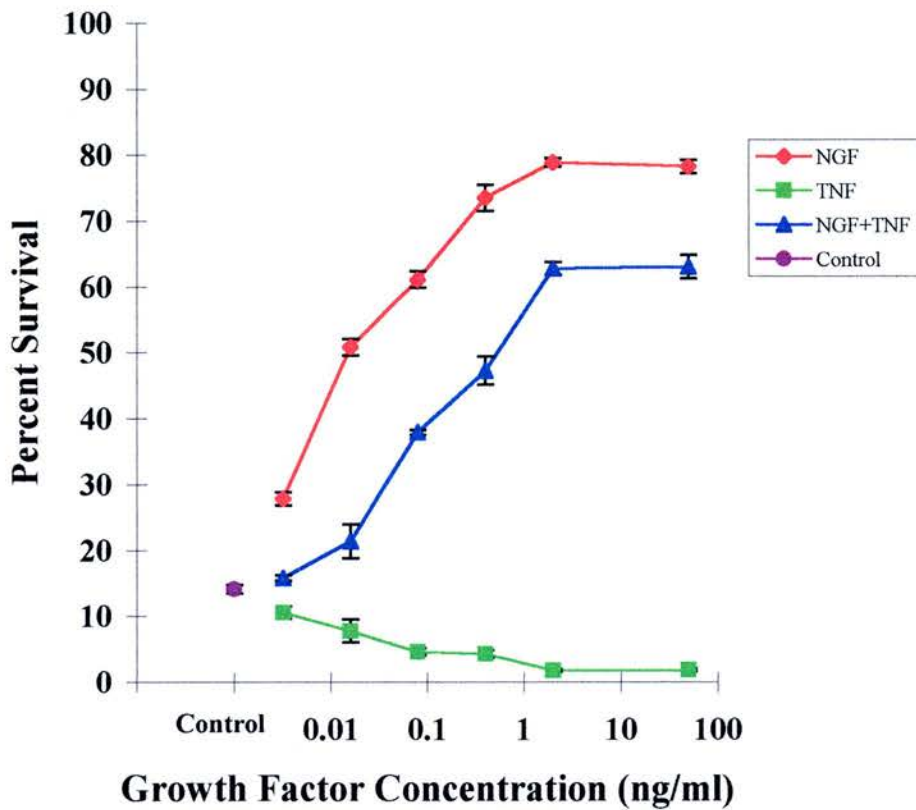


**Figure 5.7: Photomicrograph showing the expression of TNF $\alpha$  and TNFR1 by cultured SCG neurons.** E16 SCG neurons from wildtype embryos were stained for TNF $\alpha$  (a) and TNFR1 (b); E16 SCG neurons (arrowheads) from TNF $\alpha$   $-/-$  embryos did not stain for TNF $\alpha$  (c), and no neuronal staining was observed in wildtype neurons (arrowheads) if primary antibodies were omitted (d). The few non-neuronal cells in these cultures showed a low level of TNF $\alpha$  immunoreactivity (arrowhead in a). (Scale bar, 20 $\mu$ m).

### 5.2.3 TNF $\alpha$ Reduces Survival of Neurons *in vitro*

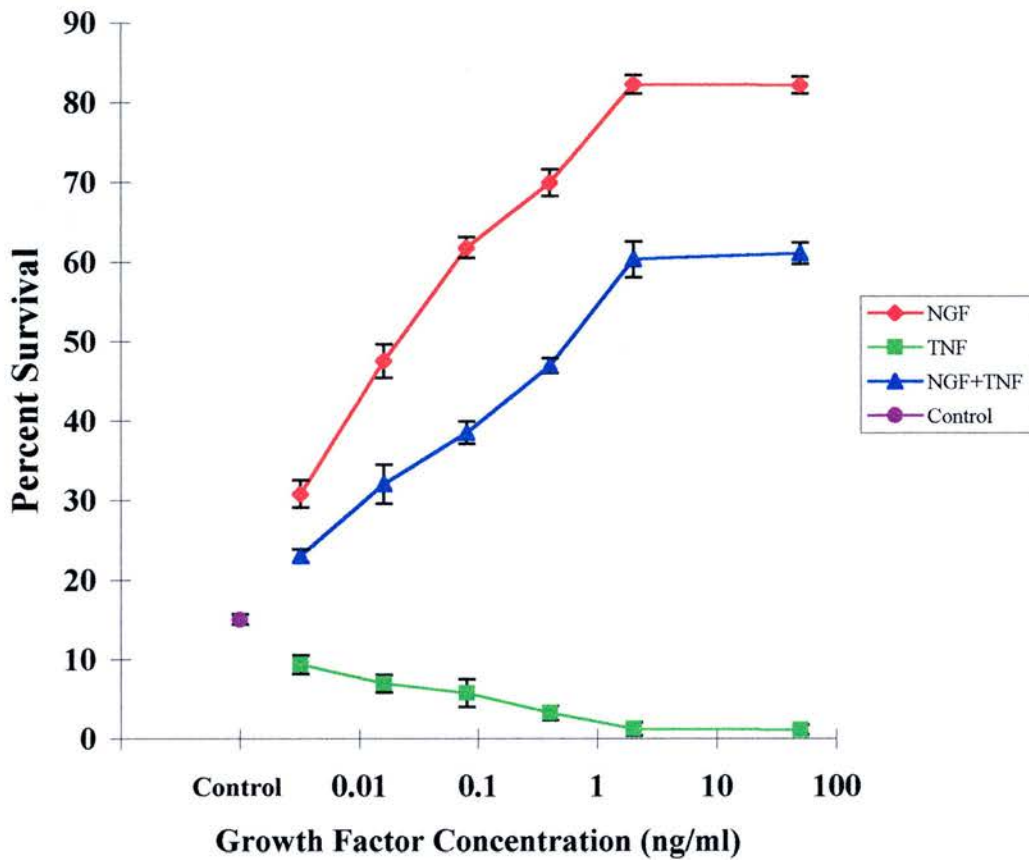
TNF $\alpha$  treatment also reduced the survival of sympathetic and sensory neurons grown either with or without neurotrophic factor (NGF for SCG and trigeminal and BDNF for nodose neurons) (figures 5.8, 5.9 and 5.10). Dose–response analysis revealed that as little as 3.2 pg/ml TNF $\alpha$  caused significant reductions in the survival of neurons grown in either the presence or absence of neurotrophic factor (SCG -  $p < 0.0001$ , unpaired t-test; trigeminal -  $p < 0.005$ , unpaired t-test; nodose-  $p < 0.01$ , unpaired t-test). This cytotoxic effect of TNF $\alpha$  reached a plateau at a concentration of 2 ng/ml, and no additional death was observed at a 25-fold higher concentration. At these concentrations of TNF $\alpha$ , less than 2% of the neurons were remaining in NGF-free cultures (compared to between 7% and 15% survival in NGF-free cultures without TNF $\alpha$ ) and there was an approximately 20% drop in survival of neurons grown with TNF $\alpha$  plus NGF relative to neurons grown with NGF alone (figures 5.8, 5.9 and 5.10).

## E16 SCG Neurons



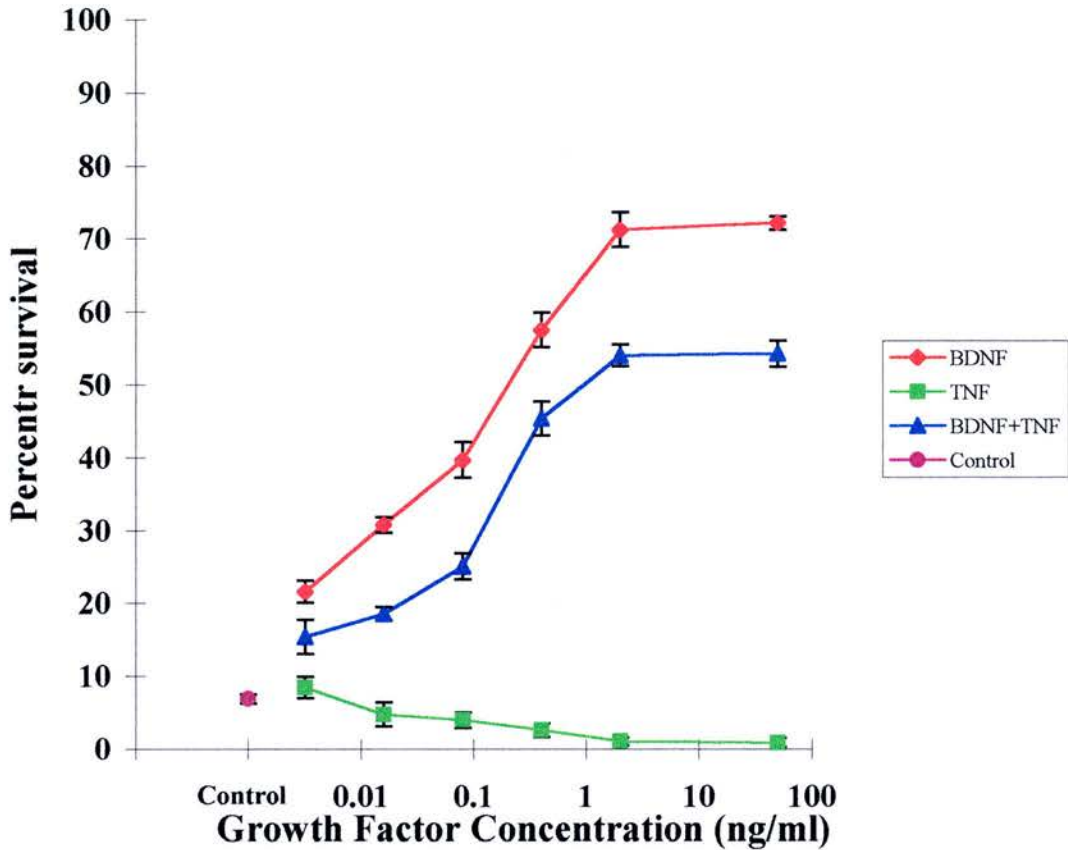
**Figure 5.8: Dose response of E16 SCG neurons cultured for 48 hours with NGF, TNF $\alpha$  or NGF plus TNF $\alpha$  following NGF deprivation.** The percent survival was quantified following treatment with increasing concentrations of reagent, in order to determine whether any effects seen occurred in a dose dependent manner. Control cultures received no reagents following NGF deprivation. The means and standard errors of six separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. TNF $\alpha$  can be seen to reduce the percent survival of SCG neurons and also reduce the neurotrophin induced survival response by approximately 20%. TNF $\alpha$  produced these effects in a dose dependent manner. The cytotoxic effect of TNF $\alpha$  reached a plateau at 2ng/ml.

## E16 Trigeminal Neurons



**Figure 5.9: Dose response of E16 trigeminal neurons cultured for 48 hours with NGF, TNF $\alpha$  or NGF plus TNF $\alpha$  following NGF deprivation.** The percent survival was quantified following treatment with increasing concentrations of reagent, in order to determine whether any effects seen occurred in a dose dependent manner. Control cultures received no reagents following NGF deprivation. The means and standard errors of six separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. TNF $\alpha$  can be seen to reduce the percent survival of trigeminal neurons and also reduce the neurotrophin induced survival response by approximately 20%. TNF $\alpha$  produced these effects in a dose dependent manner. The cytotoxic effect of TNF $\alpha$  reached a plateau at 2ng/ml.

## E16 Nodose Neurons



**Figure 5.10: Dose response of E16 nodose neurons cultured for 48 hours with BDNF, TNF $\alpha$  or BDNF plus TNF $\alpha$  following BDNF deprivation.** The percent survival was quantified following treatment with increasing concentrations of reagent, in order to determine whether any effects seen occurred in a dose dependent manner. Control cultures received no reagents following BDNF deprivation. The means and standard errors of six separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. TNF $\alpha$  can be seen to reduce the percent survival of nodose neurons and also reduce the neurotrophin induced survival response by approximately 20%. TNF $\alpha$  produced these effects in a dose dependent manner. The cytotoxic effect of TNF $\alpha$  reached a plateau at 2ng/ml.

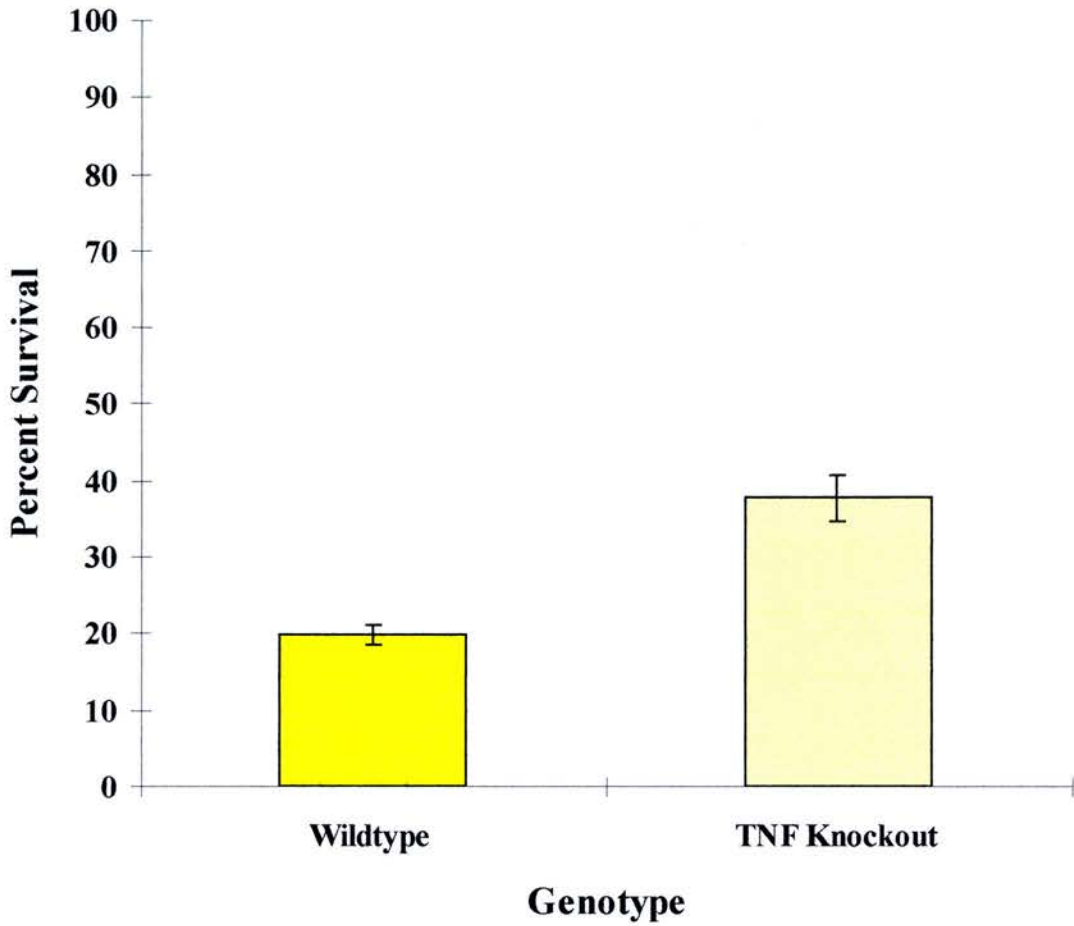
These results suggest that endogenously produced TNF $\alpha$  is not functioning to maximum effect in promoting neuronal death and that only a proportion of the neurons are susceptible to the cytotoxic actions of TNF $\alpha$  at this stage of development as the addition of TNF $\alpha$  to SCG, trigeminal and nodose cultures supported by neurotrophin only reduces cell survival partially and does not eliminate it completely.



#### 5.2.4 Enhanced Survival of TNF $\alpha$ -Deficient Neurons *in vitro*

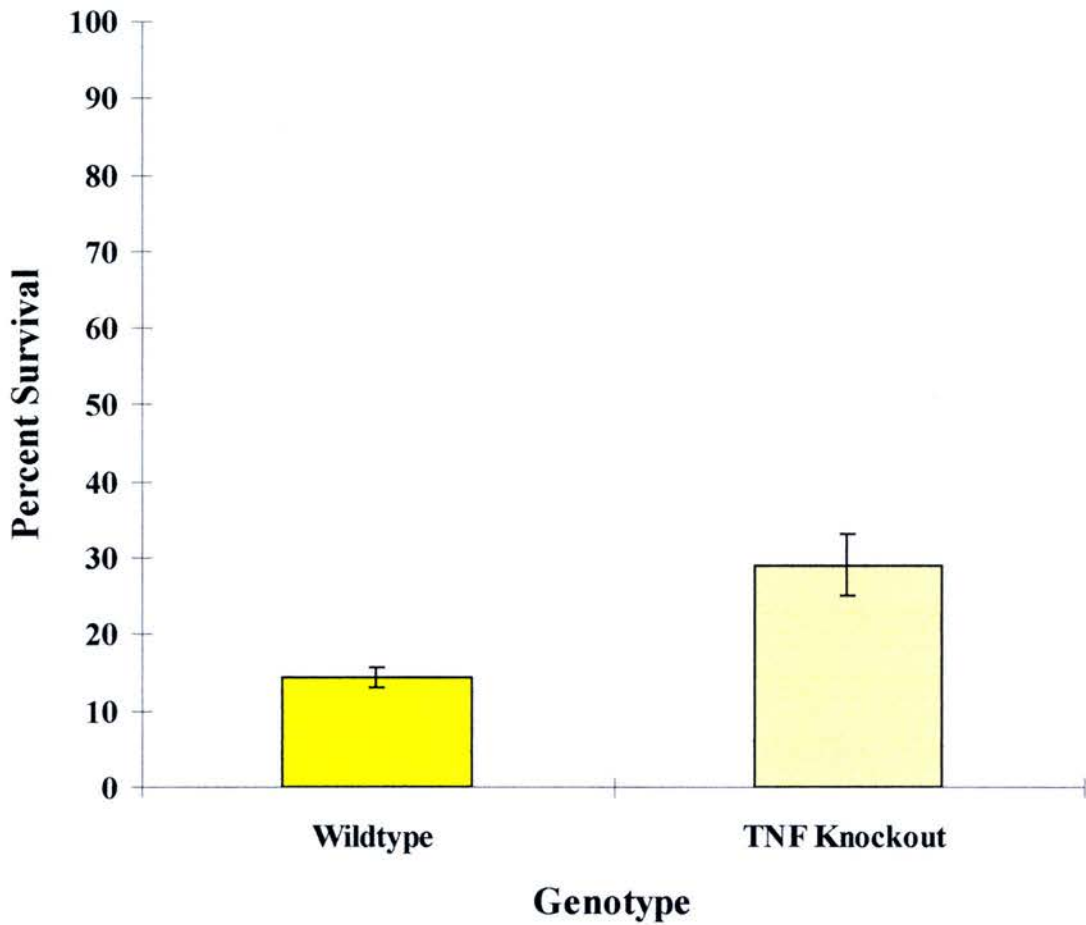
To provide additional evidence for the involvement of TNF $\alpha$  in bringing about neuronal death following NGF deprivation, I compared the survival of embryonic SCG, trigeminal and nodose neurons cultured from wildtype mice and mice that are homozygous for a null mutation in the *tnf $\alpha$*  gene. After 48 hours incubation, there were approximately twice as many sympathetic and sensory neurons surviving in cultures established from E16 *tnf $\alpha$ <sup>-/-</sup>* mice compared with neurons from wildtype embryos in the same litters (figures 5.11-5.13 and table 5.2) in the absence of NGF (significant, SCG -  $p < 0.001$ , unpaired t-test; nodose -  $p < 0.0001$ , unpaired t-test; trigeminal -  $p < 0.001$ , unpaired t-test). These results further support the idea that endogenously produced TNF $\alpha$  is involved in bringing about the death of embryonic sympathetic and sensory neurons in the absence of NGF.

## E16 SCG Neurons



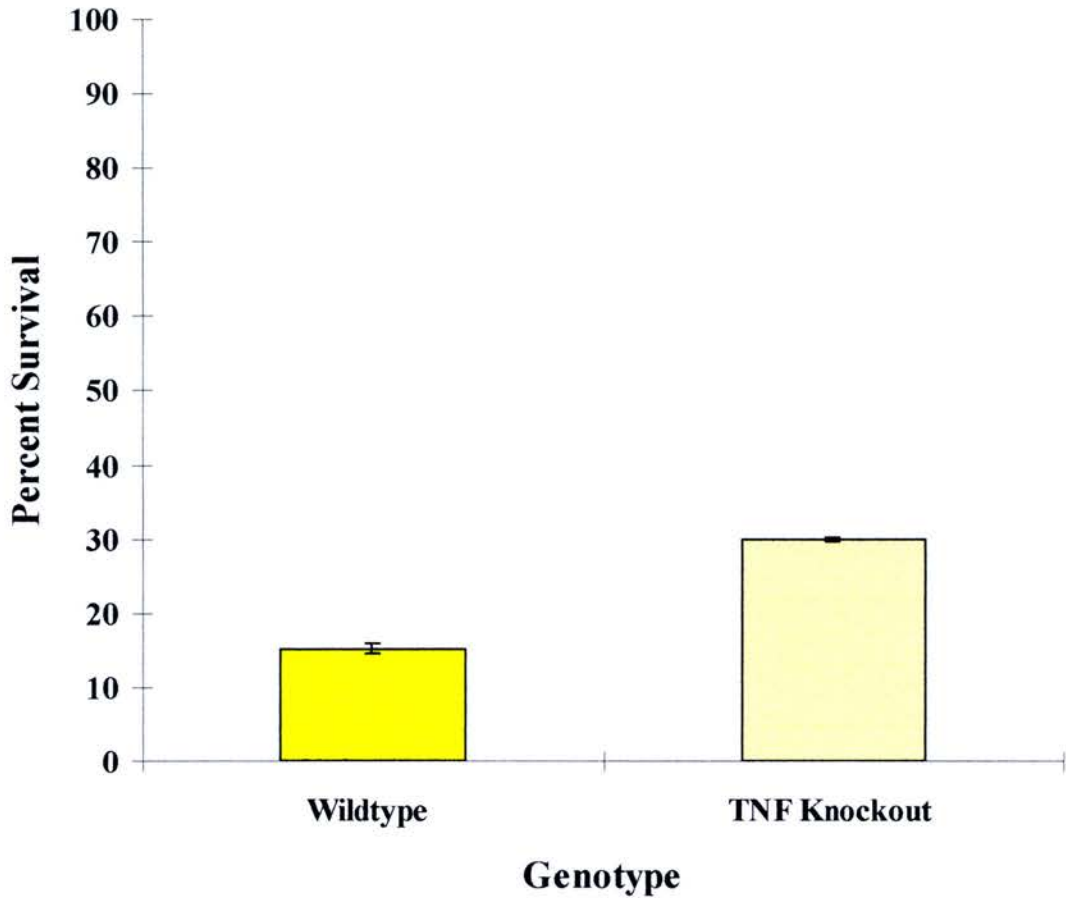
**Figure 5.11: Bar chart showing the percent survival of SCG neurons from wildtype and TNF $\alpha$  deficient embryos after 48 hours in culture.** The means and standard errors of three separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. The percent survival of SCG neurons was approximately doubled compared to the wildtypes when *tnf $\alpha$*  is knocked out, thus removing the cytotoxic activity of TNF $\alpha$ .

## E16 Trigeminal Neurons



**Figure 5.12:** Bar chart showing the percent survival of trigeminal neurons from wildtype and TNF $\alpha$ -deficient embryos after 48 hours in culture. The means and standard errors of three separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. The percent survival of trigeminal neurons was approximately doubled compared to the wildtypes when *tnf $\alpha$*  is knocked out, thus removing the cytotoxic activity of TNF $\alpha$ .

## E16 Nodose Neurons



**Figure 5.13: Bar chart showing the percent survival of nodose neurons from wildtype and TNF $\alpha$ -deficient embryos after 48 hours in culture.** The means and standard errors of three separate experiments are shown. Survival is expressed as a percentage of a count taken 6 hours after plating. The percent survival of nodose neurons was approximately doubled compared to the wildtypes when *tnf $\alpha$*  is knocked out, thus removing the cytotoxic activity of TNF $\alpha$ .

Ganglia	Wildtype		TNF $\alpha$ -/-	
	% Survival	Std Error	% Survival	Std Error
SCG	19.8	1.28	37.8	3
Trigeminal	14.32	1.24	29.09	4
Nodose	15.119	0.72	29.85	0.27

**Table 5.2: Summary of transgenic *in vitro* results.** The mean percent survival and standard errors of SCG, trigeminal and nodose neurons from wildtype and TNF $\alpha$  deficient embryos are shown. The percent survival of SCG, trigeminal and nodose neurons was approximately doubled compared to the wildtypes when *tnf $\alpha$*  is knocked out, thus removing the cytotoxic activity of TNF $\alpha$ .



### 5.2.5 Reduced Neuronal Death in TNF $\alpha$ -Deficient Embryos

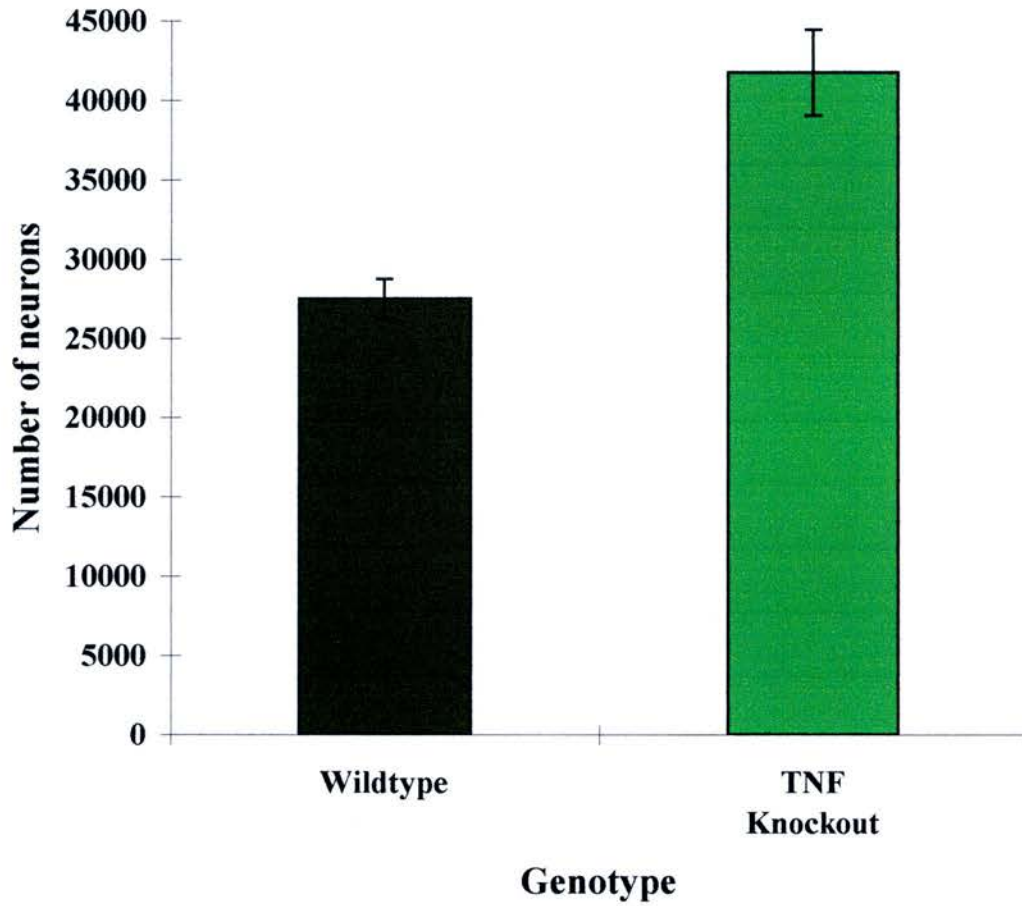
To ascertain the physiological relevance of these *in vitro* observations, I estimated the number of surviving neurons and the extent of neuronal death in the SCG, nodose and trigeminal ganglia of wildtype and *tnf $\alpha$ <sup>-/-</sup>* mice at two stages during the period of naturally occurring neuronal death by counting the total number of neurons and the number of pyknotic neurons in serial sections (Figures 5.14, 5.16, 5.18, 5.20, 5.22, 5.24 and 5.15, 5.17, 5.19, 5.21, 5.23, 5.25 and table 5.3). At E16, the SCG, trigeminal and nodose ganglia of *tnf $\alpha$ <sup>-/-</sup>* embryos contained only a quarter as many dying neurons as in wildtype embryos, and there were significantly more neurons in the SCG (52% increase,  $p < 0.0005$ , unpaired t-test), trigeminal ganglia (23% increase,  $p < 0.05$ , unpaired t-test) and nodose (37% increase,  $p < 0.01$ , unpaired t-test) of *tnf $\alpha$ <sup>-/-</sup>* embryos. By the first postnatal day (P1), the SCG, trigeminal and nodose ganglia of *tnf $\alpha$ <sup>-/-</sup>* embryos contained less than half as many dying neurons as wildtype embryos, and neurons were still significantly more abundant in the SCG (14% increase  $p < 0.05$ , unpaired t-test), trigeminal ganglia (26% increase,  $p < 0.05$ , unpaired t-test) and nodose ganglia (27% increase,  $p < 0.05$ , unpaired t-test) of these TNF $\alpha$  deficient embryos compared with wildtype embryos. These results demonstrate that TNF $\alpha$  is required to stimulate the death of a proportion of neurons in the developing SCG, trigeminal and nodose ganglia during the period of naturally occurring neuronal death.

It should be noted that there are some differences in the numbers of neurons and pyknotic in the SCG of E16 embryos and in the numbers of neurons at P1 in this set of



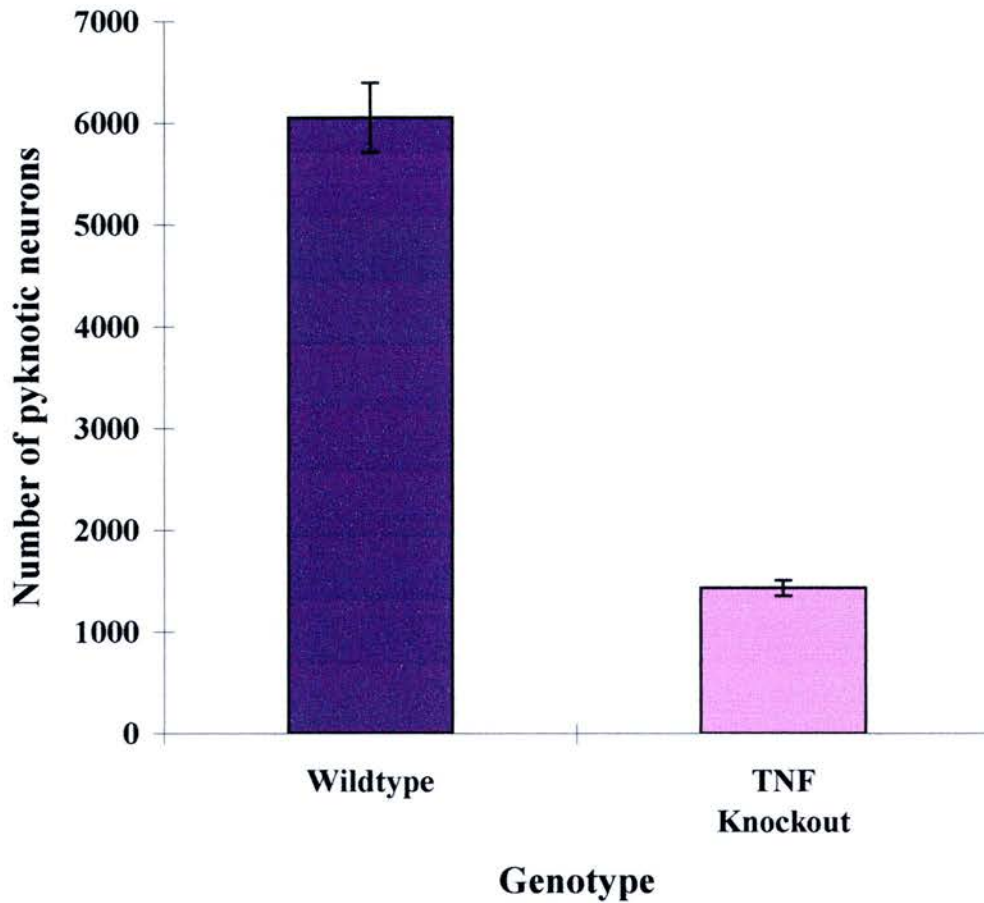
experiments and those in chapter 3 (figure 3.13 and 3.14). This did not affect the results as comparisons in these experiments were made independently of each other between wildtype and knockout embryos.

## E16 SCG: Total Neurons



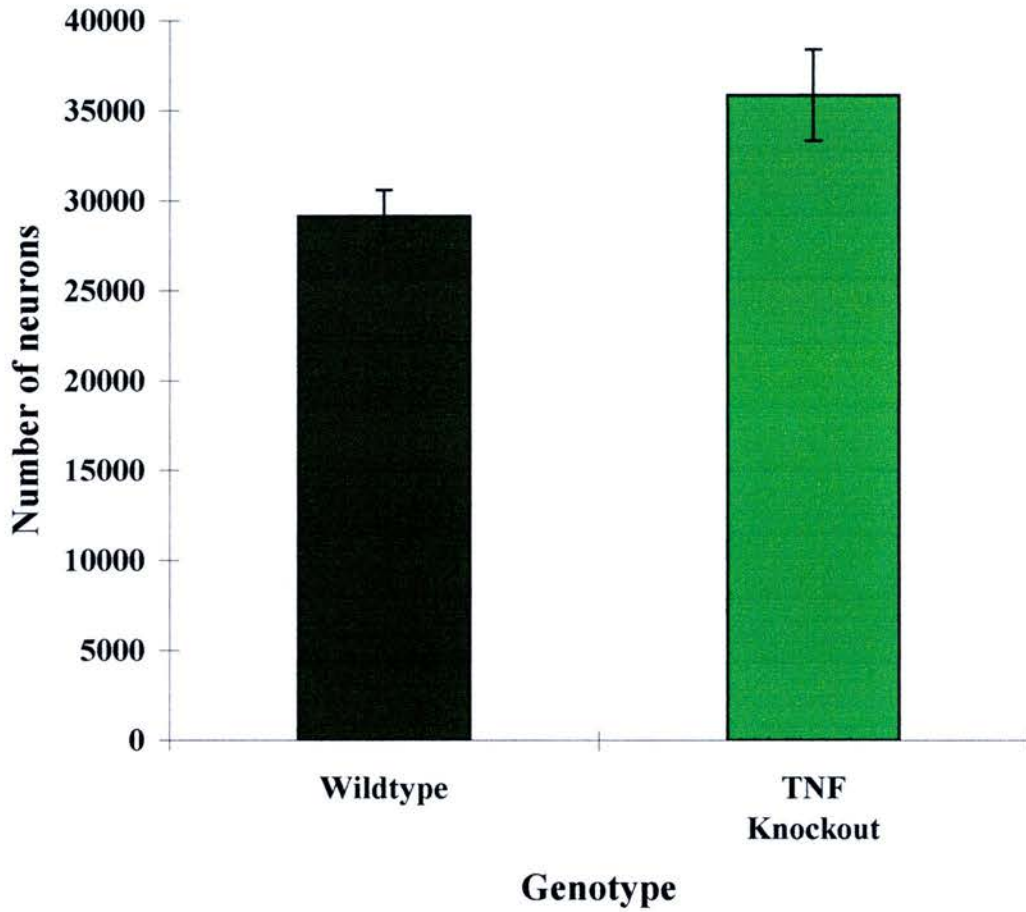
**Figure 5.14: Bar chart showing the total number of neurons in the SCG of E16 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors of the data obtained from both sets of ganglia from 3 E16 TNF $\alpha$ <sup>+/+</sup> and from 4 E16 TNF $\alpha$ <sup>-/-</sup> mice are shown. The numbers of neurons in the SCG were increased by 52% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.

## E16 SCG: Pyknotic Neurons



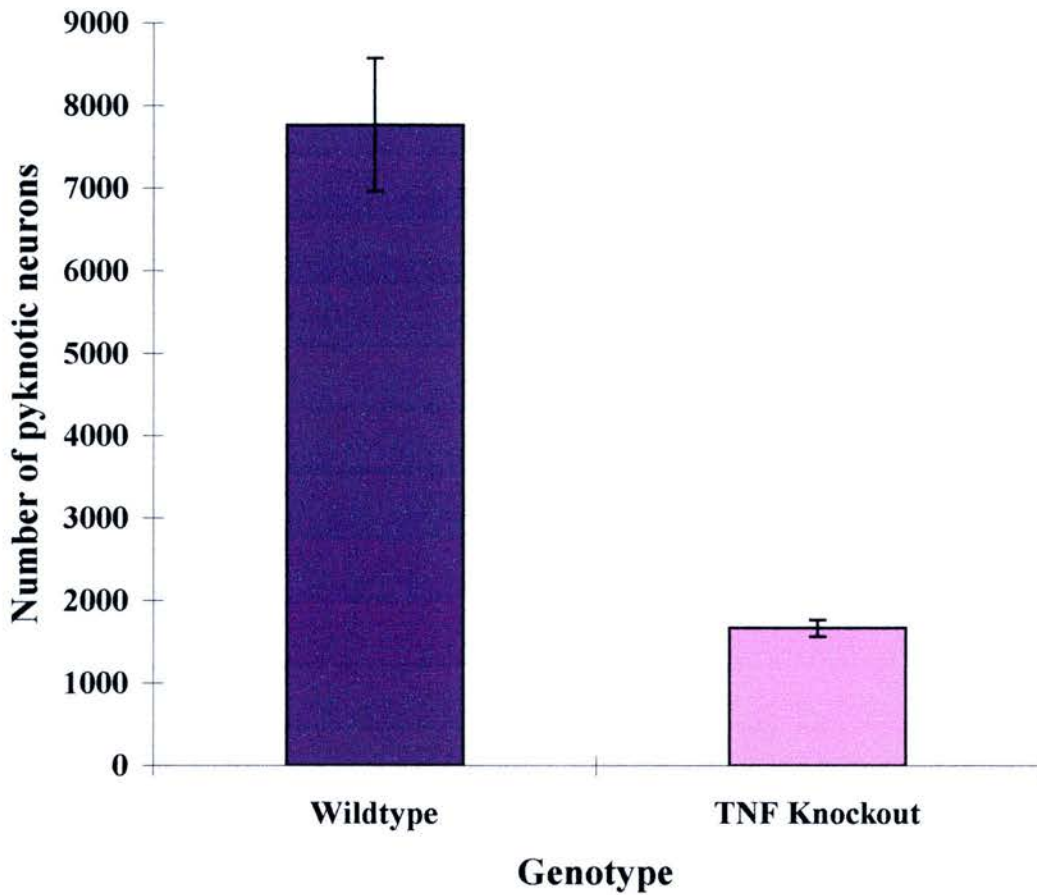
**Figure 5.15: Bar chart showing the number of pyknotic neurons in the SCG of E16 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors of the data obtained from both sets of ganglia from 3 E16 TNF $\alpha$ <sup>+/+</sup> and from 4 E16 TNF $\alpha$ <sup>-/-</sup> mice are shown. The numbers of pyknotic neurons, and therefore cell death, in the SCG were decreased by approximately 75% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.

### E16 Trigeminal: Total Neurons



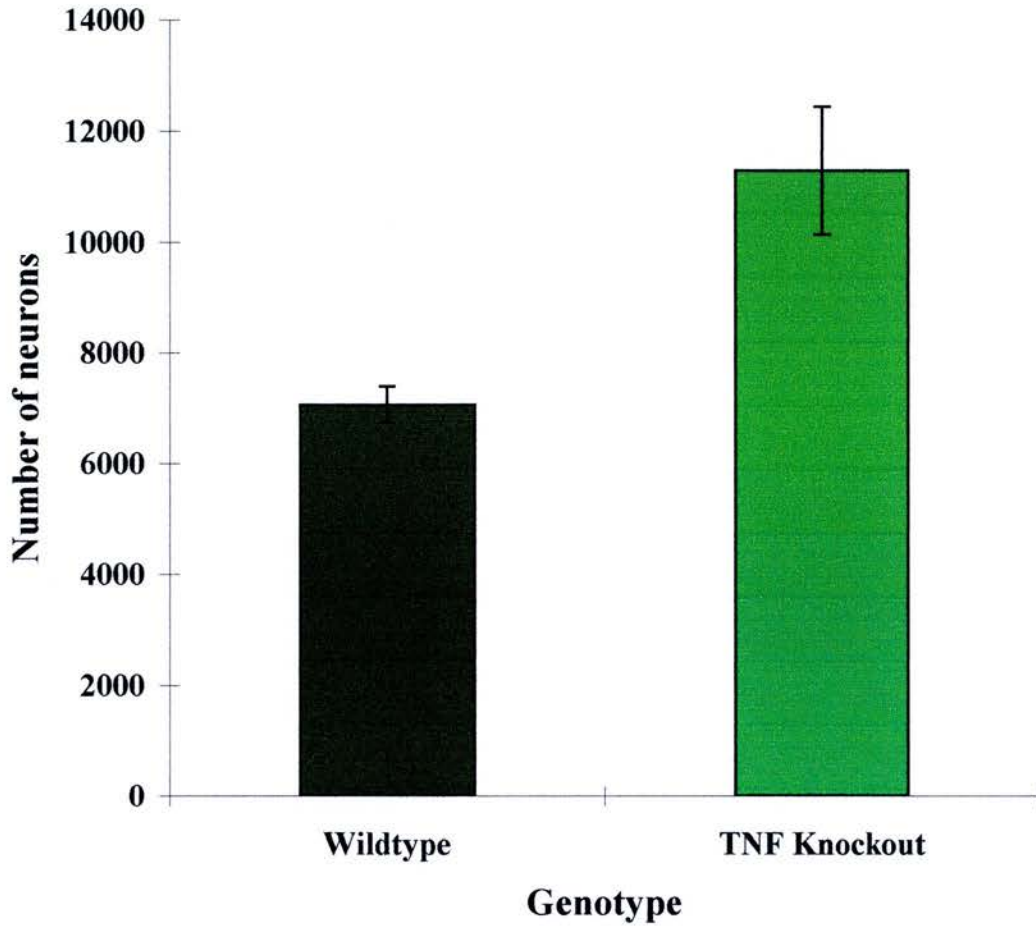
**Figure 5.16: Bar chart showing the total number of neurons in the trigeminal of E16 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors of the data obtained from both sets of ganglia from 3 E16 TNF $\alpha$ <sup>+/+</sup> and from 4 E16 TNF $\alpha$ <sup>-/-</sup> mice are shown. The numbers of neurons in the trigeminal ganglia were increased by 23% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.

## E16 Trigeminal: Pyknotic Neurons



**Figure 5.17: Bar chart showing the number of pyknotic neurons in the trigeminal of E16 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors of the data obtained from both sets of ganglia from 3 E16 TNF $\alpha$ <sup>+/+</sup> and from 4 E16 TNF $\alpha$ <sup>-/-</sup> mice are shown. The numbers of pyknotic neurons, and therefore cell death, in the trigeminal ganglia were decreased by approximately 75% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.

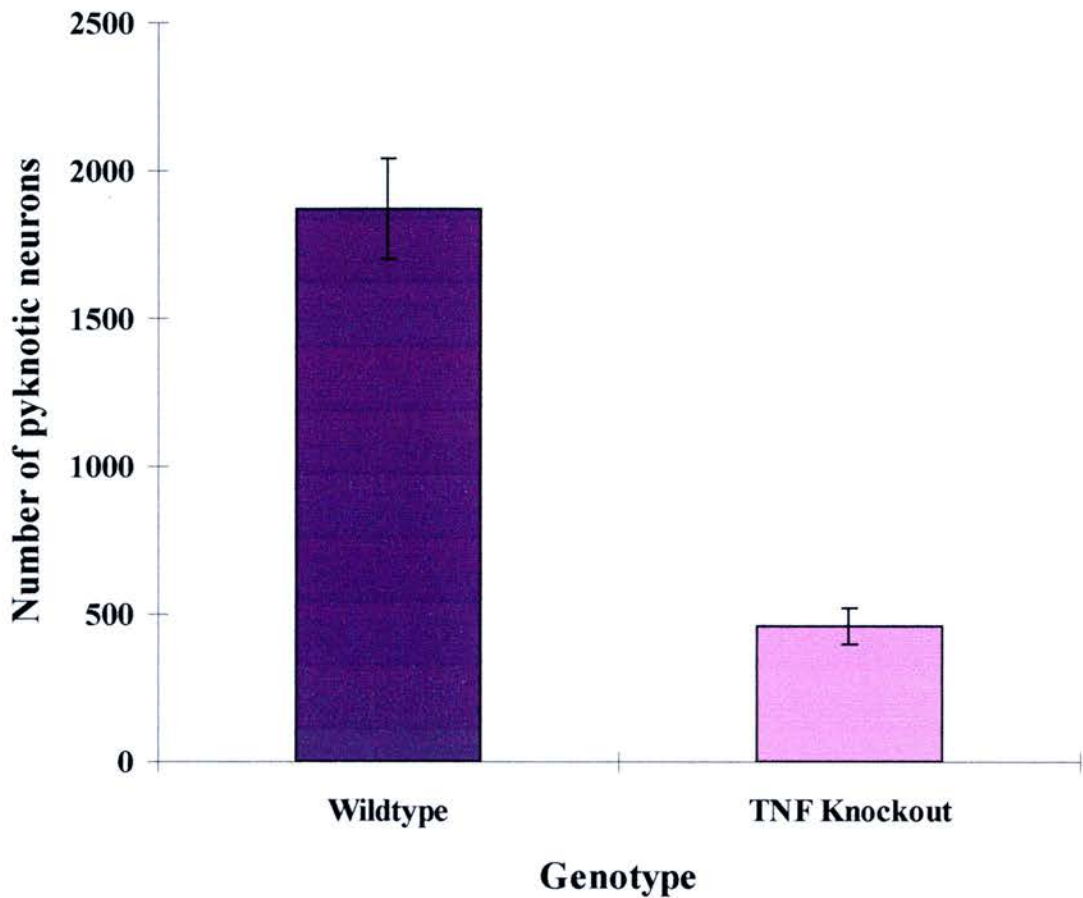
### E16 Nodose: Total Neurons



**Figure 5.18: Bar chart showing the total number of neurons in the nodose of E16 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors of the data obtained from both sets of ganglia from 3 E16 TNF $\alpha$ <sup>+/+</sup> and from 4 E16 TNF $\alpha$ <sup>-/-</sup> mice are shown. The numbers of neurons in the nodose ganglia were increased by 37% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.

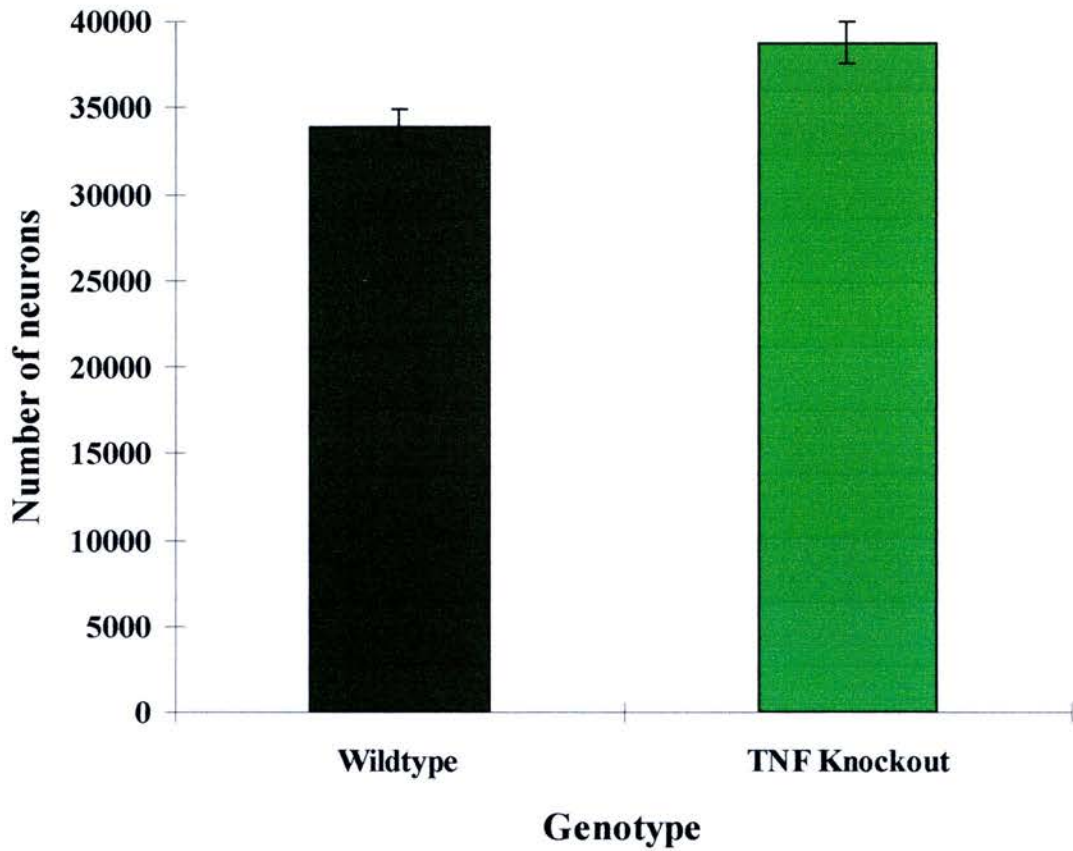


## E16 Nodose: Pyknotic Neurons



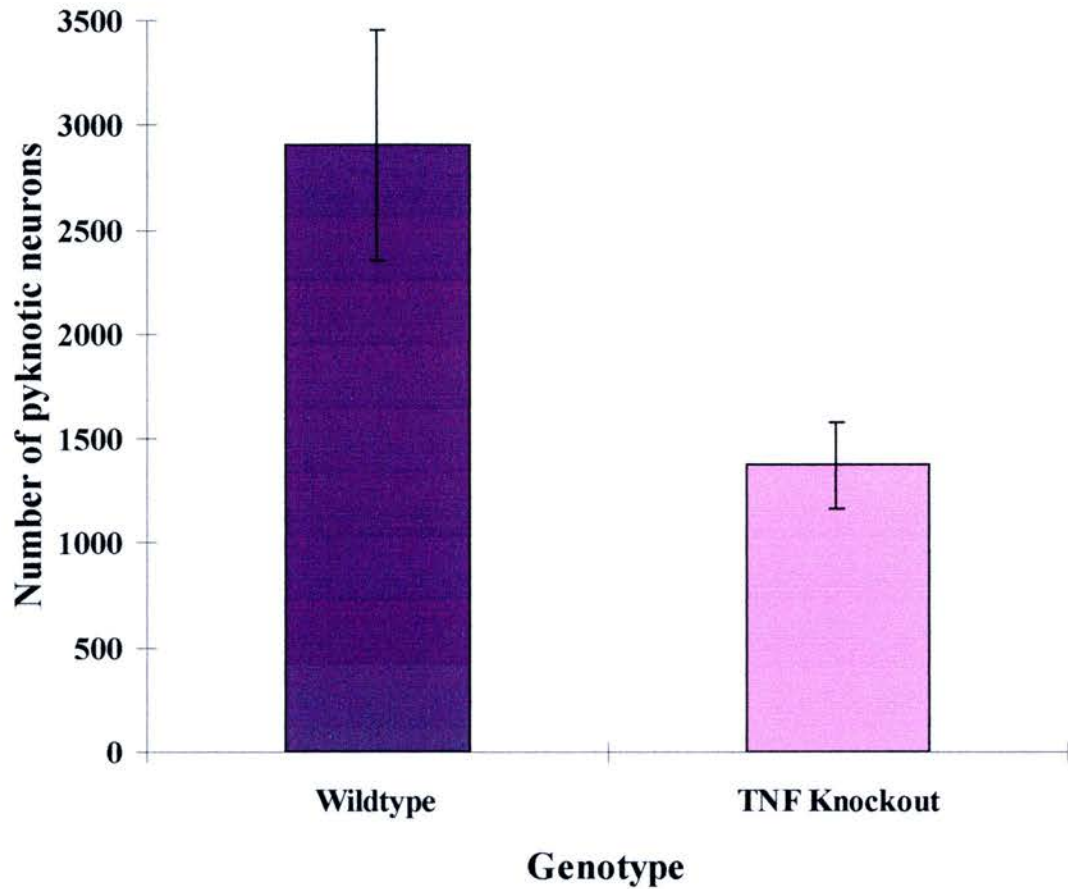
**Figure 5.19: Bar chart showing the number of pyknotic neurons in the nodose of E16 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors of the data obtained from both sets of ganglia from 3 E16 TNF $\alpha$ <sup>+/+</sup> and from 4 E16 TNF $\alpha$ <sup>-/-</sup> mice are shown. The numbers of pyknotic neurons, and therefore cell death, in the nodose ganglia were decreased by approximately 75% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.

### P1 SCG: Total Neurons



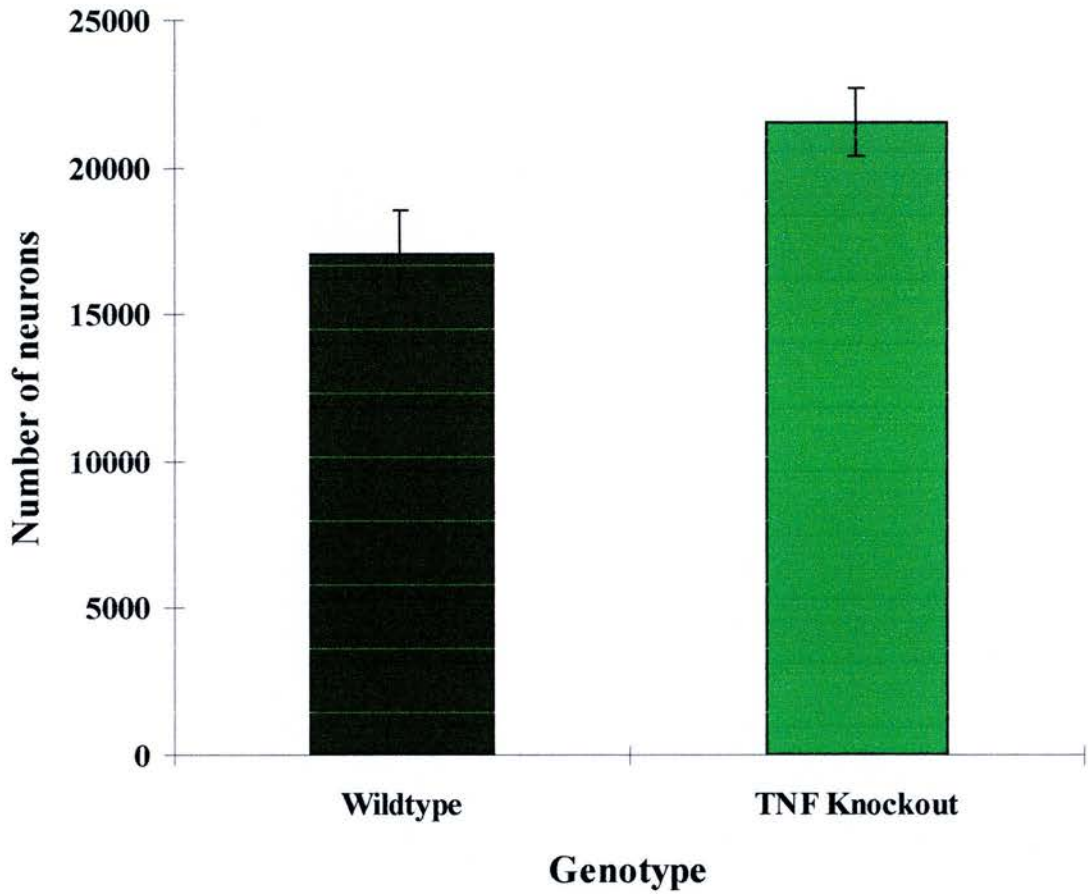
**Figure 5.20: Bar chart showing the total number of neurons in the SCG of P1 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors of the data obtained from both sets of ganglia from 4 P1 TNF $\alpha$ <sup>+/+</sup> and from 5 P1 TNF $\alpha$ <sup>-/-</sup> mice are shown. The numbers of neurons in the SCG were increased by 14% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.

### P1 SCG: Pyknotic Neurons



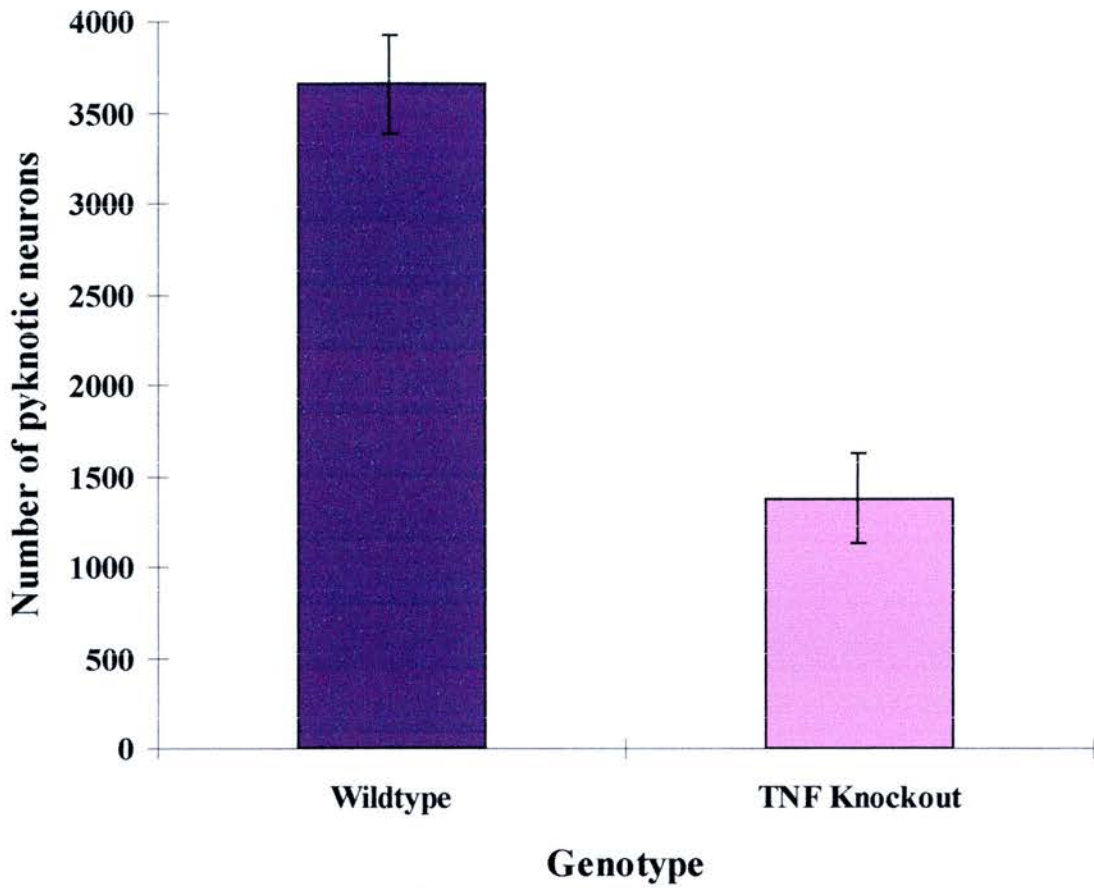
**Figure 5.21: Bar chart showing the number of pyknotic neurons in the SCG of P1 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors of the data obtained from both sets of ganglia from 4 P1 TNF $\alpha$ <sup>+/+</sup> and from 5 P1 TNF $\alpha$ <sup>-/-</sup> mice are shown. The numbers of pyknotic neurons, and therefore cell death, in the SCG were decreased by approximately 50% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.

### P1 Trigeminal: Total Neurons



**Figure 5.22: Bar chart showing the total number of neurons in the trigeminal of P1 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors of the data obtained from both sets of ganglia from 4 P1 TNF $\alpha$ <sup>+/+</sup> and from 5 P1 TNF $\alpha$ <sup>-/-</sup> mice are shown. The numbers of neurons in the trigeminal ganglia were increased by 26% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.

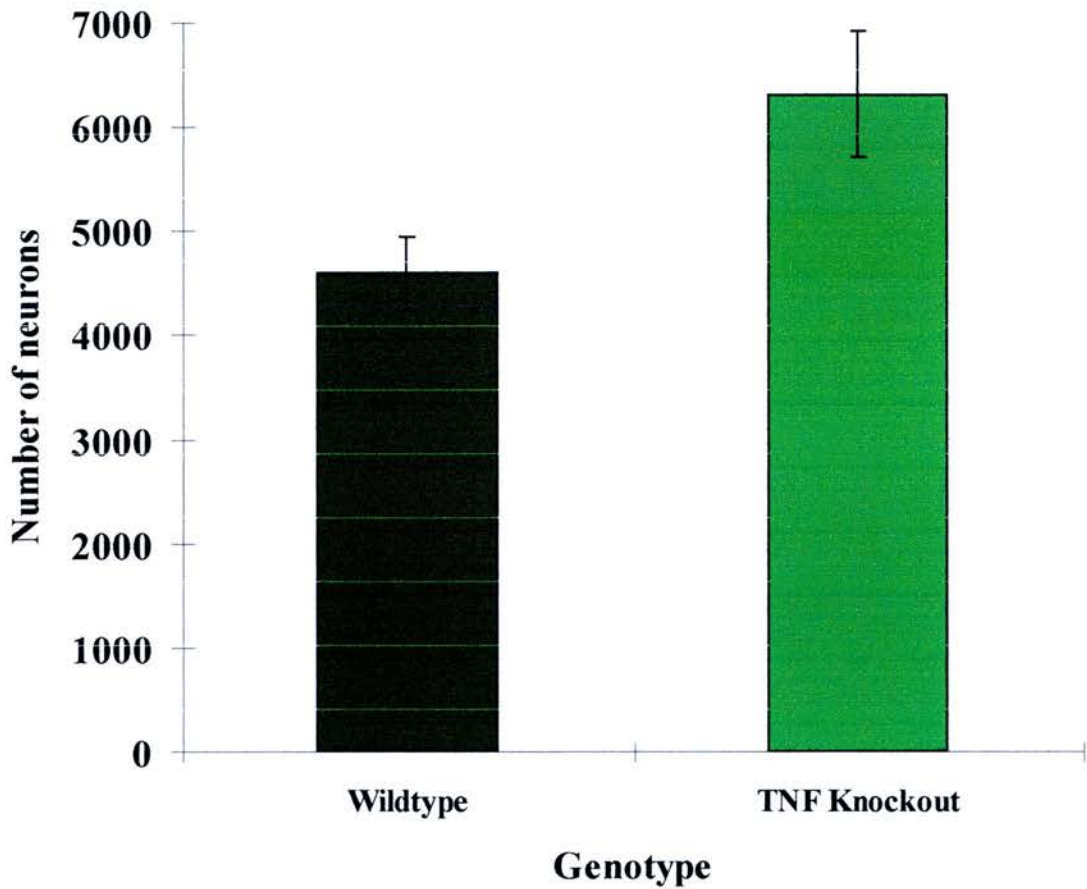
### P1 Trigeminal: Pyknotic Neurons



**Figure 5.23:** Bar chart showing the number of pyknotic neurons in the trigeminal of P1 wildtype and TNF $\alpha$  knockout mice. The means and standard errors of the data obtained from both sets of ganglia from 4 P1 TNF $\alpha$ <sup>+/+</sup> and from 5 P1 TNF $\alpha$ <sup>-/-</sup> mice are shown. The numbers of pyknotic neurons, and therefore cell death, in the trigeminal ganglia were decreased by approximately 50% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.



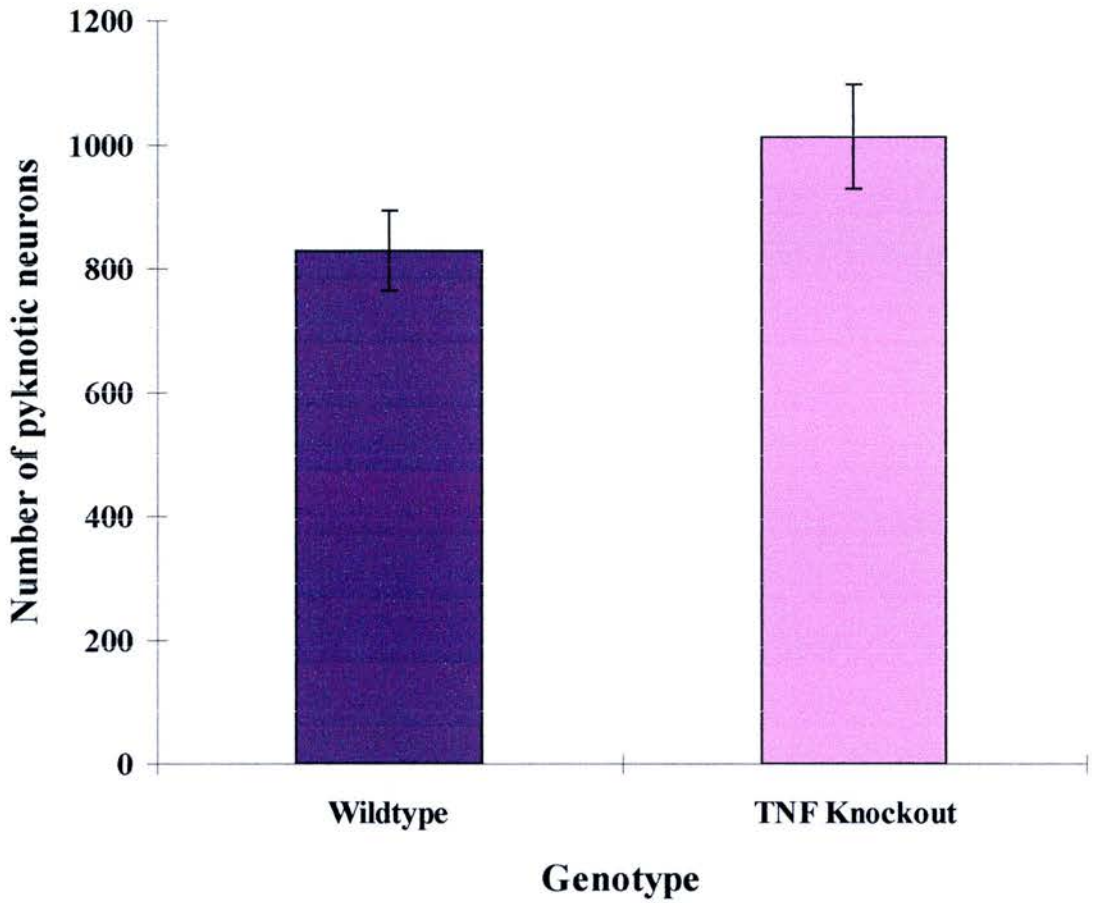
### P1 Nodose: Total Neurons



**Figure 5.24:** Bar chart showing the total number of neurons in the nodose of P1 wildtype and TNF $\alpha$  knockout mice. The means and standard errors of the data obtained from both sets of ganglia from 4 P1 TNF $\alpha$ <sup>+/+</sup> and from 5 P1 TNF $\alpha$ <sup>-/-</sup> mice are shown. The numbers of neurons in the nodose ganglia were increased by 27% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.



### P1 Nodose: Pyknotic Neurons



**Figure 5.25: Bar chart showing the number of pyknotic neurons in the nodose of P1 wildtype and TNF $\alpha$  knockout mice.** The means and standard errors of the data obtained from both sets of ganglia from 4 P1 TNF $\alpha$ <sup>+/+</sup> and from 5 P1 TNF $\alpha$ <sup>-/-</sup> mice are shown. The numbers of pyknotic neurons, and therefore cell death, in the nodose ganglia were decreased by approximately 50% in TNF $\alpha$  deficient embryos compared to their wildtype counterparts.

<b>E16</b>		<b>Neurons</b>		
<b>Ganglia</b>	<b>Wildtype</b>	<b>Std Error</b>	<b>TNF<math>\alpha</math> -/-</b>	<b>Std Error</b>
<b>SCG</b>	27523.67	1260.8	41800.33	2704.44
<b>Trigeminal</b>	29157.5	1457.23	35898.79	2541.78
<b>Nodose</b>	7071.92	335.97	11292.07	1149.39
<b>E16</b>		<b>Pyknotic Neurons</b>		
<b>Ganglia</b>	<b>Wildtype</b>	<b>Std Error</b>	<b>TNF<math>\alpha</math> -/-</b>	<b>Std Error</b>
<b>SCG</b>	6057.42	342.15	1432.46	77.06
<b>Trigeminal</b>	7768.55	804.34	1669.02	98.38
<b>Nodose</b>	1871.02	169.47	462.03	60.67
<b>P1</b>		<b>Neurons</b>		
<b>Ganglia</b>	<b>Wildtype</b>	<b>Std Error</b>	<b>TNF<math>\alpha</math> -/-</b>	<b>Std Error</b>
<b>SCG</b>	33888.97	1025.3	38753.95	1203.45
<b>Trigeminal</b>	17052.97	1517.5	21535.5	1150.26
<b>Nodose</b>	4600.48	355.65	6311.01	604.5
<b>P1</b>		<b>Pyknotic Neurons</b>		
<b>Ganglia</b>	<b>Wildtype</b>	<b>Std Error</b>	<b>TNF<math>\alpha</math> -/-</b>	<b>Std Error</b>
<b>SCG</b>	2903	550.04	1376.32	205.23
<b>Trigeminal</b>	3656.88	266.87	1376.45	249.41
<b>Nodose</b>	828.64	64.6	1013.45	83.42

**Table 5.3: Summary of *in vivo* results from wildtype and TNF $\alpha$  deficient embryos.** The means and standard errors for counts of neurons and pyknotic neurons in the SCG, trigeminal and nodose ganglia of wildtype and TNF $\alpha$  deficient embryos at E16 and P1 are shown. The numbers of neurons are increased and the levels of pyknotic neurons are decreased in embryos deficient in TNF $\alpha$  as compared to wildtype embryos at both E16 and P1.

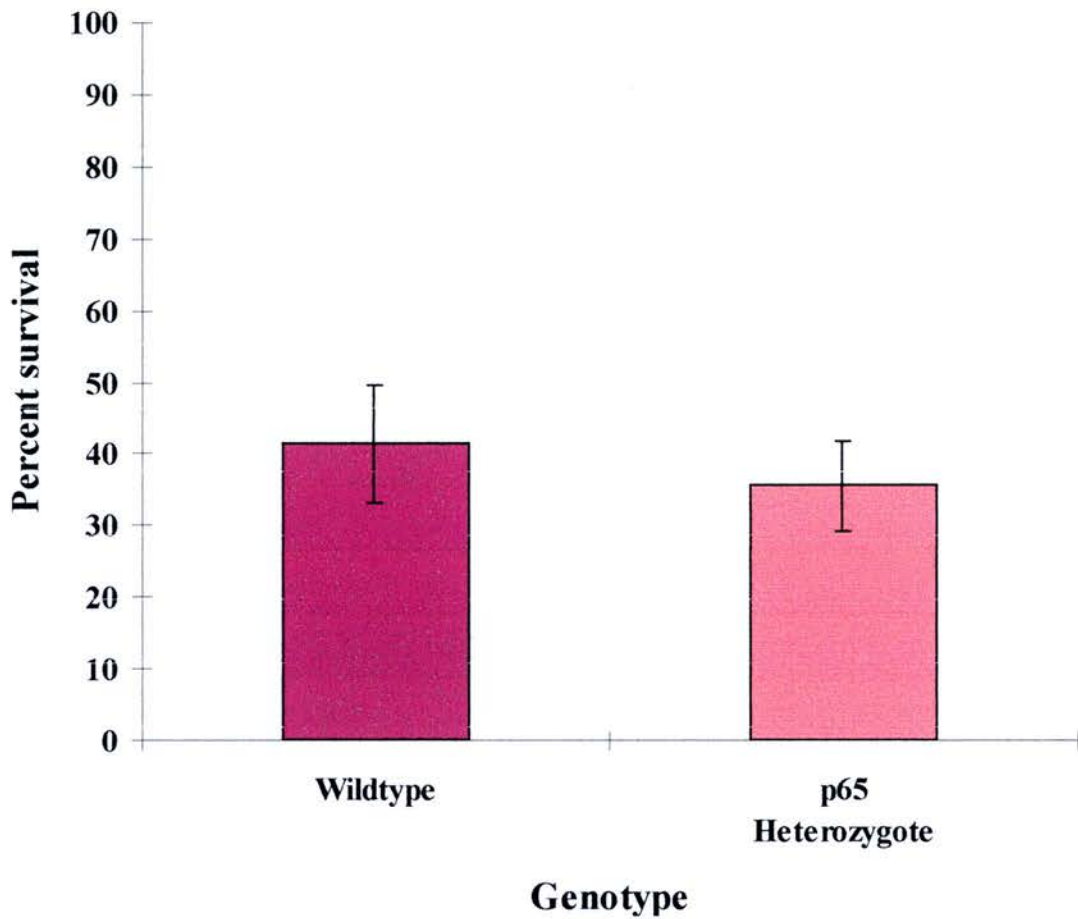
### 5.2.6 p65 is not Downstream of TNF $\alpha$ in Mediating Apoptosis.

It has been found that TNF $\alpha$  treatment promotes nuclear translocation of the p65 subunit of NF- $\kappa$ B (personal communication, Gayle Middleton). This suggests that TNF $\alpha$  induces NF- $\kappa$ B activation. It is uncertain if this signalling pathway is involved in mediating the cytotoxic action of TNF $\alpha$  on these neurons. Thus, in order to determine whether the p65 subunit of NF- $\kappa$ B is involved in mediating cell death in response to TNF $\alpha$  signalling I examined the effect of saturating concentrations of TNF $\alpha$  on the neurotrophin response of wildtype embryos and their litter-mates heterozygous for a null mutation in *p65* at E19. Low density dissociated cultures of SCG, trigeminal and nodose ganglion neurons were set up. Neurons were grown in chemically defined serum-free medium with 2ng/ml of neurotrophin plus TNF $\alpha$ . The survival of the neurons was assessed after 48 hours incubation and expressed relative to the number of neurons attached 6 hours after plating.

If TNF $\alpha$  was acting via p65 to induce cell death it would be expected that cell death in response to TNF $\alpha$  would be abrogated in neuronal cultures from p65 deficient mice. This was not the case. There was no significant difference between percent survival of wildtype and p65 deficient neurons of the SCG, trigeminal or nodose ganglia when cultured with saturating levels of TNF $\alpha$  (all cases non-significant, unpaired T-test) (figures 5.26-5.28). Neurotrophin support maintained survival of these neurons at high levels so that the effects of TNF $\alpha$  could be assessed. The addition of TNF $\alpha$  decreased

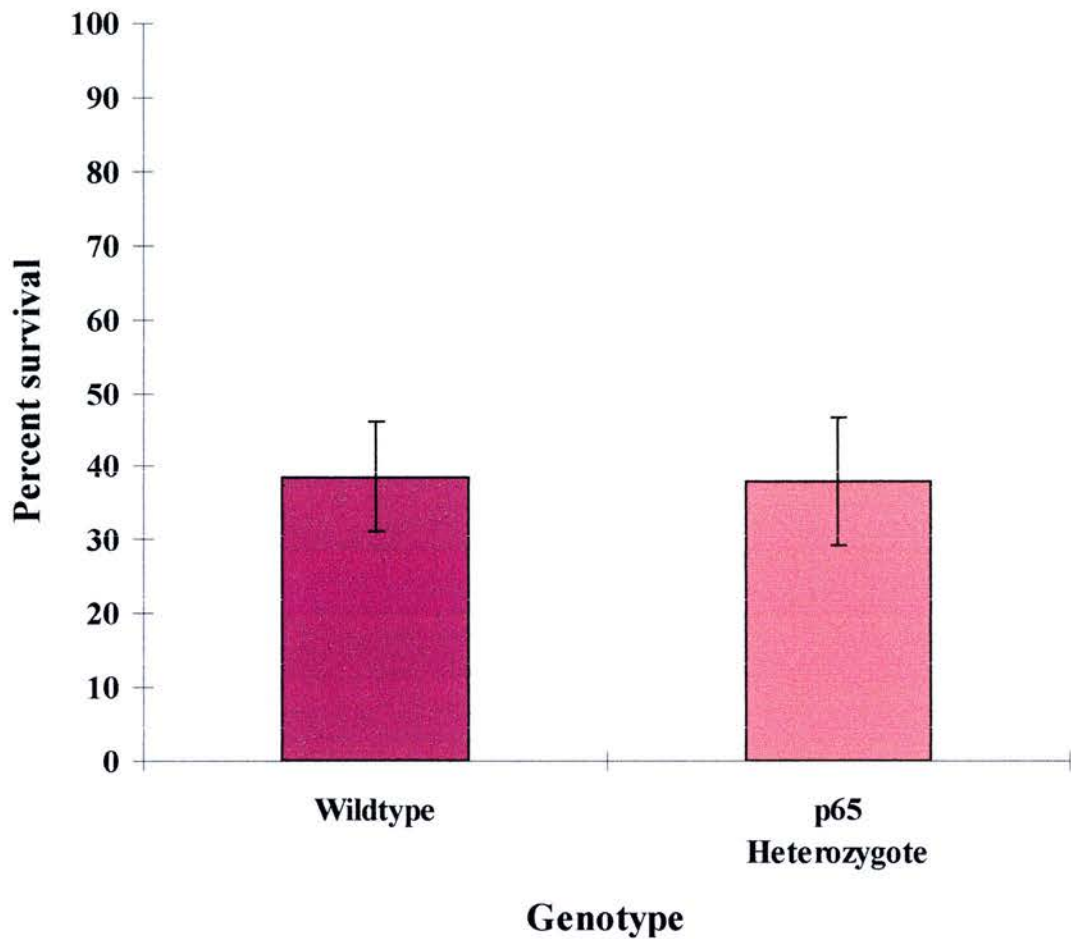
both wildtype and p65 deficient SCG, trigeminal and nodose neuronal survival to approximately 50% that usually seen in cultures of these neurons with neurotrophin alone. indicating that TNF $\alpha$  was inducing cell death but that this apoptosis is not mediated by p65.

## E19 SCG Neurons



**Figure 5.26: Bar chart showing the percent survival of E19 wildtype and p65 deficient SCG neurons.** Neurons were grown in vitro for 48 hours with 2ng/ml of neurotrophin plus 2ng/ml of TNF $\alpha$ . The means and standard errors of 3 separate experiments are shown. No significant difference can be seen between the percent survival of wildtype and *p65* knockout SCG neurons in response to TNF $\alpha$ . The percent survival of the SCG neurons is reduced to approximately 50% that seen in cultures with neurotrophin alone. This indicates that TNF $\alpha$  induces cell death but that it is not mediated by NF- $\kappa$ B.

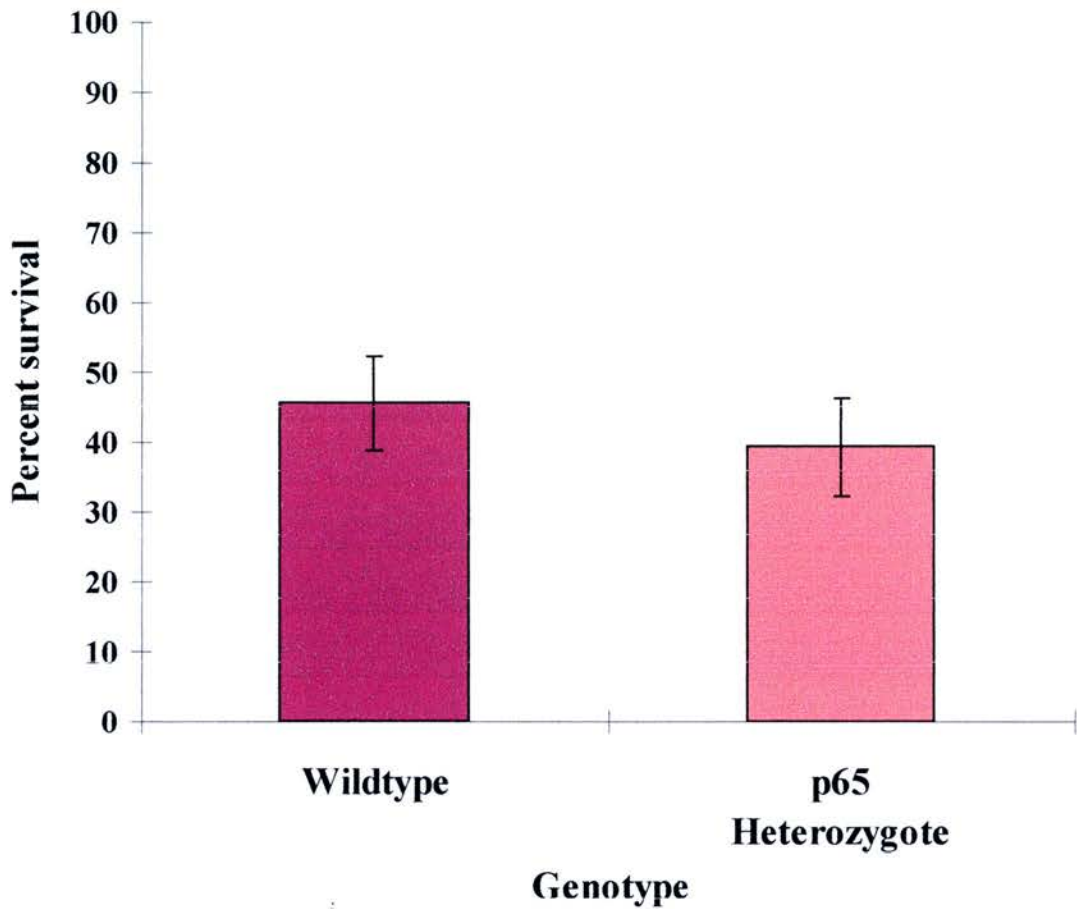
## E19 Trigeminal Neurons



**Figure 5.27: Bar chart showing the percent survival of E19 wildtype and p65 deficient trigeminal ganglion neurons.** Neurons were grown in vitro for 48 hours with 2ng/ml neurotrophin plus 2ng/ml TNF $\alpha$ . The means and standard errors of 3 separate experiments are shown. No significant difference can be seen between the percent survival of wildtype and *p65* knockout trigeminal neurons in response to TNF $\alpha$ . The percent survival of the trigeminal neurons is reduced to approximately 50% that seen in cultures with neurotrophin alone. This indicates that TNF $\alpha$  induces cell death but that it is not mediated by NF- $\kappa$ B.



## E19 Nodose Neurons

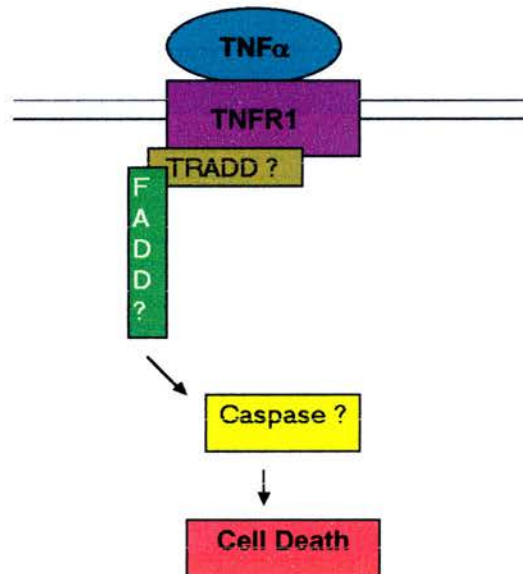


**Figure 5.28: Bar chart showing the percent survival of E19 wildtype and p65 deficient nodose ganglion neurons.** Neurons were grown in vitro for 48 hours with 2ng/ml of neurotrophin plus 2ng/ml of TNF $\alpha$ . The means and standard errors of 3 separate experiments are shown. No significant difference can be seen between the percent survival of wildtype and p65 knockout nodose neurons in response to TNF $\alpha$ . The percent survival of the nodose neurons is reduced to approximately 50% that seen in cultures with neurotrophin alone. This indicates that TNF $\alpha$  induces cell death but that it is not mediated by NF- $\kappa$ B.

### 5.3 Discussion

I have used several complementary experimental approaches to demonstrate that TNF $\alpha$  is involved in bringing about the death of sympathetic and sensory neurons during the phase of programmed cell death in the developing peripheral nervous system (see figure 5.29). Function-blocking antibodies to either TNF $\alpha$  or TNFR1 but not control antibodies to an unrelated protein prevented the death of a significant proportion of these neurons following NGF deprivation *in vitro* (figures 5.1 - 5.3). TNF $\alpha$  treatment promoted the death of cultured sympathetic and sensory neurons in a dose-dependent manner (figures 5.8-5.10). The sympathetic and sensory ganglia of TNF $\alpha$  deficient mice contained substantially fewer dying neurons and significantly greater numbers of neurons than the ganglia of wildtype litter-mates during the phase of programmed cell death *in vivo* (Figures 5.14-5.25) and sympathetic and sensory neurons from TNF $\alpha$  deficient embryos survived better in culture than neurons from wildtype embryos (figures 5.11-5.13).

**Figure 5.29: Summary Diagram of the findings in chapter 5.** The findings of this study suggest that TNF $\alpha$  binding to TNFR1 induces apoptosis of developing SCG, nodose and trigeminal neurons during the phase of programmed cell death. This cell death is not mediated by the activation of the transcription factor NF- $\kappa$ B. It may be mediated by the TRADD-FADD-caspase cell death pathway.



There are some parallels between our findings and recent work on Fas, (a member of the TNF receptor family) and its ligand Fas-L, which have a well-established role in triggering apoptosis in lymphocytes (Nagata, 1997). Blocking interaction between Fas and Fas-L reduces cell death induced by neurotrophic factor deprivation in pheochromocytoma cells, cerebellar granule cells and spinal motoneurons (Le-Niculescu et al., 1999; Brunet et al., 1999; Raoul et al., 1999). Fas is constitutively expressed in these cells and Fas-L is induced following neurotrophic factor deprivation. Treatment with Fas-L causes the death of many neurons even in the presence of optimal concentrations of neurotrophic factors. Thus, Fas signaling is triggered by neurotrophic factor withdrawal and this in turn brings about the subsequent demise of many of the neurons.

Immunocytochemical analysis of TNF $\alpha$  and TNFR1 expression on cultured embryonic sympathetic neurons suggests that virtually all of these neurons express this ligand/receptor combination (figure 5.7). This observation, together with the demonstration that inhibition of neuronal death by function-blocking anti-TNF $\alpha$  and anti-TNFR1 antibodies was observed in low dissociated density cultures that contained only a very small percentage of non-neuronal cells, suggest that TNF $\alpha$  contributes to the death of NGF-deprived neurons by an autocrine mechanism. However, the low level of

TNF $\alpha$  immunoreactivity observed in non-neuronal cells raises the possibility that the neurons may additionally obtain some TNF $\alpha$  from other cells.

In summary, these findings add a twist to the molecular mechanisms that bring about the death of developing NGF/BDNF-dependent neurons that fail to obtain adequate NGF/BDNF to support their survival. The demise of these neurons seems to be caused in part by the cytotoxic actions of TNF $\alpha$  that they produce themselves. In future work, it will be important to ascertain the extent to which TNF $\alpha$ , Fas L and possibly other cytotoxic cytokines are involved in bringing about naturally occurring neuronal death in these and other populations of neurons in the developing vertebrate nervous system.

These results are in stark contrast to my findings that at E14 TNF $\alpha$  is involved in enhancing the survival of sensory and sympathetic neurons. It is clear that there is a switch from the neurotrophic actions of TNF $\alpha$  observed at E14 to the cytotoxicity of TNF $\alpha$  observed at older embryonic ages. The reasons for this switch are unclear. The stage of maturation is different for all three ganglia at this point and yet the timing of the switch is the same. The peaks of naturally occurring neuronal death are at E13.5 in the trigeminal ganglion, E16 in the nodose ganglion and E18 in the SCG. Thus, the switch is occurring after, during and before the peak of neuronal death in each of these ganglia respectively.

The switch in function from neurotrophin to cytotoxic agent by TNF $\alpha$  will most likely be mediated by activation of different signalling pathways for each cell fate. It is widely postulated that TNF $\alpha$  signals via FADD and TRADD to activate caspases and bring about neuronal death (Sanz et al., 1999; Muzio et al., 1998) and by a pathway leading to NF- $\kappa$ B activation to bring about cell survival (Baud and Karin, 2001). The findings that p65 acts downstream from TNF $\alpha$  induced cell survival (figures 4.19-4.21) but not in TNF $\alpha$  induced cell death (figures 5.26-5.28) supports this theory as does the fact that when NF- $\kappa$ B activity is removed TNF $\alpha$  appears to have a cytotoxic activity (see section 4.2.6). Unfortunately, the small size of the neurons and amount of tissue obtained from these ganglia at these ages precludes any further biochemical or molecular study at this time. It should be noted that since the *p65* knockout mice die *in utero* by the time that TNF $\alpha$  is cytotoxic it is only possible to examine the effect caused by the deletion of a single allele which will necessarily be less than if two alleles were deleted. Therefore it is still possible that p65 acts downstream of TNF $\alpha$  in stimulating apoptosis but that its effects were not eliminated when only one allele was deleted. A further experiment that would indicate whether p65 is involved in TNF $\alpha$  cytotoxic signalling is to grow E16 or older wildtype neurons with TNF $\alpha$  and the NF- $\kappa$ B cell-permeable inhibitor SN50 (Calbiochem) as this inhibits NF- $\kappa$ B translocation and therefore activity.

TNF $\alpha$  has been shown to bring about neuronal cell survival in a range of situations including protection from glutamate toxicity (Houzen et al., 1997; Carlson et al., 1998).

Likewise it has been shown to bring about neuronal cell death in response to other stimuli such as ischaemia (Dawson et al., 1996) and axotomy (New et al., 1998). The data presented here give the first demonstration of TNF $\alpha$  switching from a neuroprotective to a cytotoxic function within the same populations of cells over a defined period in embryonic development. These findings raise many questions about how neuronal survival is controlled during embryonic development.

One such question is, what are the reasons for this switch in function? It is possible to hypothesise as to the reason for the switch. One hypothesis is that at E14 neurons that die as they grow towards their target field give off TNF $\alpha$ . This supports the survival of surrounding neurons enhancing their chances of reaching their target field. At E16 these surviving neurons produce TNF $\alpha$  in order to eliminate competing neurons. It is possible that those that have not reached sufficient support are more susceptible to the cytotoxicity of TNF $\alpha$  and therefore die more readily. This theory explains the requirement for these neurons to express both TNF $\alpha$  and TNFR1. This survival of the fittest results in the strongest possible neural connections being formed. In order to investigate this theory it will be necessary to examine whether dying neurons express TNF $\alpha$ .

An alternative hypothesis is based on the fact that neurons have been found to switch neurotrophin dependence, e.g., trigeminal neurons (Buchman and Davies, 1993). Early in development when the neurons are dependent on BDNF they express TrkB, whereas



later when they are NGF responsive they express TrkA. TNF $\alpha$  production may be a method of enhancing the neurons that express TrkB in the early stages of development when they rely on BDNF for survival. It may then stimulate the death of neurons that express TrkB but not TrkA after the neurotrophin switch has occurred in the later stages of development. Thus it will be important to examine the effects TNF $\alpha$  in trigeminal neurons over-expressing TrkB and those that have null mutations in the *TrkB* gene.

# CHAPTER 6: BAD IS INVOLVED IN NEURONAL CELL DEATH DURING DEVELOPMENT AND FOLLOWING NEUROTROPHIC FACTOR DEPRIVATION

## 6.1 Introduction

The members of the Bcl-2 protein family play an important role in the regulation of cell survival and can loosely be subdivided into two categories according to whether they repress (Bcl-2, Bcl-x<sub>L</sub>, Bcl-w, Mcl-1, A1) or trigger apoptotic cell death (Bax, Bad, Bak, Bik, Bok) (Adams et al., 2001). The pro-apoptotic category can be further subdivided into two groups, one which shares three of the four conserved BH-sequence motifs with the pro-apoptotic group and can take on similar conformations (Suzuki et al., 2000; Sattler et al., 1997; Muchmore et al., 1996), and a second that shares only the 9-16 amino acid sequence of the BH3 domain (Kaufmann and Hengartner, 2001). BH3-only proteins may induce death by binding to a Bcl-2-related death suppressor such as Bcl-2 or Bcl-x<sub>L</sub> and disrupting the complex of the death suppressor with a caspase activator, such as Apaf-1. This in turn leads to the activation of caspases (such as caspase-9) and the death of the cell (Lutz, 2000). BH3-only proteins can bind directly to anti-apoptotic members such as Bcl-x<sub>L</sub> (Sattler et al., 1997) and inhibit their interaction with death effector members e.g., Bax and Bak *in vitro* (Ottillie et al., 1997; Diaz et al., 1997), thus inducing apoptosis. This however does not take into account the presence of pro-apoptotic Bcl-2 family members such as Bax and Bak, which may be activated by the BH3-only proteins (Shimizu et al., 1999; Li et al., 1997).

Bad is one such 'BH3-only' death promoting member of this protein family and exerts its influence on cell death by binding to and activating Bax or Bak. Activated Bax or Bak undergoes a conformational change and translocates to the nuclear membrane where it oligomerises forming a pore in the membrane allowing the release of mitochondrial cytochrome c. This in turn activates caspases which are the machinery that disassembles the cell bringing about apoptosis (Li et al., 1997; Shimizu et al., 1999).

It has been found that high levels of BH3-only proteins can induce cytochrome c release (Cosulich et al., 1997), indicating cytochrome c release as a potential mechanism of BH3-only protein induced cell death. Very little is known about the role played by BH3-only proteins in the apoptotic cascade. Although considering the significant involvement of Bax in developmental cell death it is possible that BH3-only proteins also play a role in this process. In fact they may be responsible for the residual cell death found when the *bax* gene is knocked out as seen in the trigeminal, nodose ganglia (Middleton et al., 2000b) and to a small extent in the SCG (Deckwerth et al., 1996).

The anti-apoptotic members of the Bcl-2 family can act at several points in the cascade to inhibit apoptosis by binding to and inactivating the pro-apoptotic molecules. In addition Bcl-x<sub>L</sub> can promote cell survival by directly binding to Apaf1 which acts downstream of cytochrome c to activate caspases (Hu et al., 1998; Pan et al., 1998)

suggesting that the Bcl-2 protein family is able to act in a variety of ways to influence whether a cell lives or dies by apoptosis.

During the development of the peripheral nervous system, in which neurons are generated in excess and a large proportion are subsequently lost by apoptosis during the phase of naturally occurring cell death, the availability of limiting amounts of target-derived neurotrophic factors is thought to determine the magnitude of apoptosis. There is growing evidence that suggests that Bcl-2 family members play important roles in regulating cell survival downstream of these trophic influences in the developing nervous system. Many of the anti-apoptotic proteins of the Bcl-2 family are able to rescue peripheral neurons from cell death following neurotrophin deprivation including Bcl-2 (Garcia et al., 1992;Allsopp et al., 1993), Bcl-x<sub>L</sub> (Gonzalez-Garcia et al., 1995; Middleton et al., 1996) and Bcl-w (Middleton et al., 2001). Accordingly, *in vitro* reduction or elimination of Bcl-2 expression reduces neuronal survival (Allsopp et al., 1995) and mice that are homozygous for a null mutation in the *bcl-2* or *bcl-x<sub>L</sub>* genes show significant neuronal deficits in their peripheral ganglia (Motoyama et al., 1995; Michaelidis et al., 1996; Pinon et al., 1997). In contrast, mice that are homozygous for a null mutation in the pro-apoptotic *bax* gene show decreased cell death in their cranial sensory ganglia (Middleton et al., 2000b) and a virtual elimination of cell death in the sympathetic neurons of the SCG (Deckwerth et al., 1996) whilst overexpression of Bax *in vitro* is able to accelerate neuronal death following neurotrophin withdrawal

(Vekrellis et al., 1997; Martinou et al., 1998), indicating that Bax is involved in mediating developmental cell death.

Bad is known to be widely expressed in the peripheral nervous system (Merry and Korsmeyer, 1994) and Bad mRNA has been detected in both the central and peripheral nervous systems (Yang et al., 1995; Merry and Korsmeyer, 1997), suggesting a role for it in the nervous system. The over-expression of Bad in NGF-supported sympathetic neurons induced apoptosis indicating that Bad may be involved in the regulation of cell death in these neurons (Hamner et al., 2001). The role of Bad in regulating the survival of peripheral neurons suggested by these findings remains to be fully elucidated. To this end, I studied the survival of SCG, trigeminal and nodose ganglion neurons derived from Bad deficient animals both *in vitro* and *in vivo*. The hypothesis under examination is that Bad is involved in mediating cell death during the development of these areas. It would thus be expected that a decrease in cell death would be observed in mice deficient in Bad, with a concurrent increased number of neurons in these areas in Bad deficient mice.

## 6.2 Results

### 6.2.1 Enhanced Survival of Bad Deficient Neurons *in vitro*

To elucidate the role of Bad in bringing about cell death in absence of neurotrophic factors, I established low density cultures of SCG, trigeminal and nodose ganglion neurons from Bad deficient embryos and wildtype litter-mates at both E14 and P1 and grew them in chemically defined serum-free medium either with or without neurotrophic support. The survival of the neurons was assessed after 48 hours incubation and expressed as a percentage of the number of neurons attached 6 hours after plating.

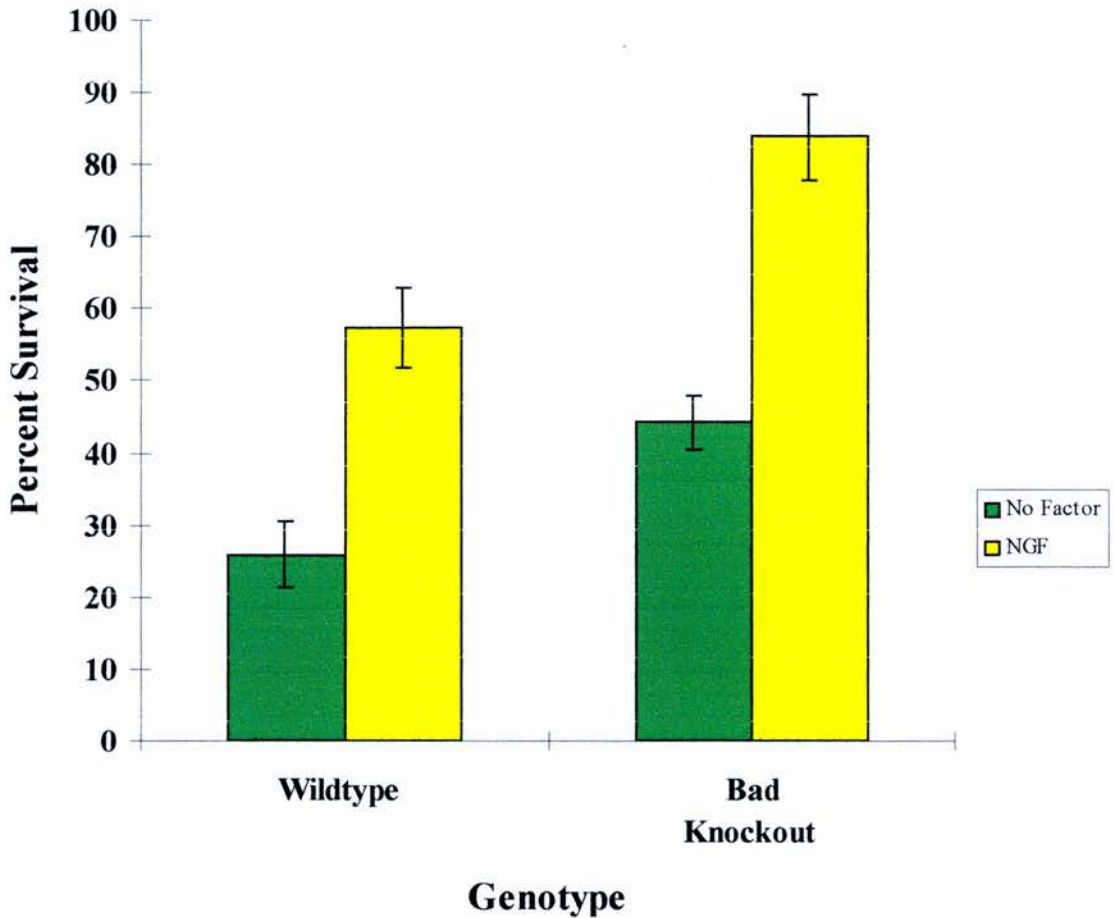
When grown with neurotrophins, Bad deficient E14 neurons showed a significant enhancement in cell survival as compared to wild type neurons. After 48 hours incubation, there was a 26% increase for SCG neurons grown with a saturating concentration of NGF ( $p < 0.05$ , unpaired t-test), and a 32% increase in neuronal survival after 48 hours incubation for trigeminal neurons grown with a saturating concentration of NGF ( $p < 0.0001$ , unpaired t-test). Similarly, there was a 16% increase in neuronal survival for Bad deficient nodose neurons grown with a saturating concentration of BDNF as compared to the survival of wildtype neurons in parallel cultures ( $p < 0.0001$ , unpaired t-test) (figures 6.1-6.3 and table 6.1). Similar results were found in the P1 cultures (figures 6.4-6.6 and table 6.1). After 48 hours there were 20% more SCG



( $p < 0.0005$ , unpaired t-test), 30% more trigeminal ( $p < 0.0001$ , unpaired t-test) and 19% more nodose neurons in the cultures from Bad deficient mice than in those from wildtype mice when grown with a saturating concentration of neurotrophins ( $p < 0.0001$ , unpaired t-test).

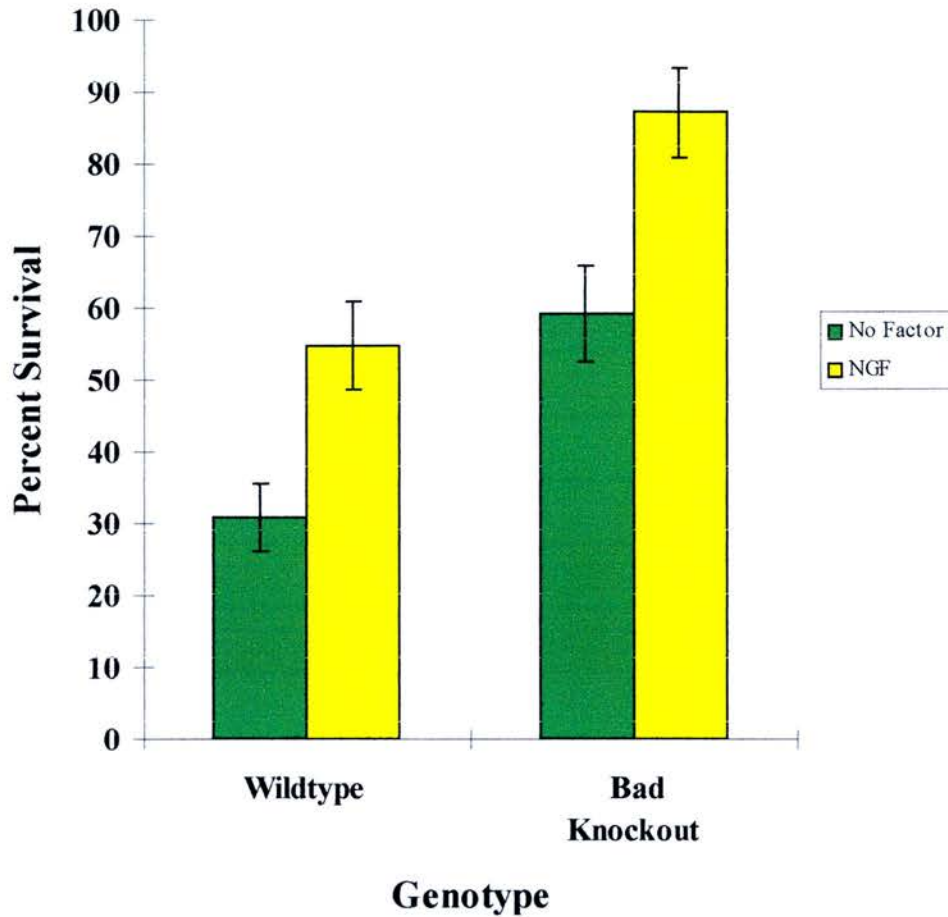
In neurotrophin-deprived cultures, there was a significant increase in cell survival in the absence of Bad for the E14 NGF-dependent trigeminal sensory and sympathetic SCG neurons (28% for trigeminal neurons [ $p < 0.001$ , unpaired t-test] and 18% for SCG neurons [ $p < 0.05$ , unpaired t-test]) (figures 6.1, 6.2 and table 6.1). In the case of nodose neurons, more than twice as many Bad deficient neurons survived for 48 hours without neurotrophic support than in wild type cultures (54% for cultures established from  $bad^{-/-}$  nodose ganglia as compared to 25% for cultures established from  $bad^{+/+}$  nodose ganglia [ $p < 0.0001$ , unpaired t-test]) (figure 6.3 and table 6.1). Similar results were found in the P1 neurotrophin deficient cultures (figures 6.4-6.6 and table 6.1). There were 12% more SCG ( $p < 0.005$ , unpaired t-test), 34% more trigeminal ( $p < 0.0001$ , unpaired t-test) and 12% more nodose neurons in  $bad^{-/-}$  culture as compared to  $bad^{+/+}$  cultures after 48 hours ( $p < 0.05$ , unpaired t-test). These results demonstrate that Bad deficient neurons show enhanced survival *in vitro* as compared to wildtype neurons and that this effect is most evident in neurotrophin-deprived cultures.

## E14 SCG Neurons



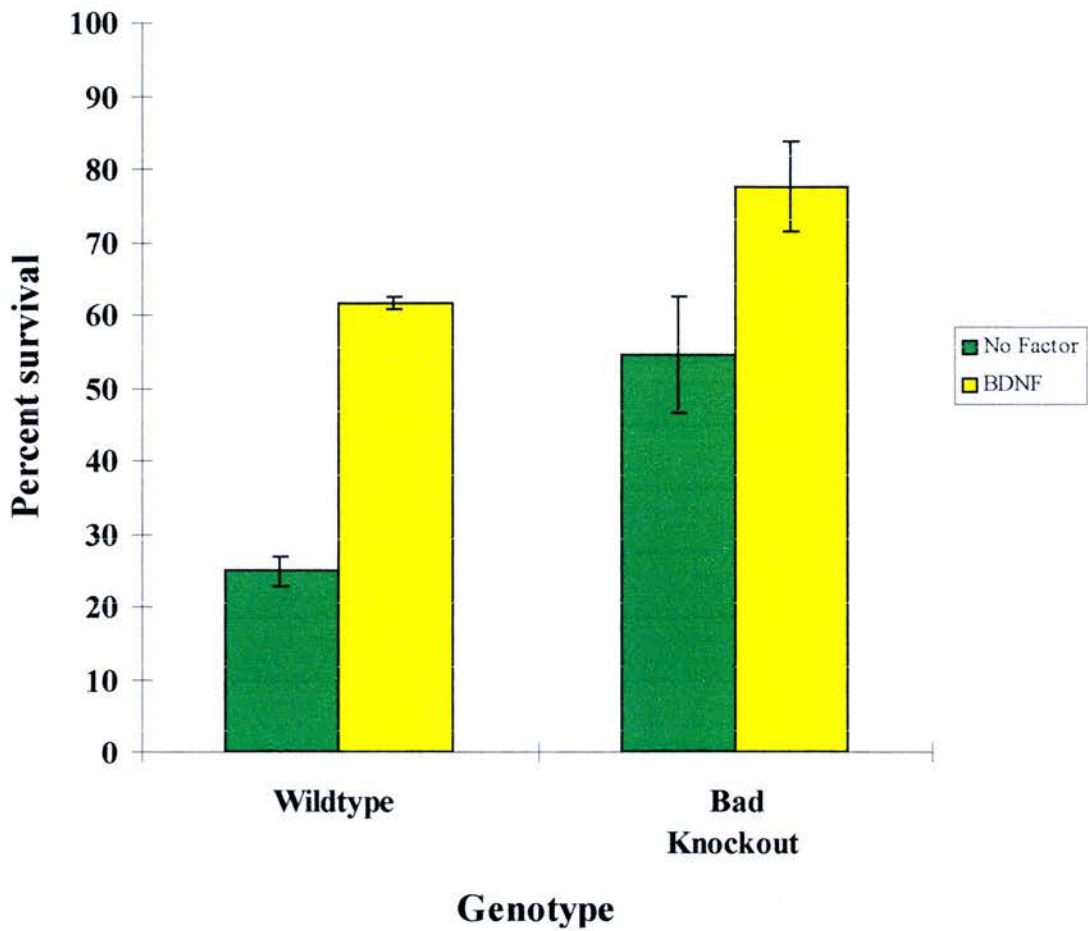
**Figure 6.1: Bar chart showing the percent survival of E14 wildtype and Bad deficient SCG neurons.** Neurons were grown in vitro for 48 hours either with neurotrophic support or without the addition of 2ng/ml neurotrophic factors. The means and standard errors of three separate experiments are shown. With saturating concentrations of neurotrophin (2ng/ml) cell survival was increased by 26% in Bad deficient cultures compared to wildtype cultures. In neurotrophin deprived cultures cell survival was increased by 18% in *bad* knockout cultures compared to wildtype cultures.

## E14 Trigeminal Neurons



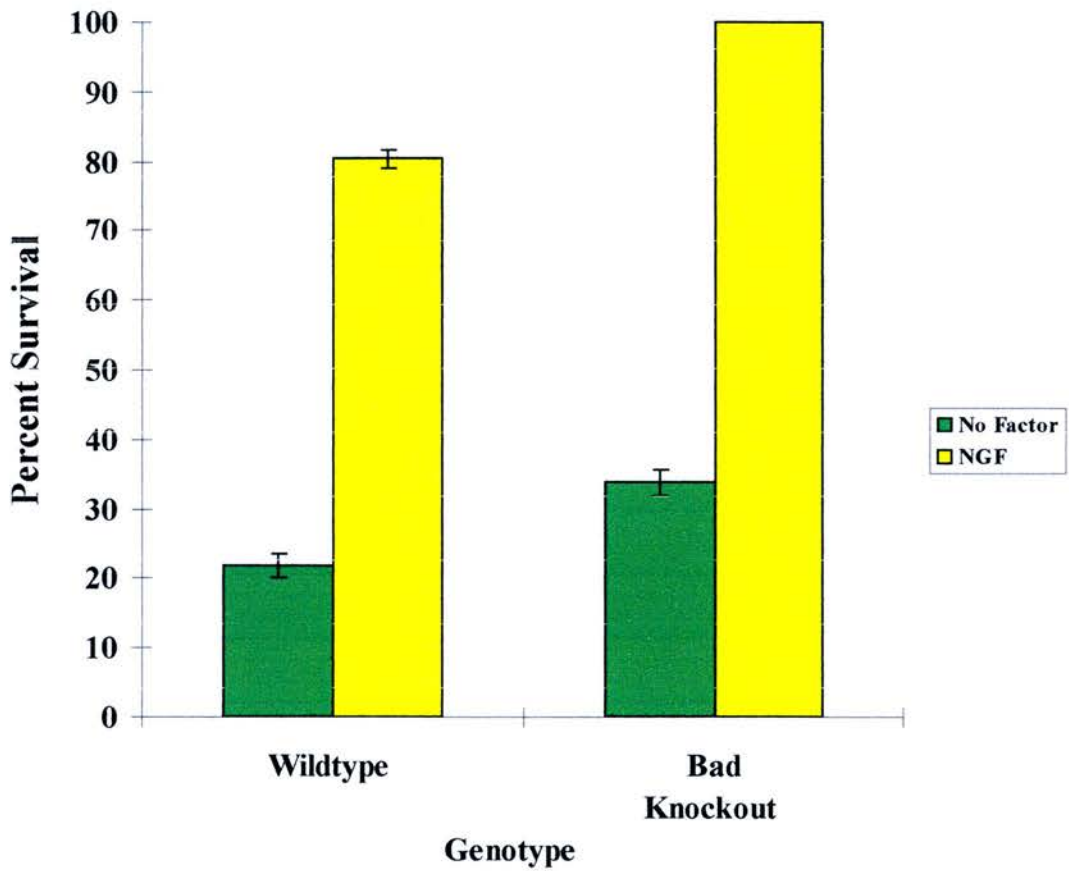
**Figure 6.2: Bar chart showing the survival of E14 wildtype and Bad deficient trigeminal ganglion neurons.** Neurons were grown in vitro for 48 hours either with 2ng/ml neurotrophin or without the addition of neurotrophic factors. The means and standard errors of three separate experiments are shown. With saturating concentrations of neurotrophin (2ng/ml) cell survival was increased by 32% in Bad deficient cultures compared to wildtype cultures. In neurotrophin deprived cultures cell survival was increased by 28% in *bad* knockout cultures compared to wildtype cultures.

### E14 Nodose Neurons



**Figure 6.3: Bar chart showing the survival of E14 wildtype and Bad deficient nodose ganglion neurons.** Neurons were grown in vitro for 48 hours either with 2ng/ml neurotrophin or without the addition of neurotrophic factors. The means and standard errors of three separate experiments are shown. With saturating concentrations of neurotrophin (2ng/ml) cell survival was increased by 16% in Bad deficient cultures compared to wildtype cultures. In neurotrophin deprived cultures cell survival was increased by 29% in *bad* knockout cultures compared to wildtype cultures.

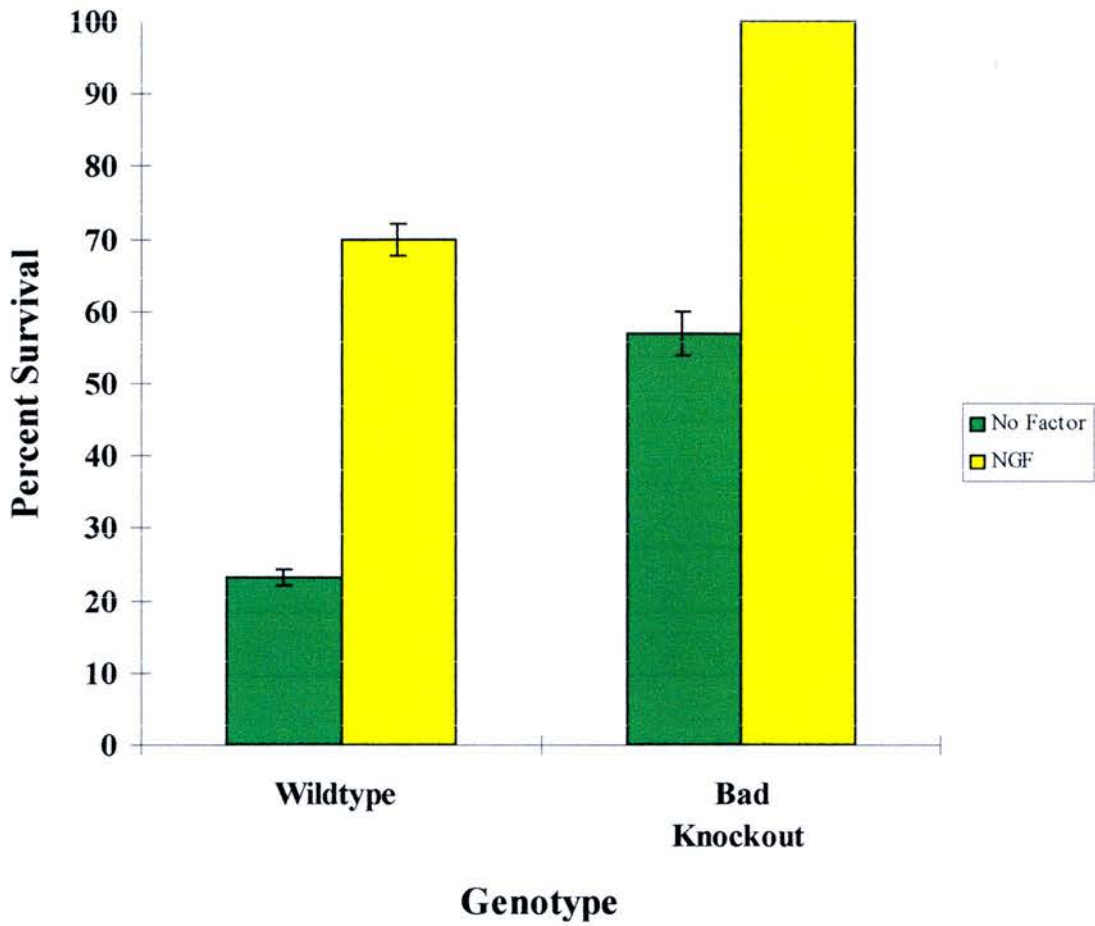
## P1 SCG Neurons



**Figure 6.4: Bar chart showing the survival of P1 wildtype and Bad deficient SCG neurons.** Neurons were grown in vitro for 48 hours either with 2ng/ml neurotrophin or without the addition of neurotrophic factors. The means and standard errors of three separate experiments are shown. With saturating concentrations of neurotrophin (2ng/ml) cell survival was increased by 20% in Bad deficient cultures compared to wildtype cultures. In neurotrophin deprived cultures cell survival was increased by 12% in *bad* knockout cultures compared to wildtype cultures.



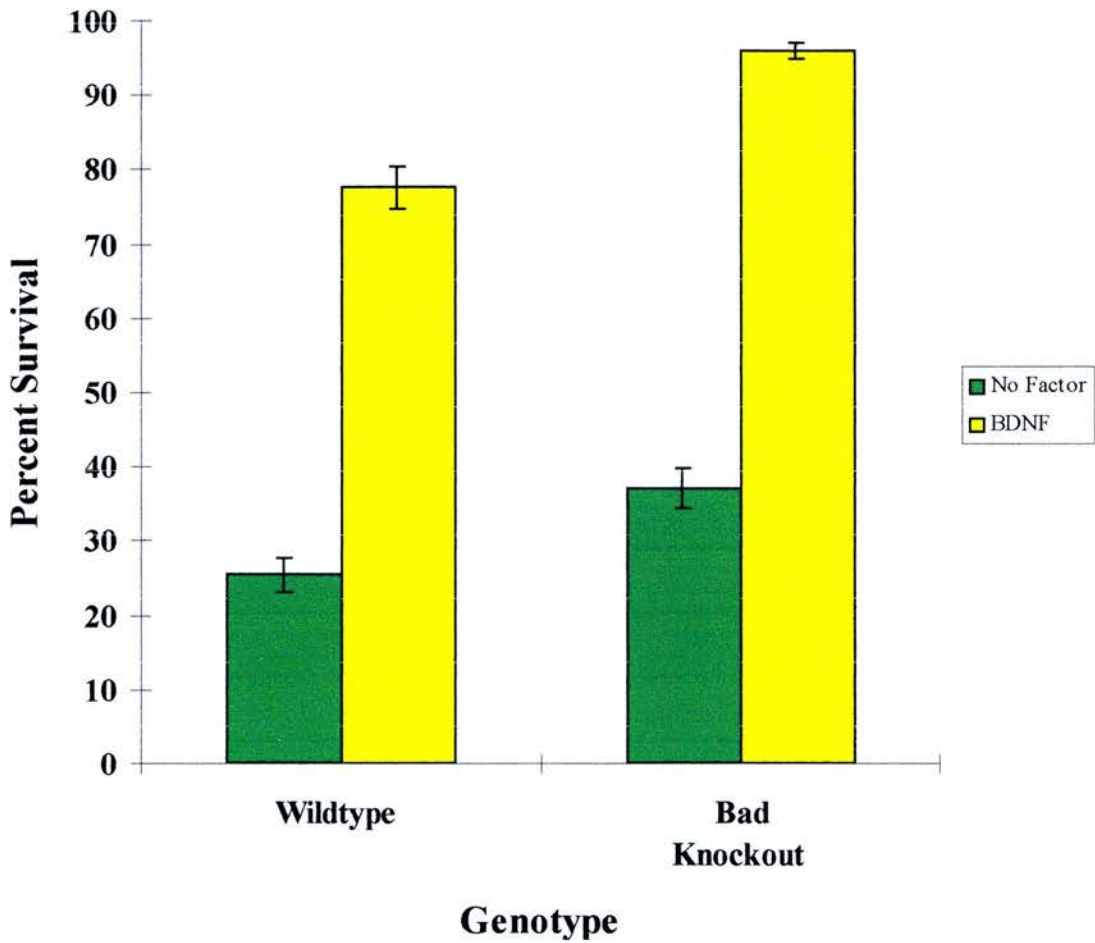
## P1 Trigeminal Neurons



**Figure 6.5: Bar chart showing the survival of P1 wildtype and Bad deficient trigeminal ganglion neurons.** Neurons were grown in vitro for 48 hours either with 2ng/ml neurotrophin or without the addition of neurotrophic factors. The means and standard errors of three separate experiments are shown. With saturating concentrations of neurotrophin (2ng/ml) cell survival was increased by 30% in Bad deficient cultures compared to wildtype cultures. In neurotrophin deprived cultures cell survival was increased by 34% in *bad* knockout cultures compared to wildtype cultures.



## P1 Nodose Neurons



**Figure 6.6: Bar chart showing the survival of P1 wildtype and Bad deficient nodose ganglion neurons.** Neurons were grown in vitro for 48 hours either with 2ng/ml neurotrophin or without the addition of neurotrophic factors. The means and standard errors of three separate experiments are shown. With saturating concentrations of neurotrophin (2ng/ml) cell survival was increased by 19% in Bad deficient cultures compared to wildtype cultures. In neurotrophin deprived cultures cell survival was increased by 12% in *bad* knockout cultures compared to wildtype cultures.

E14		Wildtype		Bad-/-	
Ganglia		% Survival	Std Error	% Survival	Std Error
SCG	NGF	57.34	5.6	83.82	5.9
	No Factor	25.9	4.6	44.5	3.7
Trigeminal	NGF	54.8	6.0	87.15	6.2
	No Factor	30.77	4.8	59.2	6.6
Nodose	BDNF	61.7	0.9	77.6	6.2
	No Factor	24.85	1.9	54.5	7.9
P1		Wildtype		Bad-/-	
Ganglia		% Survival	Std Error	% Survival	Std Error
SCG	NGF	80.44	1.3	100	0.0
	No Factor	21.7	1.7	33.79	1.78
Trigeminal	NGF	69.88		100	0.0
	No Factor	23.29	1.1	56.87	3.0
Nodose	BDNF	77.56	2.9	96.09	1.1
	No Factor	25.46	2.2	37.06	2.7

**Table 6.1: Summary of *in vitro* results from wildtype and Bad deficient embryos.** The mean percent survival and standard errors for counts of SCG, trigeminal and nodose neurons from wildtype and Bad deficient embryos at E14 and P1 are shown. The percent survival of SCG, trigeminal and nodose neurons is increased in embryos deficient in Bad as compared to wildtype embryos at both E14 and P1.

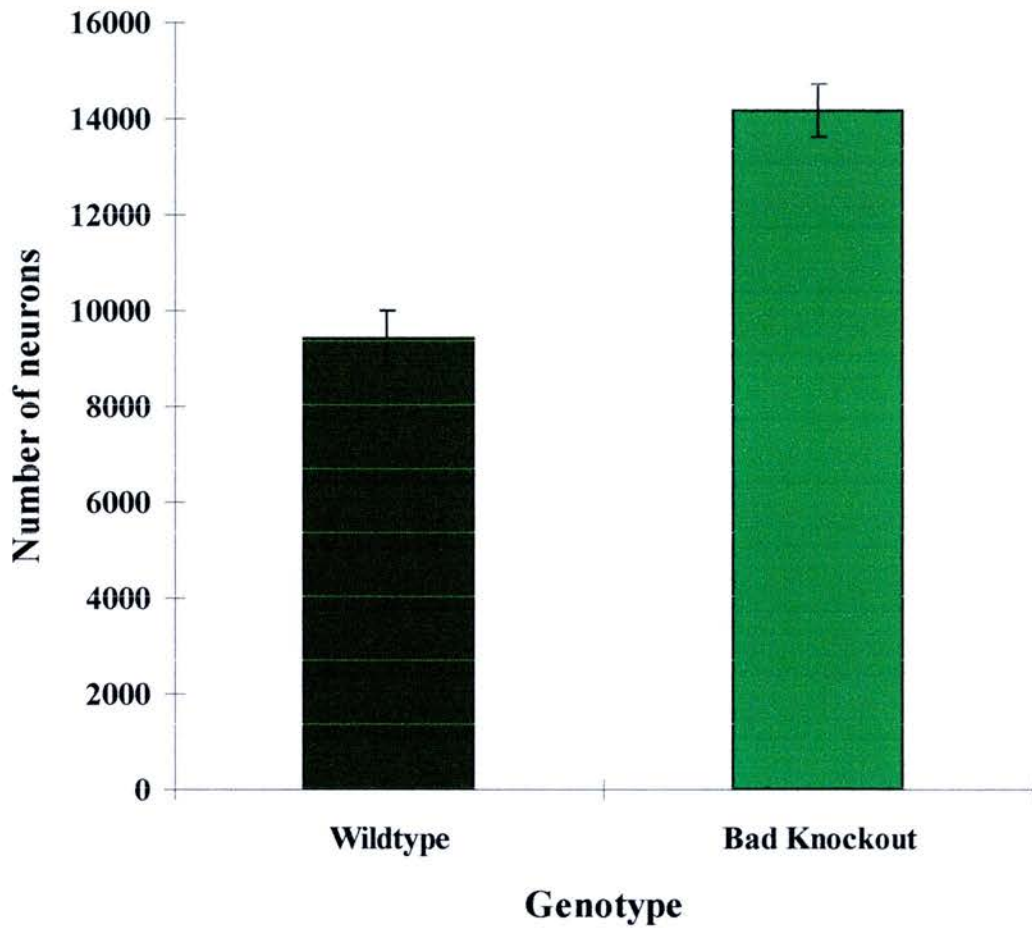
## 6.2.2 Reduced Neuronal Death in Bad Deficient Embryos

To ascertain the *in vivo* relevance of the *in vitro* findings that Bad is involved in bringing about cell death in the neurons of cranial sensory and sympathetic ganglia, I used digital stereology to determine the number of neurons and pyknotic neurons in the SCG, nodose and trigeminal ganglia at embryonic and early postnatal ages.

At E14 there is a significant difference in the number of neurons in wild type and Bad deficient SCG, trigeminal and nodose ganglia, with 33% more SCG ( $p < 0.0001$ , unpaired t-test), 11% more trigeminal ( $p < 0.05$ , unpaired t-test) and 77% more nodose neurons ( $p < 0.0001$ , unpaired t-test) in the ganglia of Bad deficient embryos as compared to their wild type litter-mates at this time (figures 6.7, 6.9, 6.11 and table 6.2). There are significantly fewer dying neurons in Bad deficient SCG (30% [ $p < 0.05$ , unpaired t-test]), nodose (47% [ $p < 0.05$ , unpaired t-test]) and trigeminal ganglia at E14 (63% [ $p < 0.005$ , unpaired t-test]) (figures 6.8, 6.10, 6.12 and table 6.2).

It should be noted that there are some differences in the numbers of neurons and pyknotic in the SCG of E14 and P1 embryos in this set of experiments and those in chapter 3 (figure 3.13 and 3.14). This did not affect the results as comparisons in these experiments were made independently of each other between wildtype and knockout embryos.

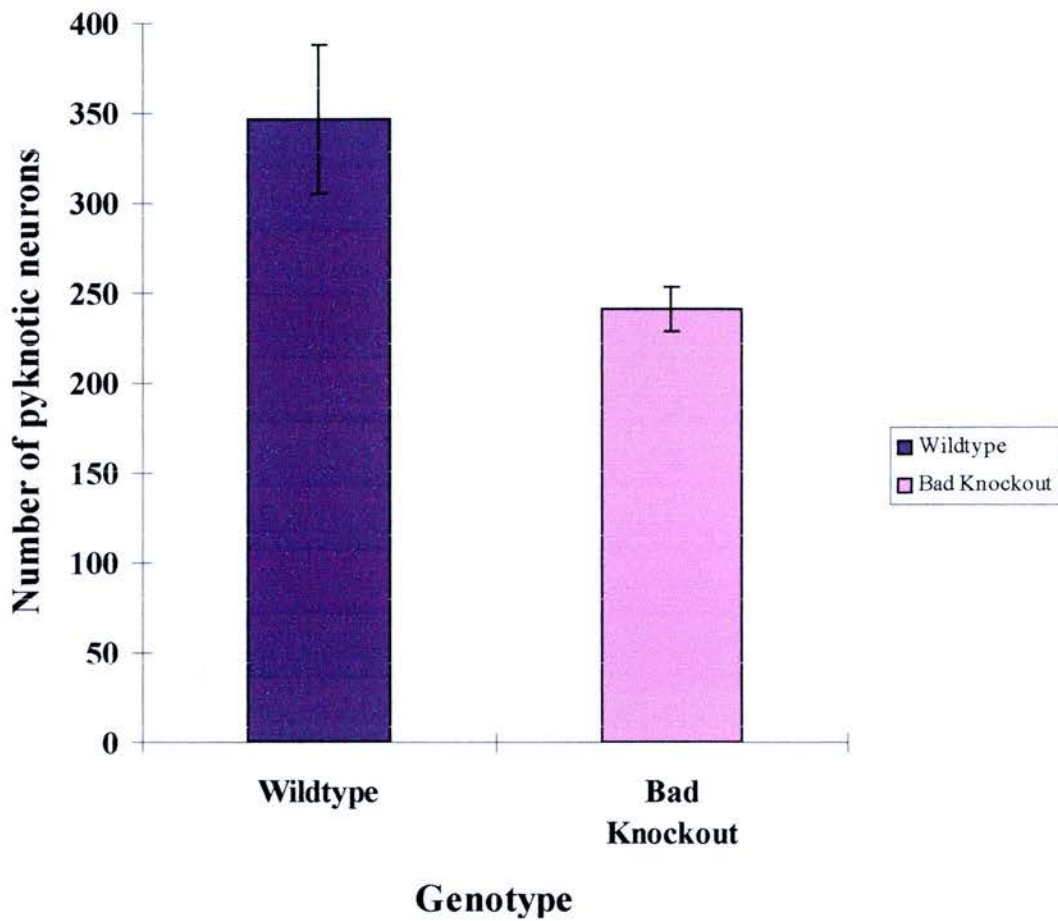
### E14 SCG: Total Neurons



**Figure 6.7: Bar chart showing the total number of neurons in the SCG of E14 wildtype and Bad deficient mice.** The means and standard errors for both ganglia from 3 wildtype and 3 knockout embryos are shown. Numbers of neurons in the SCG were increased by 33% in Bad deficient embryos compared to their wildtype counterparts.

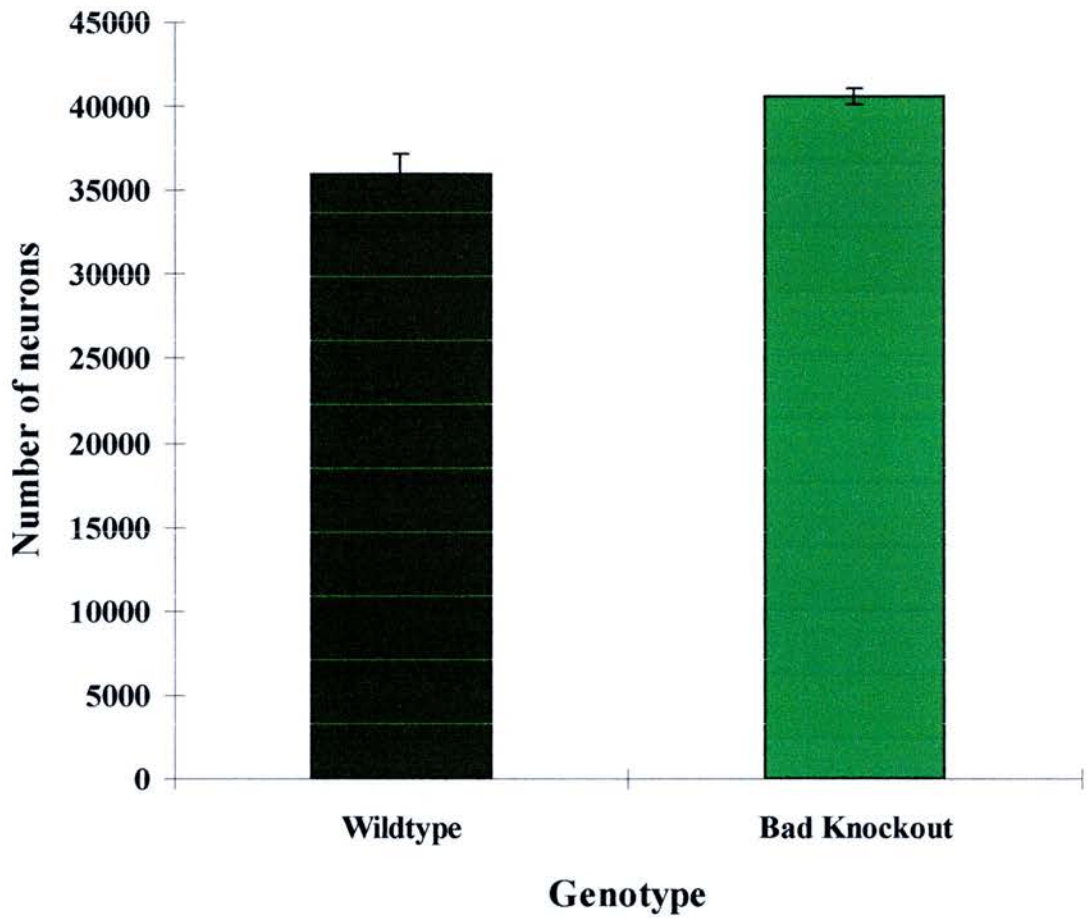


## E14 SCG: Pyknotic Neurons



**Figure 6.8: Bar chart showing the number of pyknotic neurons in the SCG of E14 wildtype and Bad deficient mice.** The means and standard errors for both ganglia from 3 wildtype and 3 knockout embryos are shown. The numbers of pyknotic neurons, and therefore cell death, in the SCG were decreased by 30% in Bad deficient embryos compared to their wildtype counterparts.

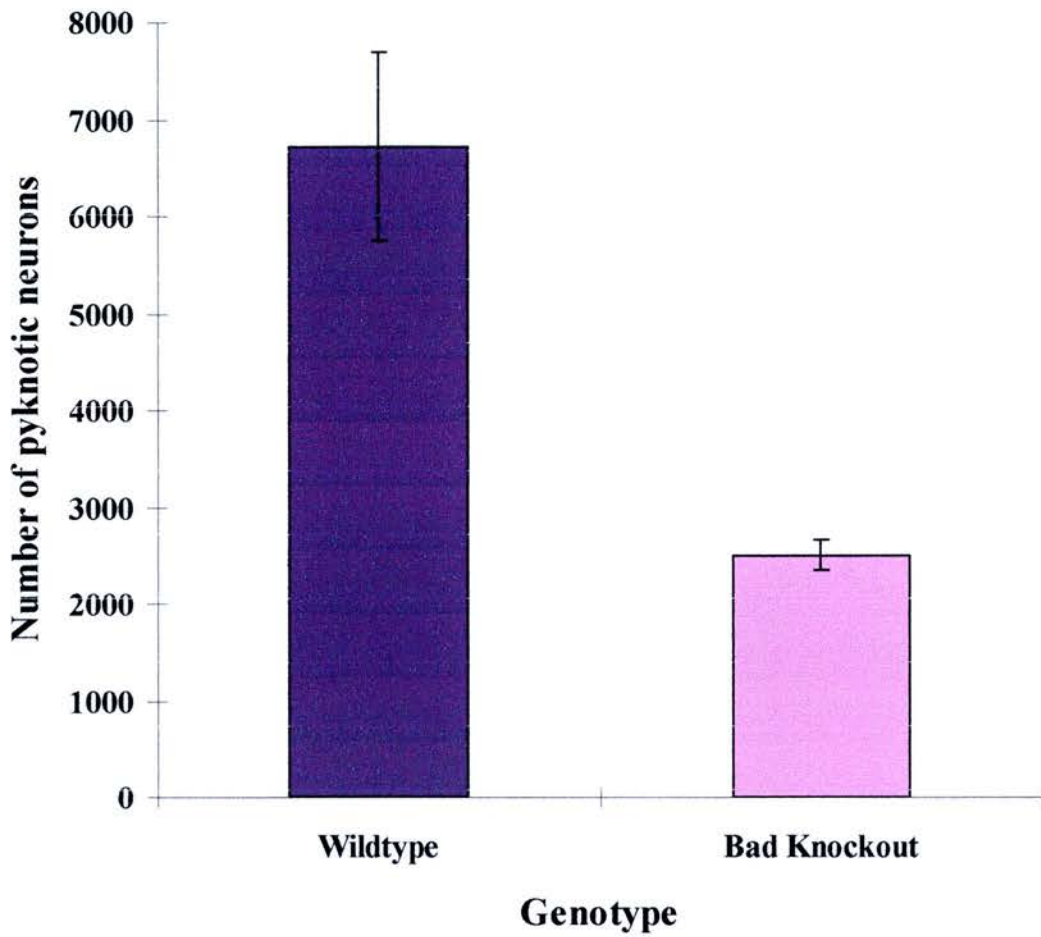
## E14 Trigeminal: Total Neurons



**Figure 6.9: Bar chart showing the total number of neurons in the trigeminal ganglia of E14 wildtype and Bad deficient mice.** The means and standard errors for both ganglia from 3 wildtype and 3 knockout embryos are shown. The numbers of neurons in the trigeminal ganglia were increased by 11% in Bad deficient embryos compared to their wildtype counterparts.

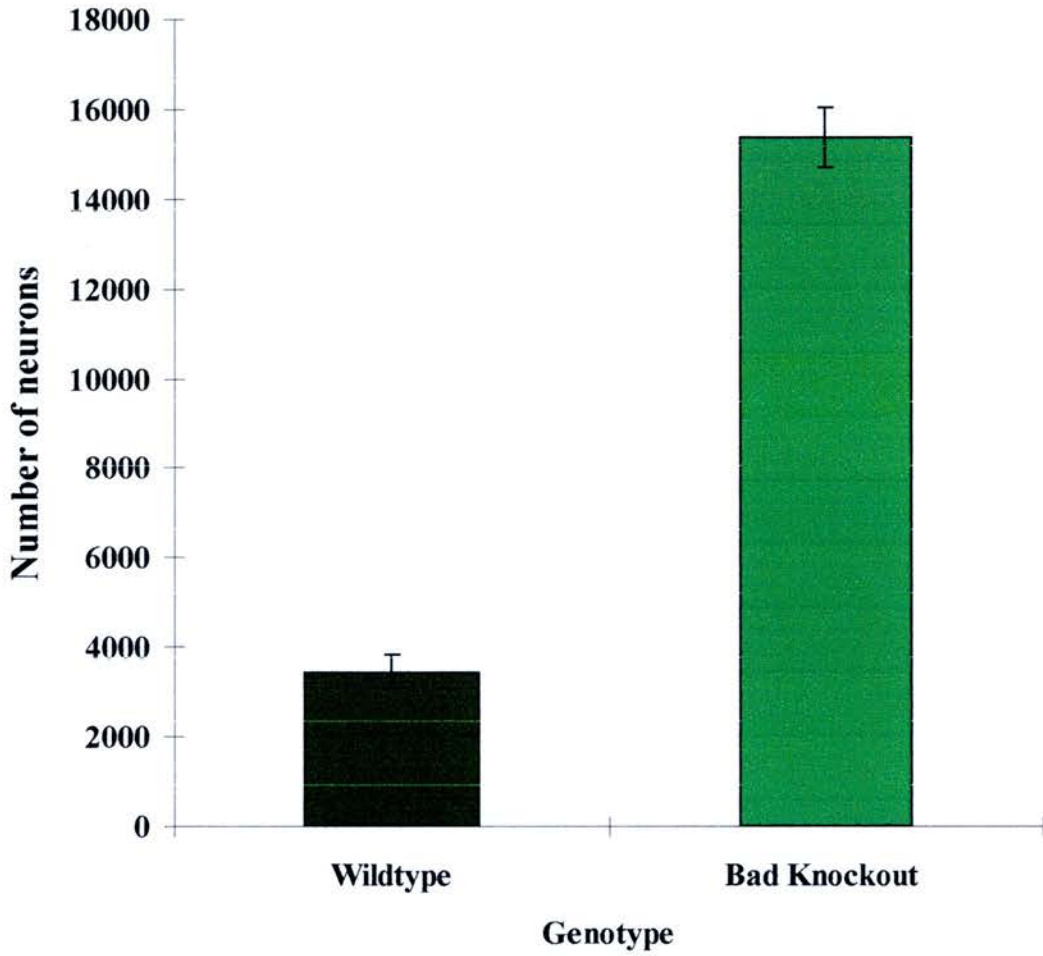


## E14 Trigeminal: Pyknotic Neurons



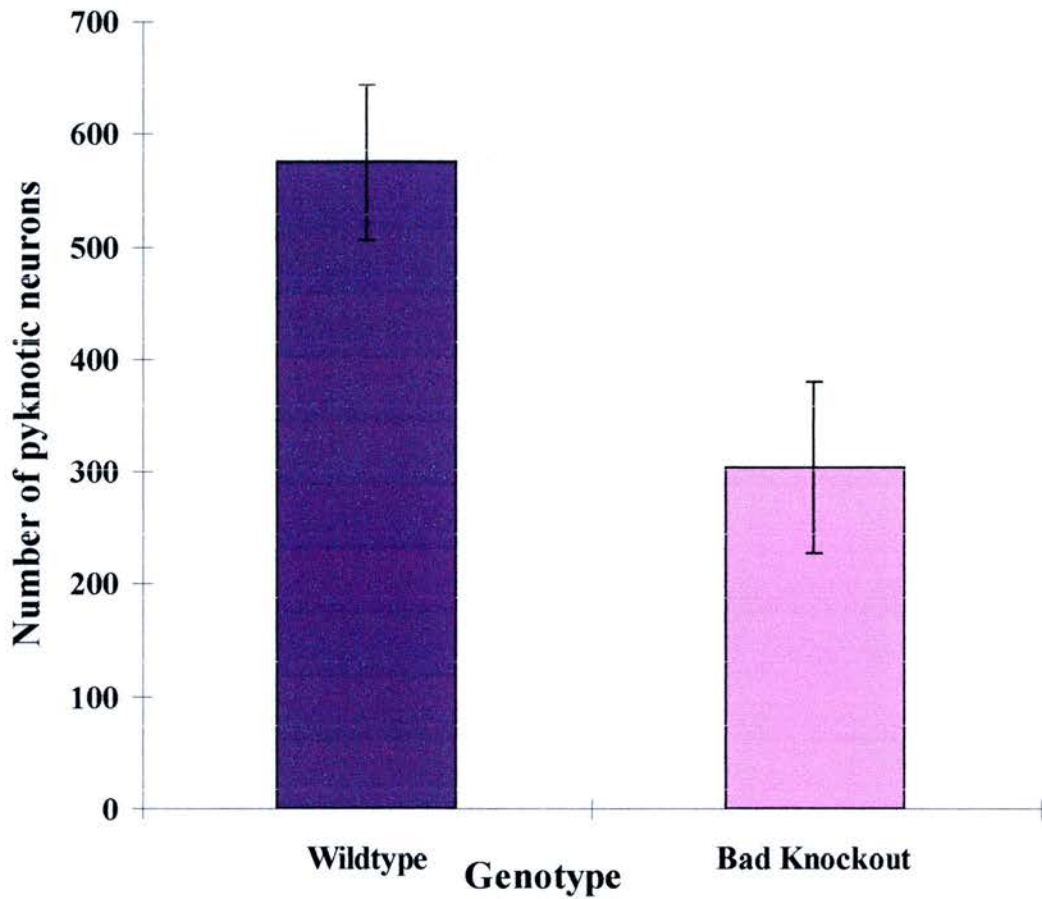
**Figure 6.10: Bar chart showing the number of pyknotic neurons in the trigeminal ganglia of E14 wildtype and Bad deficient mice.** The means and standard errors for both ganglia from 3 wildtype and 3 knockout embryos are shown. The numbers of pyknotic neurons, and therefore cell death, in the trigeminal ganglia were decreased by 63% in Bad deficient embryos compared to their wildtype counterparts.

### E14 Nodose: Total Neurons



**Figure 6.11: Bar chart showing the total number of neurons in the nodose ganglia of E14 wildtype and Bad deficient mice.** The means and standard errors for both ganglia from 3 wildtype and 3 knockout embryos are shown. The numbers of neurons in the nodose ganglia were increased by 77% in Bad deficient embryos compared to their wildtype counterparts.

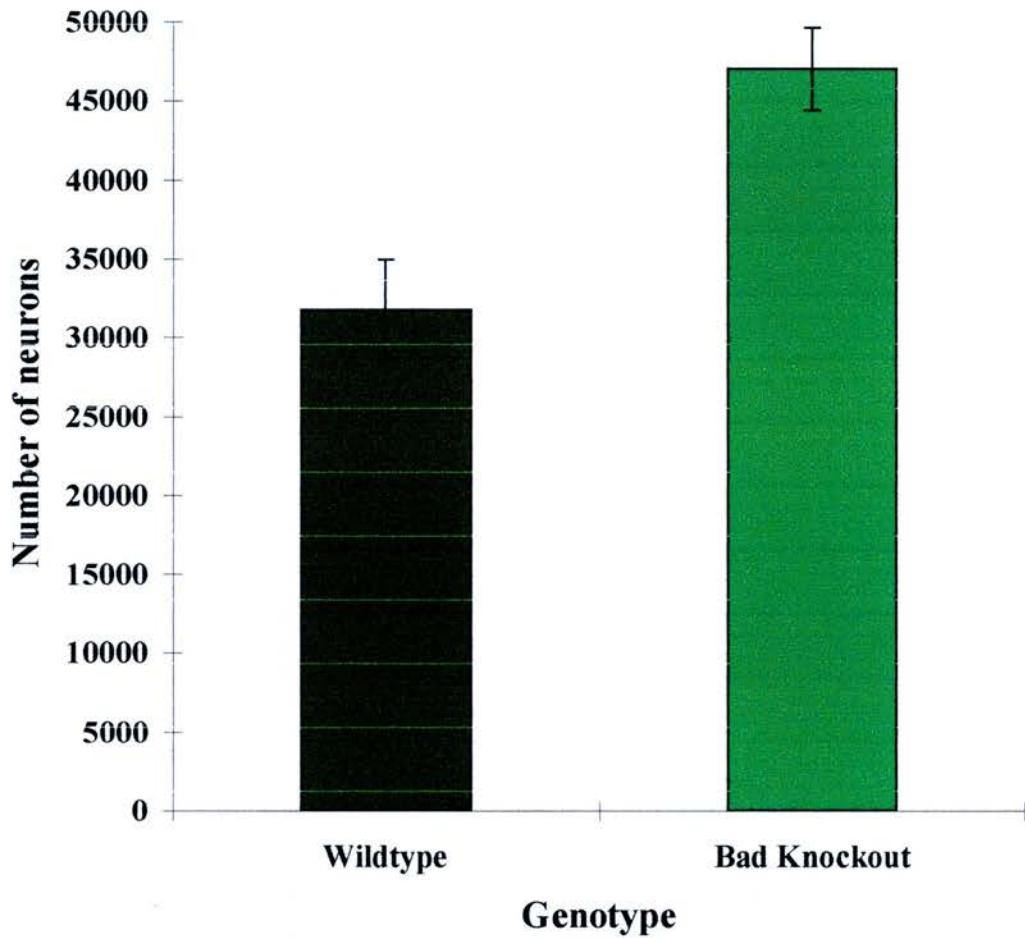
## E14 Nodose: Pyknotic Neurons



**Figure 6.12: Bar chart showing the number of pyknotic neurons in the nodose ganglia of E14 wildtype and Bad deficient mice.** The means and standard errors for both ganglia from 3 wildtype and 3 knockout embryos are shown. The numbers of pyknotic neurons, and therefore cell death, in the nodose ganglia were decreased by 47% in Bad deficient embryos compared to their wildtype counterparts.

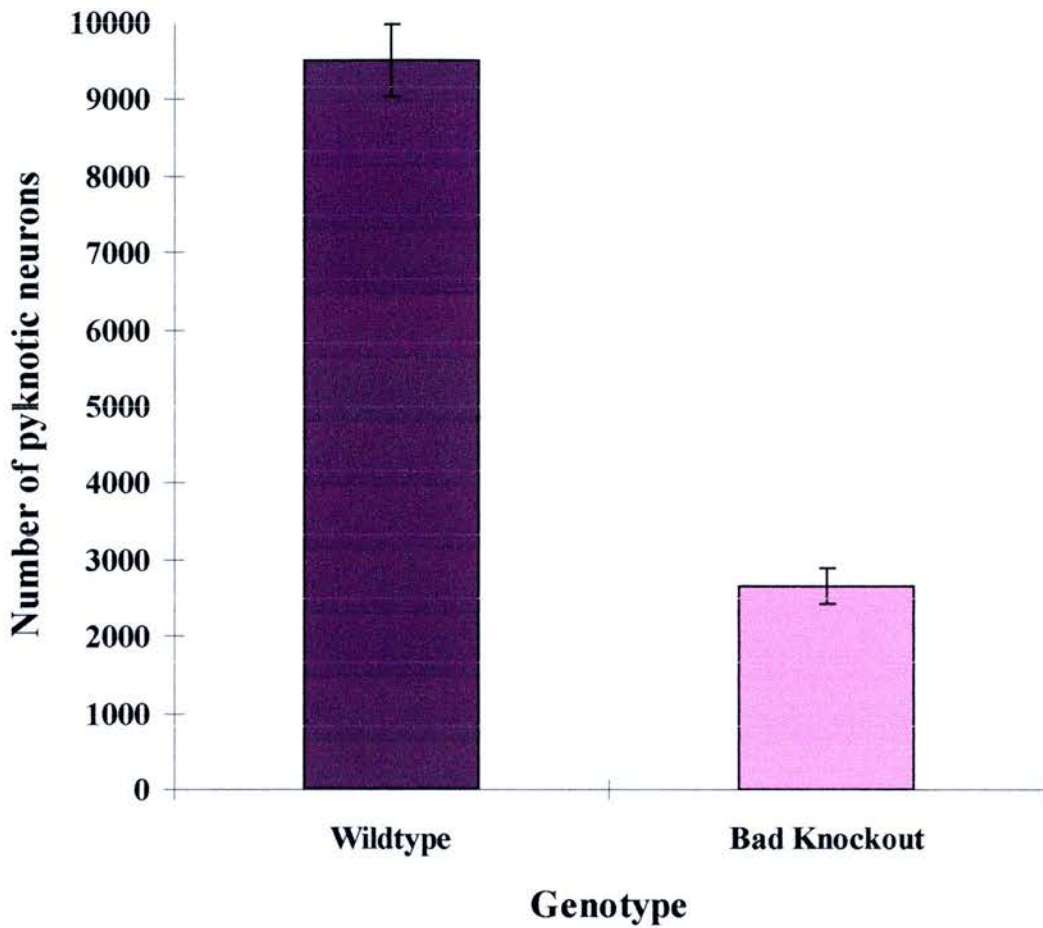
At P1 there were significantly more neurons in the SCG (32% [ $p < 0.01$ , unpaired t-test]) and nodose (64% [ $p < 0.005$ , unpaired t-test]), but not in the trigeminal ganglia (11% [non-significant - unpaired t-test]) of *bad*<sup>-/-</sup> mice as compared to their wildtype littermates (figures 6.13, 6.15, 6.17 and table 6.2). As a reflection of this there are also significantly fewer dying neurons in Bad deficient SCG (72% [ $p < 0.0001$ , unpaired t-test]), nodose (67% [ $p < 0.01$ , unpaired t-test]) and trigeminal ganglia at P1 (73% [ $p < 0.001$ , unpaired t-test]) (figures 6.14, 6.16, 6.18 and table 6.2).

### P1 SCG: Total Neurons



**Figure 6.13:** Bar chart showing the total number of neurons in the SCG of P1 wildtype and Bad deficient mice. The means and standard errors for both ganglia from 3 wildtype and 3 knockout embryos are shown. The numbers of neurons in the SCG were increased by 32% in Bad deficient embryos compared to their wildtype counterparts.

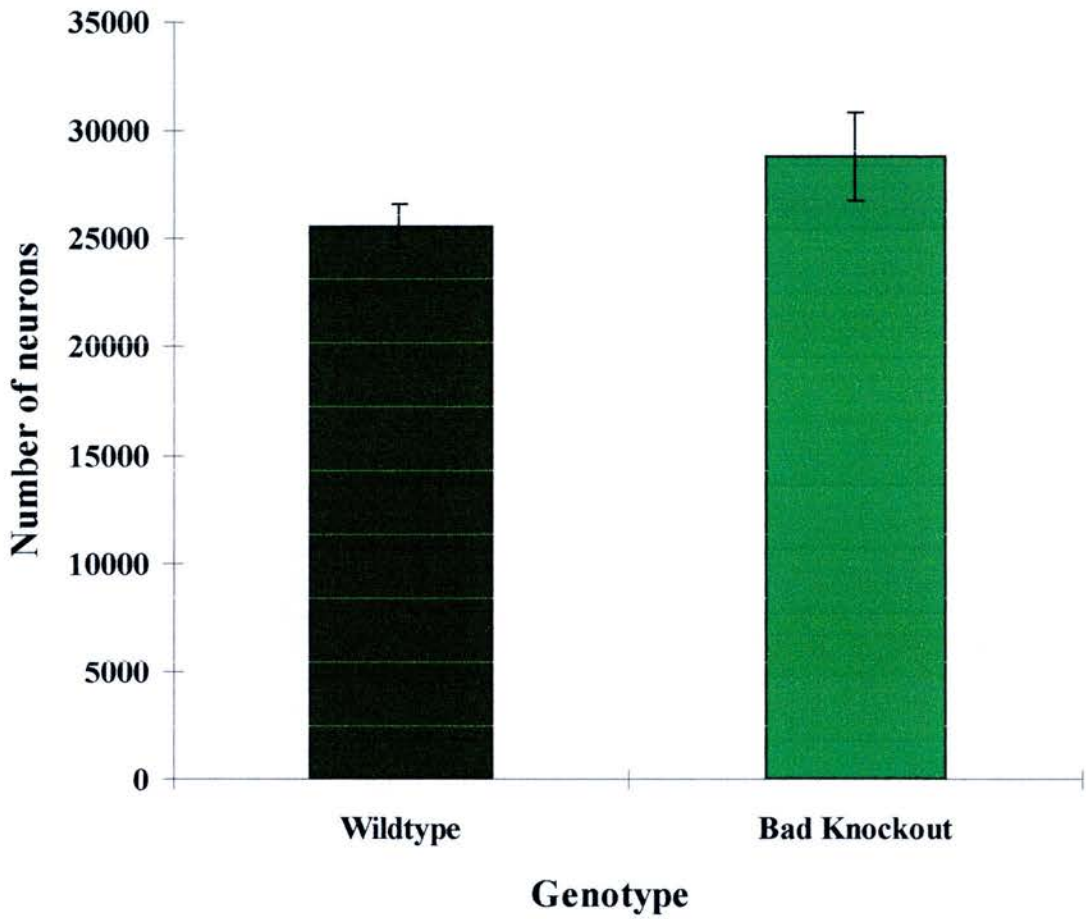
### P1 SCG: Pyknotic Neurons



**Figure 6.14: Bar chart showing the number of pyknotic neurons in the SCG of P1 wildtype and Bad deficient mice.** The means and standard errors for both ganglia from 3 wildtype and 3 knockout embryos are shown. The numbers of pyknotic neurons, and therefore cell death, in the SCG were decreased by 72% in Bad deficient embryos compared to their wildtype counterparts.

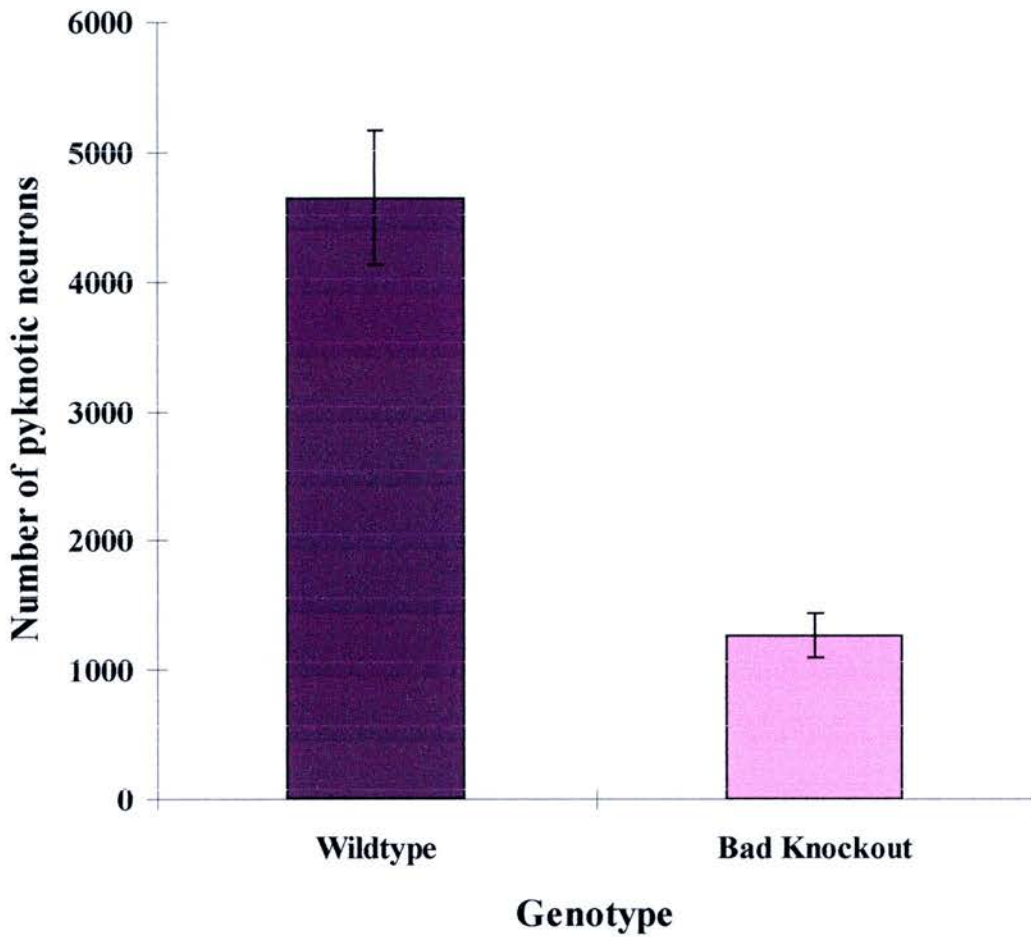


## P1 Trigeminal: Total Neurons



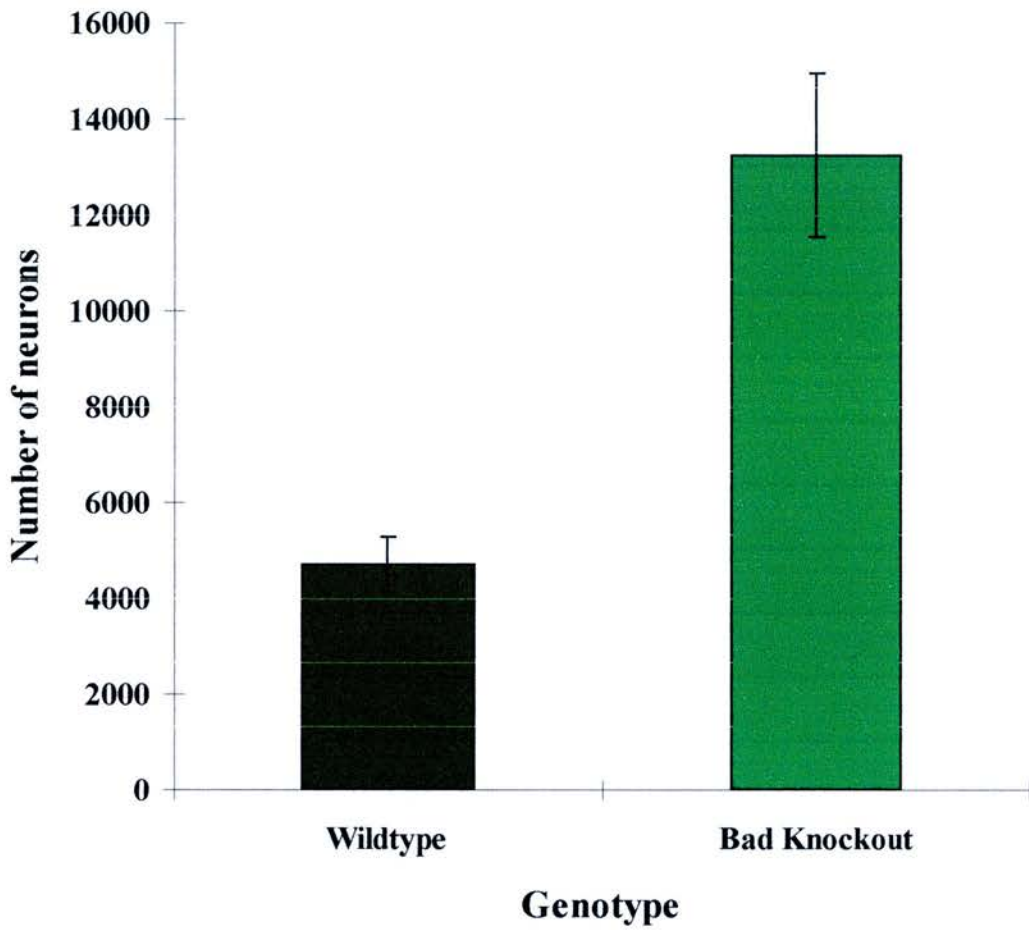
**Figure 6.15: Bar chart showing the total number of neurons in the trigeminal ganglia of P1 wildtype and Bad deficient mice.** The means and standard errors for both ganglia from 3 wildtype and 3 knockout embryos are shown. The numbers of neurons in the trigeminal ganglia were increased by 11% in Bad deficient embryos compared to their wildtype counterparts.

## P1 Trigeminal: Pyknotic Neurons



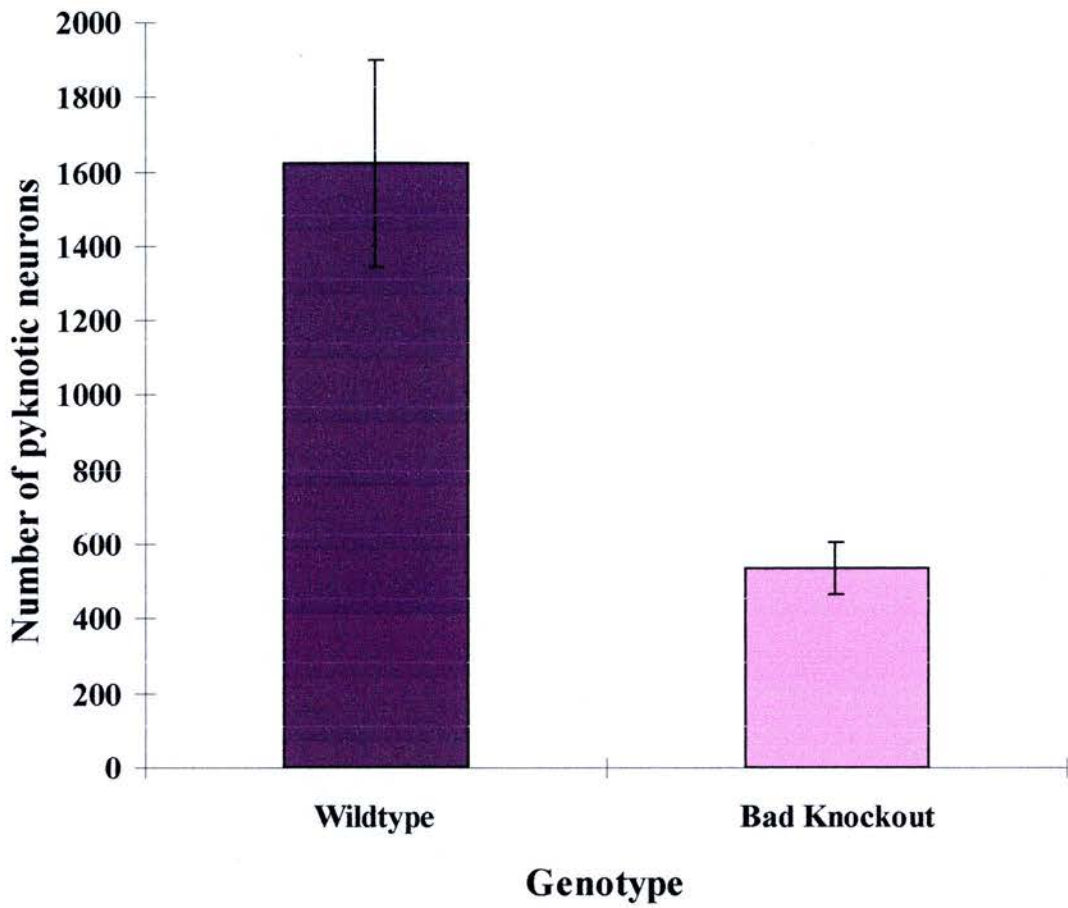
**Figure 6.16: Bar chart showing the number of pyknotic neurons in the trigeminal ganglia of P1 wildtype and Bad deficient mice.** The means and standard errors for both ganglia from 3 wildtype and 3 knockout embryos are shown. The numbers of pyknotic neurons, and therefore cell death, in the trigeminal ganglia were decreased by 73% in Bad deficient embryos compared to their wildtype counterparts.

### P1 Nodose: Total Neurons



**Figure 6.17: Bar chart showing the total number of neurons in the nodose ganglia of P1 wildtype and Bad deficient mice.** The means and standard errors for both ganglia from 3 wildtype and 3 knockout embryos are shown. The numbers of neurons in the nodose ganglia were increased by 64% in Bad deficient embryos compared to their wildtype counterparts.

## P1 Nodose: Pyknotic Neurons



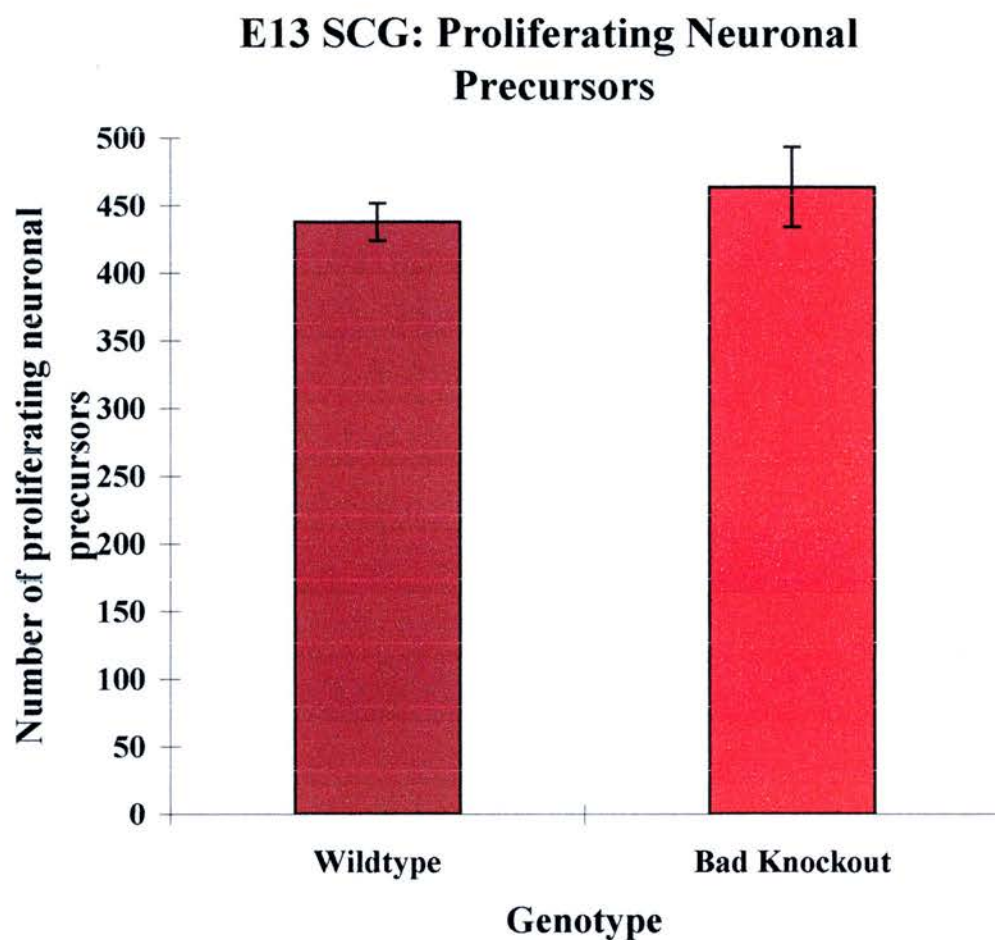
**Figure 6.18: Bar chart showing the number of pyknotic neurons in the nodose ganglia of P1 wildtype and Bad deficient mice.** The means and standard errors for both ganglia from 3 wildtype and 3 knockout embryos are shown. The numbers of pyknotic neurons, and therefore cell death, in the nodose ganglia were decreased by 67% in Bad deficient embryos compared to their wildtype counterparts.

### **6.2.3 Bad Deficiency Enhances Cell Proliferation in the Nodose Ganglion but not the Trigeminal Ganglion or SCG**

One possible explanation for the enhanced number of neurons in Bad deficient peripheral ganglia is that there is enhanced proliferation of neuroblasts in these mice. This has previously been observed in the cranial sensory ganglia of Bax deficient animals (Gayle Middleton, personal communication). In order to test this hypothesis I used digital stereology to determine the number of proliferating cell nuclear antigen (PCNA) stained and thus, dividing cells in the SCG, trigeminal and nodose ganglia at E13.

The results show that there is no significant difference between wildtype and Bad deficient animals for the NGF-dependent neurons of the SCG, trigeminal and nodose ganglia (non-significant, t-test) (figures 6.19, 6.20 and 6.21 and table 6.2). This suggests that enhanced neuroblast proliferation is probably not responsible for the increase in neurons in Bad deficient SCG, trigeminal and nodose ganglia as the increase in proliferating cell number was found not to be statistically significant. It should be noted that the use of PCNA staining does not give any indication of changes in the rate of cell cycling, which may be affecting neuronal numbers in Bad deficient embryos.

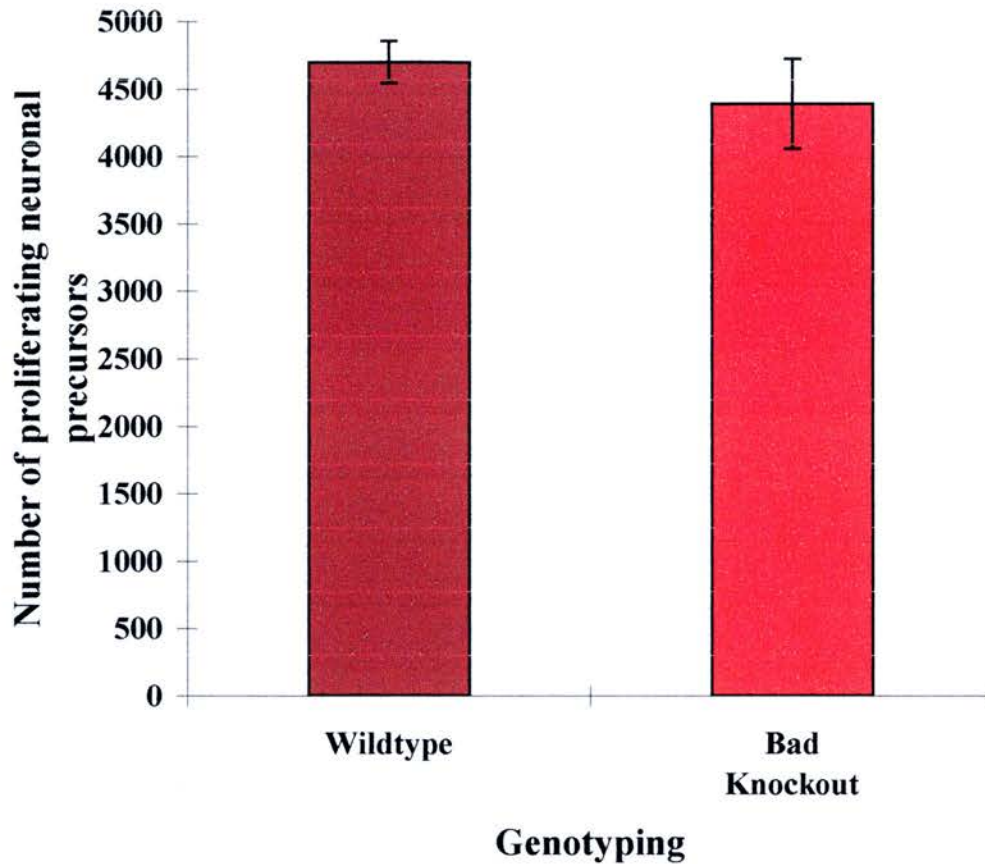




**Figure 6.19:** Bar chart showing the number of proliferating neurons in the SCG of E13 wildtype and Bad deficient mice. The means and standard errors of both ganglia in three embryos of each genotype are shown. There was no significant difference between the numbers of PCNA staining, and therefore proliferating neuroblasts, in the SCG of wildtype and Bad deficient embryos.

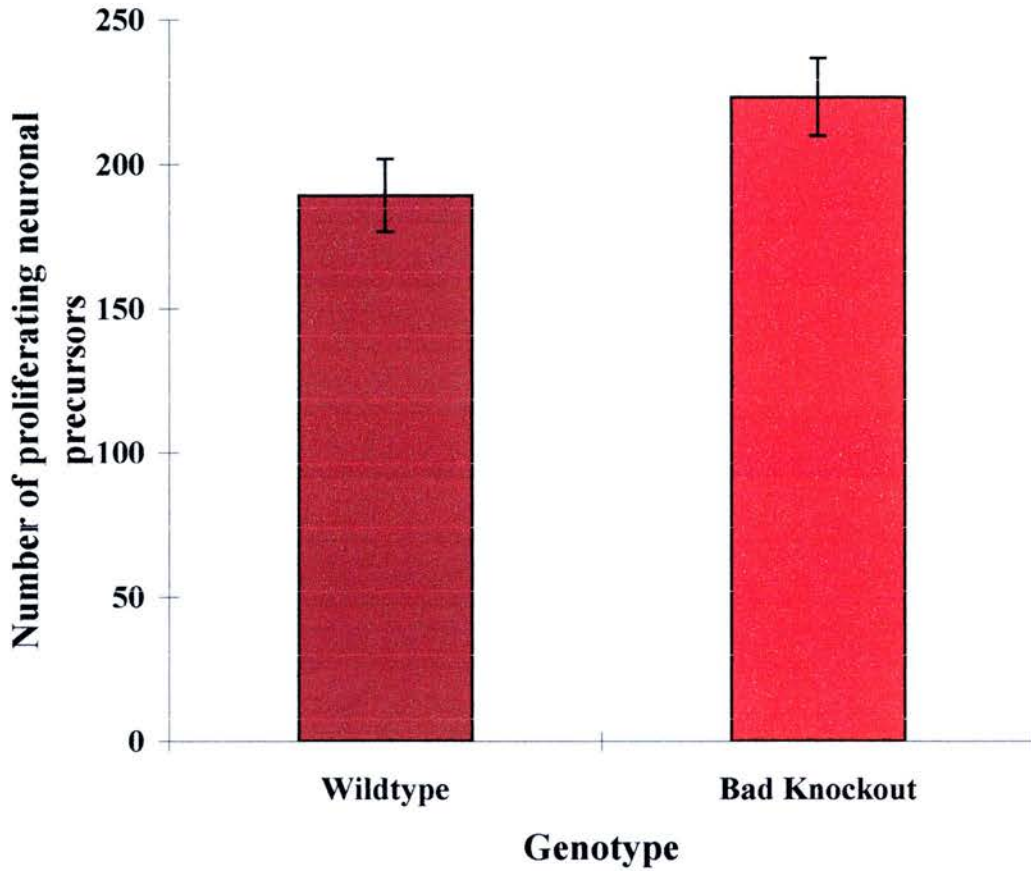


## E13 Trigeminal: Proliferating Neuronal Precursors



**Figure 6.20:** Bar chart showing the number of proliferating neurons in the trigeminal ganglia of E13 wildtype and Bad deficient mice. The means and standard errors of both ganglia in three embryos of each genotype are shown. There was no significant difference between the numbers of PCNA staining, and therefore proliferating neuroblasts, in the trigeminal ganglia of wildtype and Bad deficient embryos.

### E13 Nodose: Proliferating Neuronal Precursors



**Figure 6.21: Bar chart showing the number of proliferating neurons in the nodose ganglia of E13 wildtype and Bad deficient mice.** The means and standard errors of both ganglia in three embryos of each genotype are shown. There was no significant difference between the numbers of PCNA staining, and therefore proliferating neuroblasts, in the nodose ganglia of wildtype and Bad deficient embryos.

<b>E14</b>		<b>Neurons</b>		
<b>Ganglia</b>	<b>Wildtype</b>	<b>Std Error</b>	<b>Bad-/-</b>	<b>Std Error</b>
<b>SCG</b>	9422.8	572.13	14159	550.14
<b>Trigeminal</b>	35966.7	1245.04	40604.5	465.96
<b>Nodose</b>	3449.52	366.65	15368.17	668.8
		<b>Pyknotic Neurons</b>		
<b>Ganglia</b>	<b>Wildtype</b>	<b>Std Error</b>	<b>Bad-/-</b>	<b>Std Error</b>
<b>SCG</b>	346.16	41.12	240.85	12.3
<b>Trigeminal</b>	6720.4	975.8	2503.49	157.09
<b>Nodose</b>	575.29	68.17	303.65	76.11
<b>P1</b>		<b>Neurons</b>		
<b>Ganglia</b>	<b>Wildtype</b>	<b>Std Error</b>	<b>Bad-/-</b>	<b>Std Error</b>
<b>SCG</b>	31786.2	3136.8	47043.05	2621.68
<b>Trigeminal</b>	25576.8	979	28829.3	2028.5
<b>Nodose</b>	4718.65	585.46	13255.23	1711.3
		<b>Pyknotic Neurons</b>		
<b>Ganglia</b>	<b>Wildtype</b>	<b>Std Error</b>	<b>Bad-/-</b>	<b>Std Error</b>
<b>SCG</b>	9508.36	477.07	2648.34	238.49
<b>Trigeminal</b>	4647.9	512.34	1261.08	172.5
<b>Nodose</b>	1623.0	274.59	536.75	70.27
<b>E13</b>		<b>Proliferating Neuroblasts</b>		
<b>Ganglia</b>	<b>Wildtype</b>	<b>Std Error</b>	<b>Bad-/-</b>	<b>Std Error</b>
<b>SCG</b>	438.3	13.7	463.77	29.64
<b>Trigeminal</b>	4699.7	155.85	4392	331.5
<b>Nodose</b>	189.4	12.6	223.5	13.4

**Table 6.2: Summary of *in vivo* results from wildtype and Bad deficient embryos.**

The means and standard errors for counts of neurons and pyknotic neurons in the SCG, trigeminal and nodose ganglia of wildtype and Bad deficient embryos at E14 and P1 are shown. The means and standard errors for counts of PCNA stained cells (proliferating neuroblasts) in the SCG, trigeminal and nodose ganglia of wildtype and Bad deficient embryos at E13 are shown. The numbers of neurons are increased and the levels of pyknotic neurons are decreased in embryos deficient in Bad as compared to wildtype embryos at both E14 and P1. There was no significant difference between the numbers of PCNA staining, and therefore proliferating neuroblasts, in the SCG, trigeminal and nodose ganglia of wildtype and Bad deficient embryos.



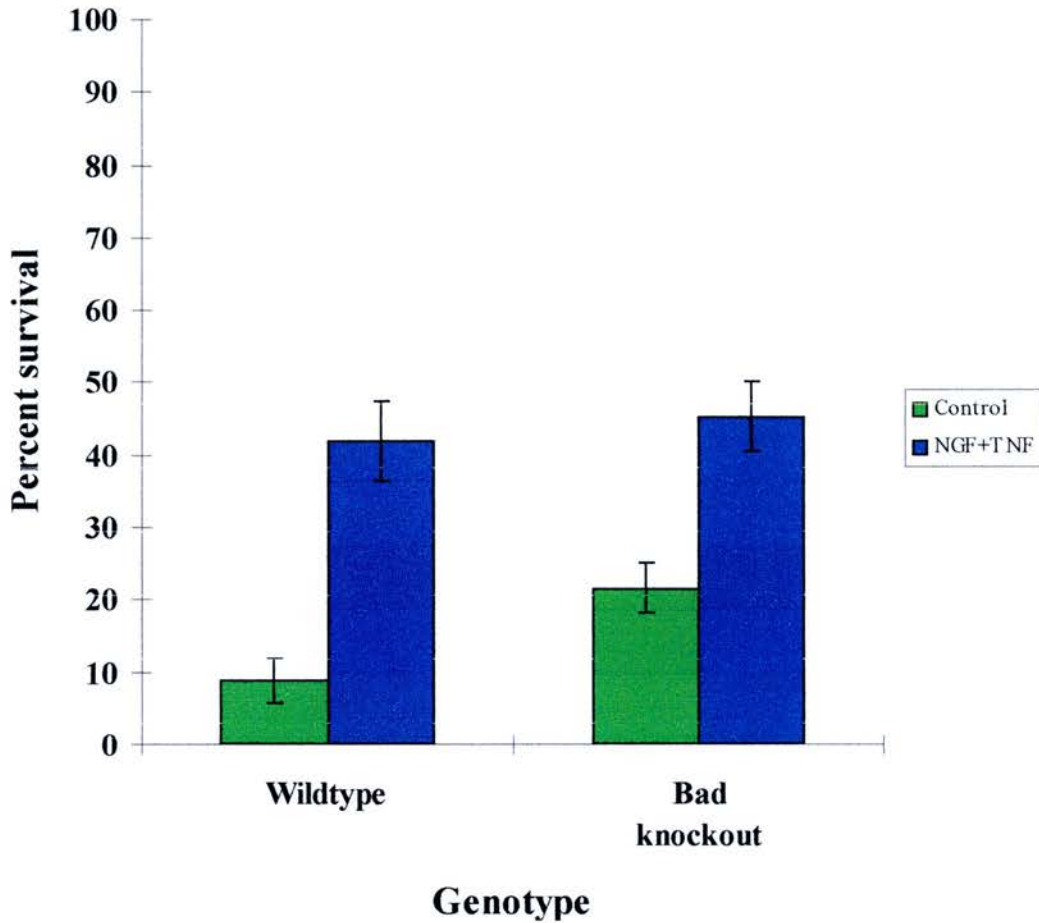
#### 6.2.4 Bad is not Downstream of TNF $\alpha$ in Mediating Apoptosis

In order to determine whether Bad is mediating cell death in response to TNF $\alpha$  signalling I examined the effect of saturating concentrations of TNF $\alpha$  on the neurotrophin response of wildtype and Bad deficient litter-mates at P1. Low density dissociated cultures of SCG, trigeminal and nodose ganglion neurons were set up. Neurons were grown in chemically defined serum-free medium with either neurotrophic support plus TNF $\alpha$  or without reagents. The survival of the neurons was assessed after 48 hours incubation and expressed relative to the number of neurons attached 6 hours after plating.

If TNF $\alpha$  were acting via Bad to induce cell death it would be expected that cell death in response to TNF $\alpha$  would be abrogated in neuronal cultures from Bad deficient mice. This was not the case. There was no significant difference between percent survival of wildtype and Bad deficient neurons of the SCG, trigeminal or nodose ganglia when cultured with saturating levels of TNF $\alpha$  (SCG, nodose and trigeminal - non-significant, unpaired t-test) (figures 6.22-6.23). Neurotrophin support maintained survival of these neurons at high levels so that the effects of TNF $\alpha$  could be assessed. The addition of TNF $\alpha$  decreased both wildtype and Bad deficient SCG, trigeminal and nodose neuronal survival to approximately 50% that normally seen in cultures with neurotrophin alone indicating that TNF $\alpha$  was inducing cell death but that this apoptosis is not mediated by Bad.

In control cultures established from neurons from the same wildtype and Bad deficient mice the removal of Bad activity was seen to enhance neuronal survival significantly. The survival of SCG neurons was 13% greater in the *bad* knockout cultures than in the wildtype cultures ( $p < 0.05$ , unpaired t-test) (6.22). Trigeminal neuron survival was increased by 14% ( $p < 0.05$ , unpaired t-test) (6.23) and nodose neurons survival was increased by 11% ( $p < 0.05$ , unpaired t-test) (6.24). This indicates that in the wildtype neurons something is inducing Bad mediated apoptosis but that it is not  $\text{TNF}\alpha$  signalling.

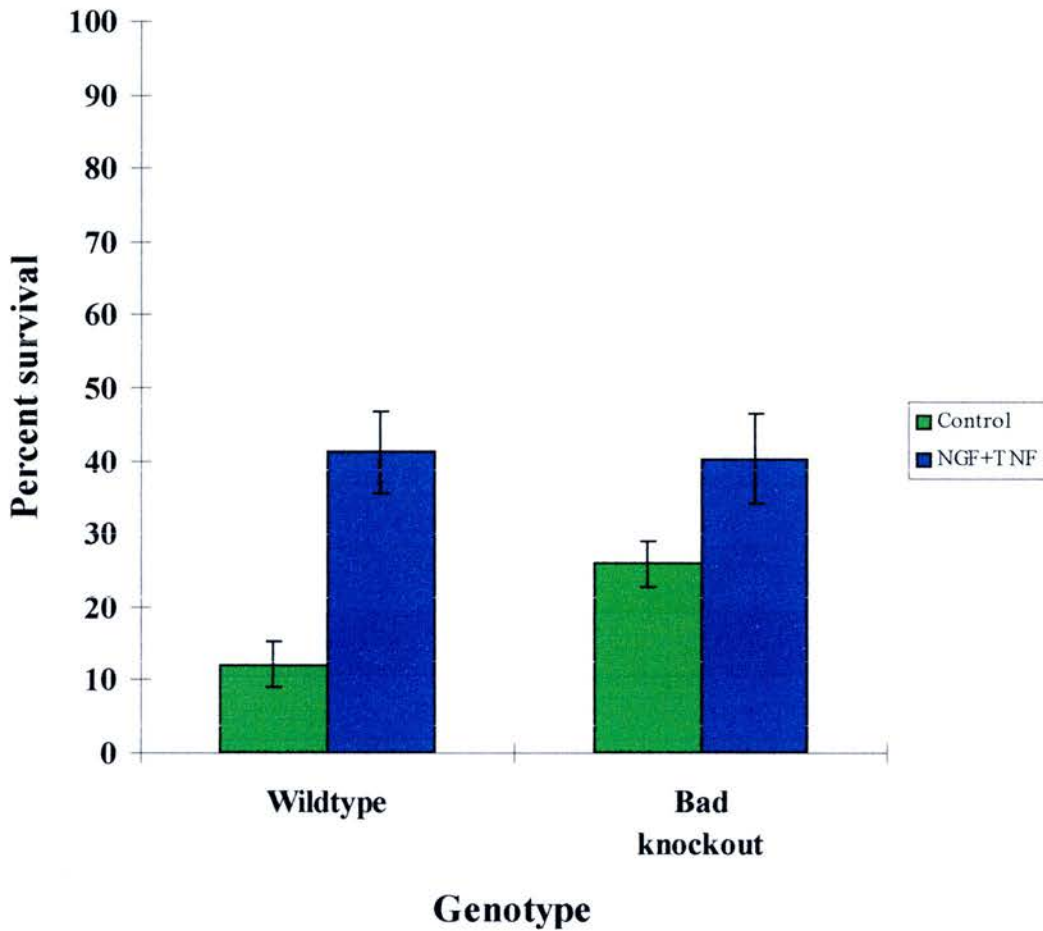
## P1 SCG Neurons



**Figure 6.22: Bar chart showing the percent survival of P1 wildtype and Bad deficient SCG neurons.** Neurons were grown in vitro for 48 hours either with neurotrophic support plus  $\text{TNF}\alpha$  or without the addition of reagents. The means and standard errors of three separate experiments are shown. The addition of  $\text{TNF}\alpha$  decreased both wildtype and Bad deficient SCG neuronal survival to approximately 50% that normally seen in cultures with neurotrophin alone indicating that  $\text{TNF}\alpha$  was inducing cell death but that this apoptosis is not mediated by Bad. The survival of SCG neurons in control cultures was 13% greater in those from *bad* knockout embryos than from their wildtype counterparts.

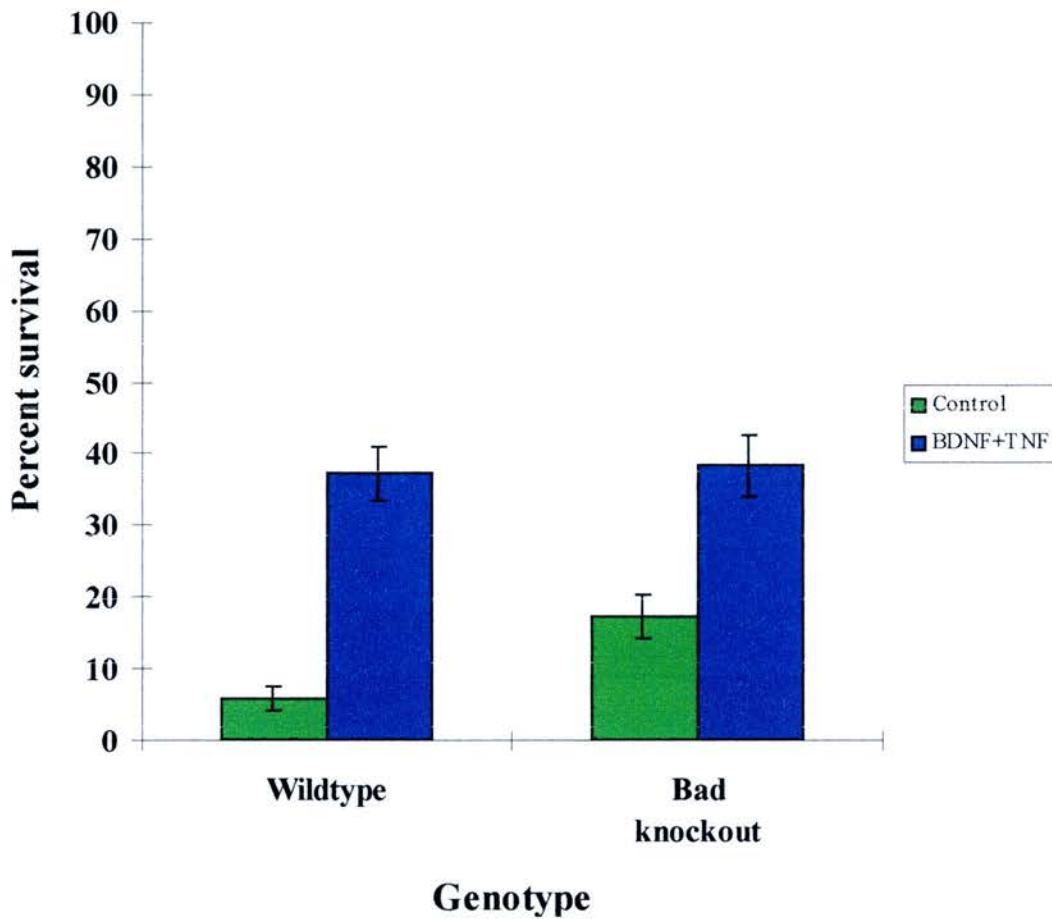


## P1 Trigeminal Neurons



**Figure 6.23: Bar chart showing the percent survival of P1 wildtype and Bad deficient trigeminal ganglion neurons.** Neurons were grown in vitro for 48 hours either with neurotrophic support plus  $\text{TNF}\alpha$  or without the addition of reagents. The means and standard errors of 3 separate experiments are shown. The addition of  $\text{TNF}\alpha$  decreased both wildtype and Bad deficient SCG neuronal survival to approximately 50% that normally seen in cultures with neurotrophin alone indicating that  $\text{TNF}\alpha$  was inducing cell death but that this apoptosis is not mediated by Bad. In control cultures trigeminal neuron survival was increased by 14% in those from *bad* knockout embryos than from their wildtype counterparts.

## P1 Nodose Neurons



**Figure 6.24: Bar chart showing the percent survival of P1 wildtype and Bad deficient nodose ganglion neurons.** Neurons were grown in vitro for 48 hours either with neurotrophic support plus  $\text{TNF}\alpha$  or without the addition of reagents. The means and standard errors of 3 separate experiments are shown. The addition of  $\text{TNF}\alpha$  decreased both wildtype and Bad deficient SCG neuronal survival to approximately 50% that normally seen in cultures with neurotrophin alone indicating that  $\text{TNF}\alpha$  was inducing cell death but that this apoptosis is not mediated by Bad. In control cultures nodose neurons survival was increased by 11% in those from *bad* knockout embryos than from their wildtype counterparts.

## 6.3 Discussion

I have shown that the pro-apoptotic protein Bad is involved in bringing about neuronal cell death of peripheral neurons during development and following neurotrophic factor deprivation. Over-expression of Bad in cultured SCG, trigeminal and nodose ganglion neurons reduces their survival when grown with neurotrophins suggesting that Bad is a potent killer of neurons and that its effects are dominant to the survival promoting effects of neurotrophic factors. SCG, trigeminal and nodose neurons were microinjected with Bad expression vector. This over-expression of Bad significantly reduced the survival of SCG, trigeminal and nodose ganglion neurons as compared to neurons injected with an empty expression plasmid or uninjected control cells. In the case of the sensory neurons of the trigeminal ganglion only 44% of Bad injected neurons survived for 48 hours after DNA microinjection as compared to 88% survival in the control neurons. In the nodose sensory neurons, 58% of neurons were surviving 48 hours after Bad over-expression as compared to 85% of control neurons and in sympathetic SCG neurons 34% of neurons survived for 48 hours after Bad injection as compared to 89% of control cells (work done by Gayle Middleton). These data clearly show that Bad over-expression brings about the death of cranial sensory and sympathetic neurons and that the effects of Bad over-expression are dominant to the survival-promoting effects of neurotrophins.

Accordingly, Bad deficient neurons grown in culture without added neurotrophic support show enhanced survival as compared to wild type neurons (see figures 6.1-6.6) and there are increased numbers of neurons and decreased numbers of dying cells in the ganglia of Bad deficient mice *in vivo* (see figures 6.7-6.18). Taken together these data suggest that Bad can bring about the death of cranial sensory and sympathetic neurons and that it plays a role in the execution of apoptosis during neuronal development. It should be noted that the increases in the levels of trigeminal neurons at both E14 and P1 in Bad deficient compared to wildtype embryos are small relative to the decreases in the levels of pyknotic neurons in this region. This may be due to a reduction in clearance of pyknotic neurons and will need to be investigated in future work.

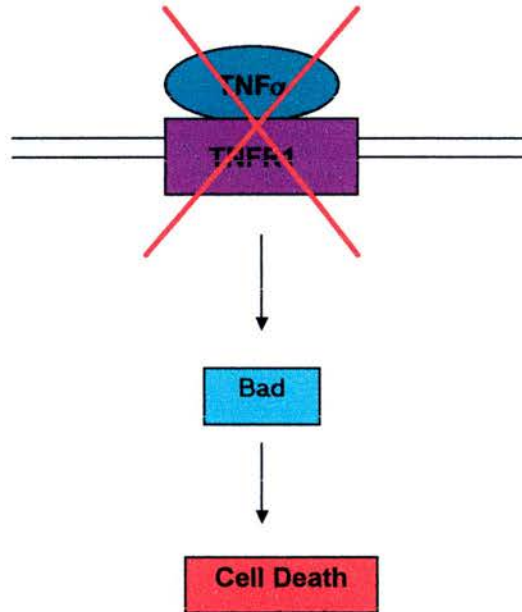
There was no enhanced proliferation of Bad deficient SCG, trigeminal or nodose ganglion neurons at E13 (see figures 6.19, 6.20 and 6.21). This indicates that the increased survival seen in Bad deficient SCG, trigeminal and nodose neurons is due to the removal of a cytotoxic effect of Bad expression rather than the removal of inhibition of neuronal precursor proliferation or increased proliferation. It should be noted that PCNA staining only determines that number of cells that are cycling but not the cycling rates of these cells. Thus it is possible that cell cycle rate may be affected in the *bad* knockout mice leading to increased production of neurons in the SCG, trigeminal and nodose ganglia.

The discovery of a phenotype in the nervous system of the Bad deficient animals argues for specificity of function amongst the BH3-only members of the Bcl-2 protein family. It is possible that Bad acts in concert with Bax to produce the apoptosis found in the peripheral nervous system during the period of programmed cell death. The incomplete removal of apoptosis seen in the SCG (Deckwerth et al., 1998), nodose and trigeminal neurons (Middleton et al., 2000) of Bax deficient mice embryos would indicate that a further apoptotic pathway is in operation. It is highly possible that this pathway is regulated by Bad. Also, in the Bad deficient mice apoptosis was reduced but not totally removed supporting the idea that Bad and Bax work in concert to bring about programmed cell death during development. Alternatively there may be a level of redundancy (see section 2.2.1b) between Bad and Bax. Future work should examine the extent of apoptosis in sympathetic and sensory ganglia of *bad/bax* double knockout mice during the period of developmental cell death.

In summary, the absence of Bad enhanced cell survival *in vitro* in sympathetic and sensory neurons without neurotrophic support as compared to wildtype neurons. *In vivo* there is an enhanced number of neurons in the SCG, trigeminal and nodose ganglia and a decrease in the number of dying cells during the peak of naturally occurring cell death. It has been shown by the over-expression of Bad in cultures of primary neurons derived from cranial sensory and sympathetic ganglia that Bad is able to kill these neurons even in the presence of saturating levels of neurotrophic support (work done by Gayle Middleton). Although these data propose a role for Bad in regulating neuronal survival



during the period of naturally occurring neuronal cell death *in vivo*, the actions of Bad during developmental cell death were found not to be downstream of TNF $\alpha$  induced cell death during this period (see figure 6.25). The generality of this finding and its relevance to other neuronal populations remains to be elucidated.



**Figure 6.25: Summary diagram of the findings in chapter 5.** The findings of this study suggest that the pro-apoptotic Bcl-2 protein Bad is involved in mediating the apoptosis of developing SCG, nodose and trigeminal neurons during development. This cell death is not stimulated by TNF $\alpha$  binding to TNFR1.

Future work involving the examination of the expression of Bad mRNA and protein following neurotrophin deprivation will be important. This will clarify the role of Bad in developmental cell death as it is uncertain as of yet what the stimulus for Bad-mediated apoptosis during the development of the peripheral nervous system is. This may then lead to an initial idea of which pathway might result in Bad-mediated cell death. Supplementary to this a study of the effects of Bad-mediated cell death in mice with null mutations in *trk* and *p75* receptors will also be relevant, as it will further elucidate whether the elimination of neurotrophin activity effects the induction of this cell death.



## Chapter 7: Final Discussion

### 7.1 Summary of Findings

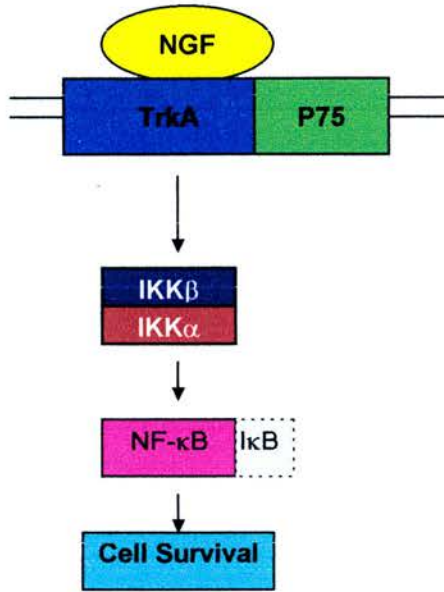
Firstly, a novel role for NF- $\kappa$ B and the IKK $\alpha$  and IKK $\beta$  subunits of the IKK complex in bringing about cell death in the developing retina and spinal cord has been demonstrated. It was also concluded that the NF- $\kappa$ B death signalling pathway is not downstream of NGF binding to p75 or TNF $\alpha$ . NGF does have an apoptotic effect in these neurons but it appears to be mediated by an alternative pathway. I have found that NF- $\kappa$ B activity also plays an important role in the mediation of the cell survival response to NGF in SCG sympathetic neurons. This suggests that NF- $\kappa$ B has dual actions during development. It mediates apoptosis in neurons that do not express TrkA and cell survival in neurons that do.

Secondly, I have found that at E14 TNF $\alpha$  is also involved in enhancing the survival of sensory and sympathetic neurons, whereas at a later stage (E16) it has been shown to have a cytotoxic effect on these same cells. The data presented here give the first demonstration of TNF $\alpha$  switching from a neuroprotective to a cytotoxic function within the same populations of cells over a defined period in embryonic development. The reasons for this switch are unclear and will need to be elucidated in future work. It is widely postulated that TNF $\alpha$  signals via FADD and TRADD to activate caspases and

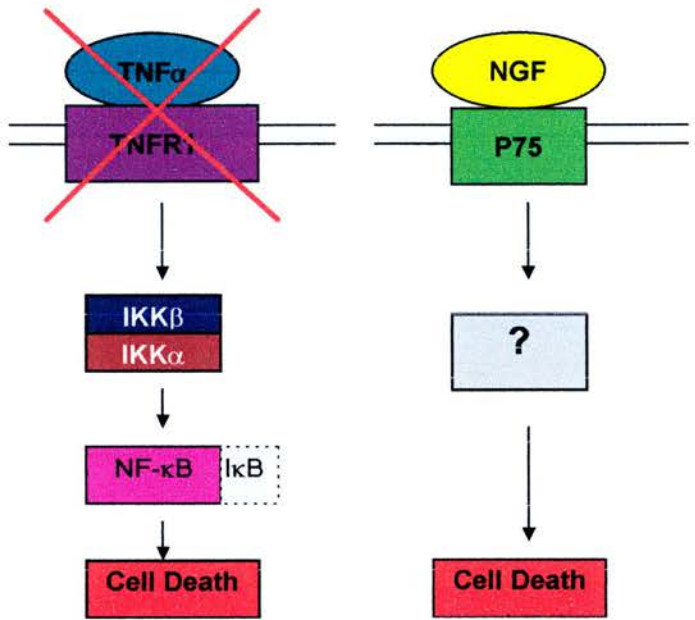
bring about neuronal death and by a pathway leading to NF- $\kappa$ B activation to bring about cell survival (Baud and Karin, 2001). The findings that p65 acts downstream from TNF $\alpha$  induced cell survival but not in TNF $\alpha$  induced cell death supports this theory. These findings raise many questions about how neuronal survival is controlled during embryonic development.

Finally, I have demonstrated for the first time that the elimination of Bad reduces the magnitude of cell death both *in vitro* and *in vivo*. Thus, Bad can bring about the death of cranial sensory and sympathetic neurons and it plays a role in the execution of apoptosis during neuronal development. The generality of this finding and its relevance to other neuronal populations remains to be elucidated. It was found that Bad did not act downstream of TNF $\alpha$  in mediating cell death, indicating that a further pathway is active in developmental cell death in response to TNF $\alpha$ . Although it is beyond the scope of this thesis, it would be of relevance to examine whether Bad is involved in mediating the apoptotic response to NGF or in the NF- $\kappa$ B mediated cell death pathway in cells of the spinal cord and retina.

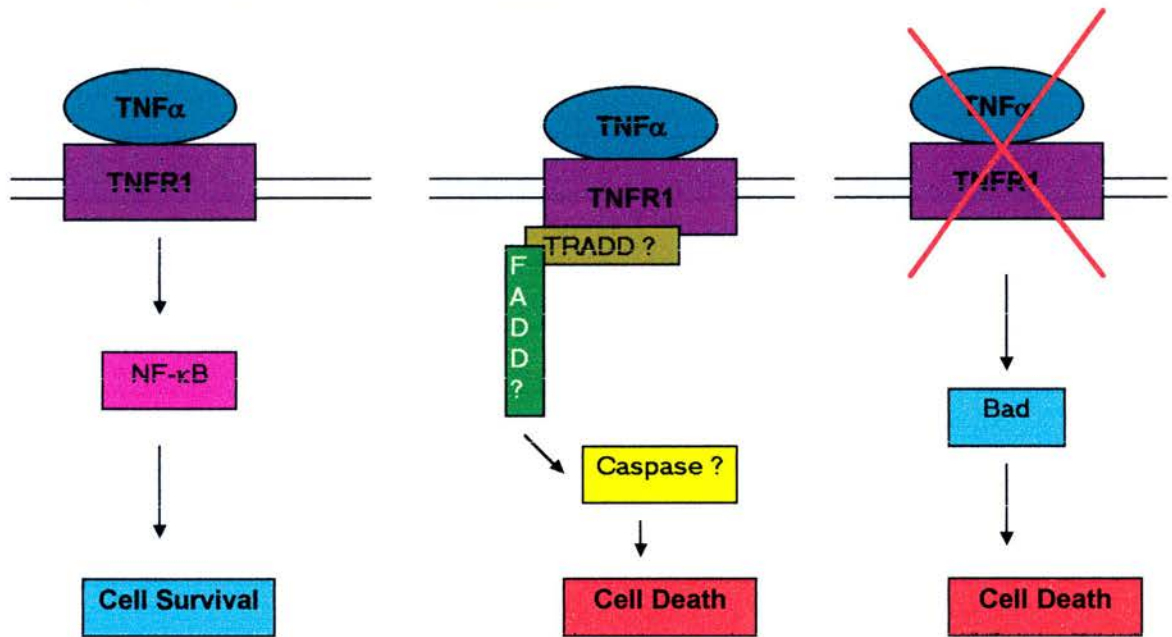
SCG:



Spinal Cord and Retina:



SCG, Trigeminal and Nodose Ganglia:



**Figure 7.1: Summary of areas of the developmental cell death and survival pathways in the nervous system elucidated in this thesis.** The binding of NGF to p75 leads to NF-κB mediated survival of SCG neurons. In the retina and spinal cord NGF binding to p75 results in apoptosis, but this is not mediated by NF-κB, although NF-κB is involved in mediating cell death in these neurons. Early in development TNFα binding to TNFR1 results in NF-κB mediated survival of SCG, trigeminal and nodose neurons. At later stages this interaction results in the induction of a cell death signal, which is not mediated by either NF-κB or Bad, although Bad is involved in mediating apoptosis in these neurons during development.

A number of questions arise from the findings of this thesis, which need to be answered if we are to fully elucidate the pathways involved in developmental cell death and survival. Firstly, what is the pathway that is initiated by NGF and leads to apoptosis in neurons of the spinal cord and retina? Secondly, what extracellular event induces NF- $\kappa$ B mediation of cell death in these same neurons? Thirdly, is the TRADD-FADD-caspase pathway involved in cell death signalling in response to TNF $\alpha$  in SCG, trigeminal and nodose ganglia in the late developmental stages? And finally, what events both intracellular and extracellular lead to apoptosis mediated by Bad? These questions need to be examined in future research.

## 7.2 Reasons for Examining Neuronal Death During Development

Programmed cell death involves the activation of intrinsic cellular pathways for example the NF- $\kappa$ B activation pathway and the expression of certain molecules such as NGF, TNF $\alpha$ , members of the Bcl-2 family or the caspases. The disruption of these pathways and the presence or over-expression of these molecules in neuronal tissue may have pathological consequences. Indeed, abnormal development may predispose individuals to a number of neuropsychiatric and behavioural disorders. It has been suggested that a decrease in developmental cell death can lead to abnormal adult behaviours (Rondi-Reig and Mariani, 2002). In humans, perturbations in developmental neuronal apoptosis leading to an excess of neurons could also be associated with neuro-developmental psychiatric disorders (Margolis et al., 1994). This has been proposed to occur in the neurodevelopmental group of psychiatric disorders. For example, neuronal deficits have been found in multiple areas of the brain of schizophrenic patients including the cerebral cortex (Benes et al., 1986,1991; Falkai et al., 1988; Jacob and Beckman, 1986; Raedler et al., 1998), the hippocampus (Falkai and Bogerts, 1986; Jeste and Lohr, 1989) and in the mediodorsal thalamic nuclei and nucleus accumbens (Pakkenberg, 1990). Abnormal neuronal distribution has been found in the prefrontal cortex and medial lobes of schizophrenics (Akbarian et al., 1993a, 1993b; Bloom, 1993), which may be the result of abnormal neuronal death. In certain limbic regions and the cerebella of autistic individuals abnormal numbers of neurons have been seen (Bauman, 1991; Courchesne



et al., 1994) and also there has been shown to be considerable neuronal disorganisation in the superficial layers of the entorhinal region in individuals with bipolar affective disorder, together with a deficit in neuronal number in a part of the rostral-ventral insula (Beckmann and Jakob, 1991). The behavioural study of genetically modified mice that present a blockade in developmental death, perhaps restricted to a particular area of the brain, could help bring us to a better understanding of the genetic onset of some of the neuro-developmental psychiatric disorders. But, in order to do this we must fully understand the mechanisms and the pathways involved in developmental cell death, together with the factors that stimulate it. For example, reduction of the Bcl-2 protein and Purkinje cell depletion has been demonstrated in the cerebellum of autistic subjects. This study indicated that the autistic cerebellum may be vulnerable to pro-apoptotic stimuli and to neuronal atrophy as a consequence of decreased Bcl-2 levels (Fatemi et al., 2000).

At the other end of the spectrum TNF $\alpha$  has been implicated in a number of neurodegenerative conditions. An example of this is multiple sclerosis, in which there are increased levels of TNF $\alpha$  mRNA in the lesions (Bitsch et al., 2000) and of TNF $\alpha$  protein in the sera (Martino et al., 1997). A dysregulation of TNF $\alpha$  in the CNS is indicated in Alzheimer's disease (AD) (Lanzrein et al., 1998) and upregulation (Perry et al 2001) and increased levels of TNF $\alpha$  have been observed in the CNS of AD patients (Fillit et al., 1991). TNF $\alpha$  is an obligatory component of dopaminergic neurodegeneration and its expression has been found to be enhanced in the substantia



nigra of patients with Parkinson's disease (PD) (Sriram et al., 2002). One hypothesis of the cause of degeneration of the nigrostriatal dopaminergic neurons in PD is that apoptosis results from increased levels of cytokines and/or decreased levels of neurotrophins. Markedly increased levels of TNF $\alpha$  and decreased levels of NGF and BDNF have been observed in the nigrostriatal dopaminergic regions and ventricular and lumbar cerebrospinal fluid of PD patients (Nagatsu et al., 2000). Furthermore, the levels of TNFR1, Bcl-2, Fas, and the activities of caspase-1 and caspase-3 were also elevated in the nigrostriatal dopaminergic regions in PD (Nagatsu et al., 2000). This indicates that the presence of such pro-apoptotic environment in the substantia nigra in PD may induce increased vulnerability of neuronal cells towards a variety of neurotoxic factors. Genetic work has recently verified that the TNF $\alpha$  pathway contributes to the pathogenesis of sporadic PD. TNF $\alpha$  signalling activates NF- $\kappa$ B, which has also been found to be activated in affected neurons in PD (Wintermeyer et al., 2002).

Immunohistochemical analysis has revealed that the nuclear p65 immunoreactivity in the dopaminergic neurons in the substantia nigra of brain sections from PD patients was 70-fold greater than that in age-matched controls (Hugnot et al., 1997). Studies of post-mortem brain tissue from patients with AD have also revealed increased NF- $\kappa$ B activity in cells involved in the neurodegenerative process. p65 immunoreactivity increases in neurons and astrocytes in the immediate vicinity of amyloid plaques in brain sections from AD patients, consistent with NF- $\kappa$ B activation in those cells (O'Neill and Kaltschmidt, 1997). Other studies have shown that amyloid  $\beta$ -peptide can activate NF-

$\kappa$ B in cultured neurons, suggesting a molecular mechanism by which amyloid may act during AD pathogenesis (Barger et al., 1995). The increased NF- $\kappa$ B activity in the affected neurons may represent an early protective response to ongoing oxidative stress and mitochondrial dysfunction implicated in the pathogenesis of AD and PD. Although activated NF- $\kappa$ B has been demonstrated in AD brain, there was also activated expression of I $\kappa$ B in a distribution that corresponded to the neurofibrillary pathology of AD (Yoshiyama et al., 2001). These observations indicate that disruption of the autoregulatory mechanism of NF- $\kappa$ B in the brain may play a role in the pathogenesis of AD. These results suggest that enhanced expression of neuronal NF- $\kappa$ B occurs in areas affected by AD pathology (hippocampal formation, entorhinal cortex, middle temporal gyrus and visual cortex)(Terai 1996). In support of this, immunohistochemical studies suggest that levels of NF- $\kappa$ B activity are increased in cholinergic neurons in the basal forebrains of AD patients, the dysfunction and degeneration of which is believed to contribute greatly to cognitive impairment in AD (Boissiere et al., 1997). NF- $\kappa$ B has been implicated in a number of other neurodegenerative diseases. In Huntington's disease (HD), striatal neurons degenerate and NF- $\kappa$ B activity is markedly increased and it has been reported that mice lacking the p50 subunit of NF- $\kappa$ B exhibit increased damage to striatal neurons (Yu et al., 2000), indicating a neuroprotective role for NF- $\kappa$ B. In amyotrophic lateral sclerosis (ALS), spinal cord motor neurons degenerate. The spinal cords of patients with ALS show increased NF- $\kappa$ B activation in astrocytes associated with degenerating motor neurons (Migheli et al., 1997). This may reflect a

similar cytotoxic role for NF- $\kappa$ B to that seen in the development of the spinal cord, as demonstrated in this thesis. It is possible that parallel pathways are activated in both developmental cell death and neurodegeneration.

The observations described above suggest that both TNF $\alpha$  and NF- $\kappa$ B play important roles in cellular responses to injury of the nervous system in neurodegenerative conditions. It is uncertain as of yet whether the increased activity of these proteins represents a pathological process or whether they are protective responses to the pathological changes of these neurodegenerative diseases. The responses in the above neurodegenerative diseases may parallel the neurodevelopmental processes in the peripheral nervous system described in this thesis. Because of this, proteins involved in TNF $\alpha$  and NF- $\kappa$ B signalling are potentially important targets for therapeutic intervention in an array of neurological disorders. The use of such therapeutic intervention in neurodegenerative disorders is complicated by the possibility that inhibition of TNF $\alpha$  or NF- $\kappa$ B in neurons may exacerbate the neurodegenerative process, and by the considerable evidence suggesting that these proteins play critical roles in normal neural functioning which may be compromised by such inhibitors. Thus, it is important to fully understand these proteins and their roles in normal neural functioning, one of which is as regulators of neuronal survival during the development.

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# Appendices

## Appendix 1:

### A. p65 PCR schedule

1. 95°C for 15 minutes
  2. 95°C for 1 minute
  3. 67°C for 45 seconds
  4. 72°C for 90 seconds
  5. 95°C for 1 minute
  6. 59°C for 45 seconds
  7. 72°C for 90 seconds
  8. 72°C for 10 minutes
  9. Soak at 4°C
- × 10
- × 22

### B. IKK $\alpha$ PCR schedule

1. 95°C for 15 minutes
  2. 95°C for 1 minute
  3. 55°C for 1 minute
  4. 72°C for 90 seconds
  5. 72°C for 10 minutes
  6. Soak at 4°C
- × 34

### C. IKK $\beta$ PCR schedule

#### **Wildtype reaction**

1. 95°C for 15 minutes
  2. 95°C for 1 minute
  3. 50°C for 1 minute
  4. 72°C for 150 seconds
  5. 72°C for 10 minutes
  6. Soak at 4°C
- × 33

### D. NGF PCR schedule

1. 94°C for 15 minutes
  2. 94°C for 1 minute
  3. 57°C for 1 minute
  4. 72°C for 90 seconds
  5. 72°C for 10 minutes
  6. Soak at 4°C
- × 36

### E. TNF PCR schedule

1. 95°C for 15 minutes
  2. 95°C for 1 minute
- × 34

3. 60°C for 1 minute
4. 72°C for 2 minutes
5. 72°C for 10 minutes —
6. Soak at 4°C

**F. (i) Bad PCR schedule**

**Wildtype reaction**

1. 94°C for 15 minutes
  2. 94°C for 1 minute
  3. 64°C for 1 minute
  4. 72°C for 90 seconds
  5. 72°C for 10 minutes
  6. Soak at 4°C
- } × 34

**F. (ii) Bad PCR schedule**

**Knockout reaction**

1. 94°C for 15 minutes
  2. 94°C for 1 minute
  3. 55°C for 1 minute
  4. 72°C for 90 seconds
  5. 72°C for 10 minutes
  6. Soak at 4°C
- } × 34

## **Appendix 2:**

### **A. Processing of E15 Embryo fixed in Carnoy's Fixative**

1. Fix in Carnoy's fluid for 30 minutes
2. 96% alcohol 2 minutes
3. 96% alcohol 2 minutes
4. 96% alcohol 2 minutes
5. 100% alcohol 5 minutes
6. 100% alcohol 5 minutes
7. 1:1 alcohol:chloroform 15 minutes
8. chloroform 15 minutes
9. chloroform 1 hour
10. wax 30 minutes
11. wax 45 minutes
12. wax 45 minutes
13. embed

### **B. Processing of E12 Embryo fixed in Carnoy's Fixative**

1. Fix in Carnoy's fluid for 15 minutes
2. 96% alcohol 1 minute
3. 96% alcohol 1 minute
4. 96% alcohol 1 minute
5. 100% alcohol 2 minutes
6. 100% alcohol 7 minutes
7. 1: 1 alcohol: chloroform 10 minutes
8. chloroform 10 minutes
9. chloroform 1 hour
10. wax 30 minutes
11. wax 30 minutes
12. wax 30 minutes
13. embed

### **Appendix 3:**

#### **A. Processing of E15 Embryos Fixed in Neutral Buffered Formalin**

1. Fix for 48 - 96 hours
2. Wash in ddH<sub>2</sub>O - 2 changes of 1 hour
3. Dehydrate in 75% alcohol overnight
4. 96% alcohol - 5 minutes
5. 96% alcohol - 10 minutes
6. 100% alcohol - 10 minutes
7. 100% alcohol - 10 minutes
8. 1:1 alcohol:chloroform - 30 minutes
9. chloroform 1 hour
10. chloroform overnight
11. wax 1 hour
12. wax 1 hour
13. wax 1 hour
14. embed

#### **B. Processing of E12 Embryos Fixed in Neutral Buffered Formalin**

1. Fix overnight
2. wash in ddH<sub>2</sub>O two changes of 30 minutes
3. 75% alcohol 30 minutes
4. 96% alcohol 5 minutes
5. 100% alcohol 5 minutes
6. 100% alcohol 5 minutes
7. 1:1 alcohol: chloroform 15 minutes
8. chloroform 15 minutes
9. chloroform 2 hours
10. wax 30 minutes
11. wax 30 minutes
12. wax 45 minutes
13. embed.

## Appendix 4

### TUNEL Staining Schedule

1. Xylene – 5 minutes
2. Xylene – 5 minutes
3. 100% - 2 minutes
4. 100% - 2 minutes
5. 96% - 2 minutes
6. 70% - 2 minutes
7. Distilled water – 2 minutes
8. TBS + 0.5% Triton X-100 – 2 minutes
9. TBS – 2 minutes
10. Proteinase K – 1:100 in 10mM Tris pH8.0 – 20 minutes
11. TBS – 5 minutes
12. TBS – 5 minutes
13. Quench – 1% Hydrogen peroxide in methanol – 10 minutes
14. TBS – 5 minutes
15. TBS – 5 minutes
16. TdT Equilibration buffer – 1:5 in distilled water – 30 minutes
17. TdT Enzyme – 1:20 in TdT Reaction mix – 2 hours at 37°C
18. TBS – 5 minutes
19. TBS – 5 minutes
20. Stop solution – 5 minutes
21. TBS – 5 minutes
22. TBS – 5 minutes
23. Blocking buffer – 10 minutes
24. Conjugate 1:50 in blocking buffer – 30 minutes at room temperature
25. TBS – 5 minutes
26. TBS – 5 minutes
27. Chromogen – as required
28. Tap water – 5 minutes
29. Distilled water – 2 minutes
30. 70% Alcohol – 2 minutes
31. 96% Alcohol – 2 minutes
32. 100% Alcohol – 2 minutes
33. 100% Alcohol – 2 minutes
34. Xylene – 5 minutes
35. Xylene – 5 minutes
36. Mount in DPX

**Note:** Gloves should be worn at all times. Fresh quenching solution should be made up as late as possible before use. All incubations should be carried out in a humidified chamber.

## Appendix 5

### Islet-1 Staining Schedule

1. Xylene – 5 minutes
2. Xylene – 5 minutes
3. 100% - 5 minutes
4. 100% - 5 minutes
5. 96% - 2 minutes
6. 70% - 2 minutes
7. Distilled water – 2 minutes
8. PBS – 2 minutes
9. Quench (1% Hydrogen peroxide in methanol) - 20 minutes
10. PBS – 2 minutes
11. PBS – 2 minutes
12. Rinse in PBS
13. Block (10% HHS and 0.4% Triton X-100 in PBS) - 120 minutes at room temperature
14. 1<sup>o</sup> Antibody (1:2 dilution in PBS) - 120 minutes at 37°C or 40°C, overnight
15. PBS – 2 minutes
16. PBS – 2 minutes
17. Rinse in PBS
18. 2<sup>o</sup> Antibody (1:200 dilution in PBS) – 3 hours at room temperature
19. PBS – 2 minutes
20. PBS – 2 minutes
21. Rinse in PBS
22. ABC – 30 minutes
23. PBS – 2 minutes
24. PBS – 2 minutes
25. Chromogen – as required
26. Tap water – 5 minutes
27. Distilled water – 2 minutes
28. 70% Alcohol – 2 minutes
29. 96% Alcohol – 2 minutes
30. 100% Alcohol – 2 minutes
31. 100% Alcohol – 2 minutes
32. Xylene – 5 minutes
33. Xylene – 5 minutes
34. Mount in DPX

**Note:** Gloves should be worn at all times. Fresh quenching solution should be made up as late as possible before use. The ABC solution should be made up at least 30 minutes



before use, mixed well and left at room temperature. All incubations and the ABC incubation should be carried out in a humidified chamber.

## Appendix 6

### βIII-Tubulin Staining Schedule

1. Xylene – 5 minutes
2. Xylene – 5 minutes
3. 100% - 5 minutes
4. 100% - 5 minutes
5. 96% - 2 minutes
6. 70% - 2 minutes
7. Distilled water – 2 minutes
8. PBS – 5 minutes
9. Quench (2% Hydrogen peroxide in methanol) - 15 minutes
10. PBS – 5 minutes
11. PBS – 5 minutes
12. Rinse in PBS
13. Block (20% HHS and 0.5% Triton X-100 in PBS) - 45 minutes at room temperature
14. 1° Antibody (1:10,000 dilution in PBS) - overnight at 4°C
15. PBS – 5 minutes
16. PBS – 5 minutes
17. Rinse in PBS
18. 2° Antibody (1:200 dilution in PBS) – 3 hours at room temperature
19. PBS – 5 minutes
20. PBS – 5 minutes
21. Rinse in PBS
22. ABC – 30 minutes at -4°C
23. PBS – 5 minutes
24. PBS – 5 minutes
25. DAB – as required
26. Tap water – 5 minutes
27. 0.5% CuSO<sub>4</sub>:0.85% NaCl – 5 minutes
28. Haematoxylin – 6 minutes
29. Acid Alcohol – 1 minute 30 seconds
30. Running Tap water – 12 minutes
31. 70% Alcohol – 2 minutes
32. 96% Alcohol – 2 minutes
33. 100% Alcohol – 2 minutes
34. 100% Alcohol – 2 minutes
35. Xylene – 5 minutes
36. Xylene – 5 minutes
37. Mount in DPX

**Note:** Gloves should be worn at all times. Fresh quenching solution should be made up as late as possible before use. The ABC solution should be made up at least 30 minutes

before use, mixed well and left at room temperature. All incubations and the ABC incubation should be carried out in a humidified chamber.

## Appendix 7

### PCNA Staining Schedule

1. Xylene – 5 minutes
2. Xylene – 5 minutes
3. 100% - 5 minutes
4. 100% - 5 minutes
5. 96% - 2 minutes
6. 70% - 2 minutes
7. Distilled water – 2 minutes
8. PBS – 5 minutes
9. Quench (2% Hydrogen peroxide in methanol) - 15 minutes
10. PBS – 5 minutes
11. PBS – 5 minutes
12. Rinse in PBS
13. Block (20% HHS and 0.5% Triton X-100 in PBS) - 45 minutes at room temperature
14. 1° Antibody anti-PCNA (1:1000 dilution in PBS) - 1 hours at room temperature
15. PBS – 5 minutes
16. PBS – 5 minutes
17. Rinse in PBS
18. 2° Antibody (1:200 dilution in PBS) – 3 hours at room temperature
19. PBS – 5 minutes
20. PBS – 5 minutes
21. Rinse in PBS
22. ABC – 30 minutes at room temperature
23. PBS – 5 minutes
24. PBS – 5 minutes
25. DAB – as required
26. 3° Antibody anti- $\beta$ III Tubulin (1:5000 dilution in PBS) - Overnight at 4°C
27. PBS – 5 minutes
28. PBS – 5 minutes
29. Rinse in PBS
30. 4° Antibody (1:200 dilution in PBS) – 3 hours at room temperature
31. PBS – 5 minutes
32. PBS – 5 minutes
33. Rinse in PBS
34. ABC – 30 minutes at room temperature
35. PBS – 5 minutes
36. PBS – 5 minutes
37. VIP – as required
38. Tap water – 5 minutes
39. 70% Alcohol – 2 minutes
40. 96% Alcohol – 2 minutes

41. 100% Alcohol – 2 minutes
42. 100% Alcohol – 2 minutes
43. Xylene – 5 minutes
44. Xylene – 5 minutes
45. Mount in DPX

**Note:** Gloves should be worn at all times. Fresh quenching solution should be made up as late as possible before use. The ABC solution should be made up at least 30 minutes before use, mixed well and left at room temperature. All incubations and the ABC incubation should be carried out in a humidified chamber.

# TNF $\alpha$ contributes to the death of NGF-dependent neurons during development

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Many sympathetic and sensory neurons depend on a supply of nerve growth factor (NGF) from their targets during development, and neurons that fail to obtain sufficient NGF die by apoptosis. Here we show that tumor necrosis factor alpha (TNF $\alpha$ ) is involved in bringing about the death of NGF-deprived neurons. Function-blocking antibodies against either TNF $\alpha$  or TNF receptor 1 (TNFR1) rescued many sympathetic and sensory neurons following NGF deprivation *in vitro*. Fewer sympathetic and sensory neurons died during the phase of naturally occurring neuronal death in TNF-deficient embryos, and neurons from these embryos survived in culture better than wild-type neurons. These neurons coexpress TNF $\alpha$  and TNFR1 during this stage of development, suggesting that TNF $\alpha$  acts by an autocrine loop.

Neurons are generated in excess in the developing vertebrate peripheral nervous system, and the superfluous neurons are lost during a phase of programmed cell death that occurs shortly after they innervate their targets. NGF is the founder of a family of structurally related secreted proteins termed neurotrophins that promote and regulate the survival of many kinds of neurons in the peripheral nervous system during this stage of development. NGF is required for the survival of sympathetic neurons and a subset of nociceptive sensory neurons. These neurons are lost in developing rodents treated with function-blocking anti-NGF antibodies<sup>1</sup> and in mice that are homozygous for null mutations in the *NGF* gene<sup>2</sup> or the *TrkA* gene, which encodes the NGF receptor tyrosine kinase<sup>3</sup>. NGF is synthesized in the peripheral target tissues of NGF-dependent neurons in proportion to their innervation density<sup>4,5</sup>, and administration of exogenous NGF prevents naturally occurring cell death within populations of NGF-dependent neurons during development<sup>1</sup>.

Because the binding of NGF to TrkA generates survival signals within the cell that prevent caspase activation and subsequent apoptosis<sup>6</sup>, it is assumed that neurons die following NGF deprivation because of the withdrawal of these survival signals. However, we show here that this death is due in part to the action of TNF $\alpha$ , a proinflammatory cytokine that induces apoptosis in some cell types. TNF $\alpha$  exerts its effects by binding to the receptors TNFR1 and TNFR2. The cytotoxic effects of TNF $\alpha$  are mediated via TNFR1, which has a cytoplasmic death domain that interacts with the adapter protein TRADD following ligand binding. TRADD in turn interacts with another adapter protein FADD, which recruits and activates pro-caspase 8 with the resultant activation of the cell death machinery<sup>7</sup>. TNFR2 lacks a death domain but synergistically enhances TNFR1-induced cytotoxicity<sup>8</sup>. In the nervous system, TNF $\alpha$  contributes to neuronal death associated

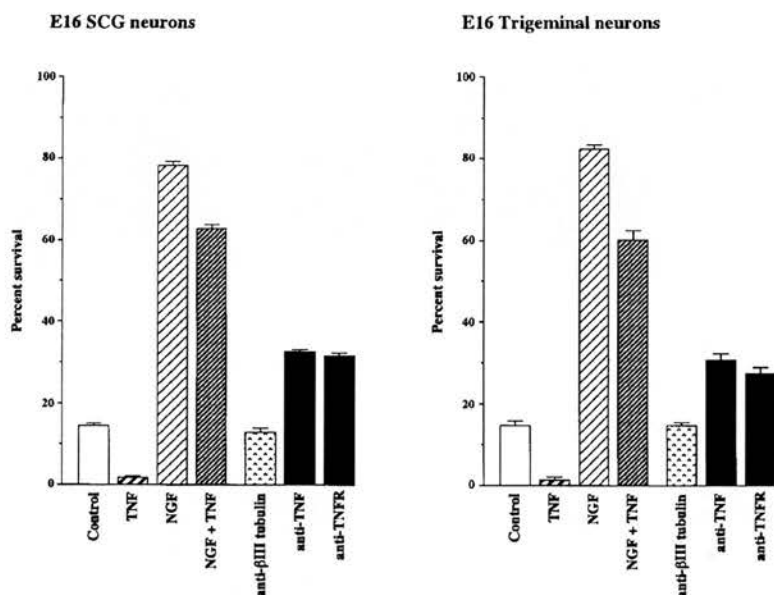
with ischemia<sup>9</sup>, HIV-1 infection<sup>10</sup> and axotomy<sup>11</sup>, and can exert a neuroprotective effect against glutamate excitotoxicity<sup>12,13</sup>. It is not known, however, if TNF $\alpha$  is involved in bringing about the death of neurons that fail to obtain adequate trophic support from their innervation targets during normal embryonic development.

## RESULTS

### TNF $\alpha$ and TNFR1 antibodies rescue neurons

To investigate if TNF $\alpha$  is involved in promoting apoptosis of embryonic neurons deprived of trophic support, we studied the effects of function-blocking anti-TNF $\alpha$  and anti-TNFR1 antibodies on the survival of NGF-dependent neurons following NGF deprivation. We established low-density, dissociated cultures of sympathetic neurons from the superior cervical ganglia (SCG) and sensory neurons from the trigeminal ganglia of mouse embryos at E16, when most of these neurons have become dependent on NGF for survival *in vitro* and naturally occurring neuronal death is occurring *in vivo*<sup>14–16</sup>. After 12 hours incubation with NGF, the cultures were washed extensively to remove this neurotrophin and were either resupplemented with NGF, or grown without NGF and treated with anti-TNF $\alpha$  or anti-TNFR1 antibodies or TNF $\alpha$ . Whereas about 80% of the sympathetic and sensory neurons survived for at least another 48 hours after resupplementation with NGF, over 80% died within 48 hours of NGF deprivation (Fig. 1). Treatment of NGF-deprived neurons with either anti-TNF $\alpha$  or anti-TNFR1 antibodies rescued a quarter to a third of the neurons that would have otherwise died (Fig. 1). Dose-response analysis revealed that this effect of the antibodies reached a plateau at a concentration of 2 ng/ml, and higher concentrations of antibodies singularly and in combination did not rescue additional neurons (data not shown). These results were not due to non-specific actions of antibodies on





**Fig. 1.** Survival of E16 SCG and trigeminal neurons cultured with NGF, TNF $\alpha$  and function-blocking TNF $\alpha$  or TNFR1 antibodies after NGF deprivation. The neurons were grown for 12 h after plating in medium containing 2 ng/ml NGF. After washing, growth factors and antibodies were added to a concentration of 2 ng/ml. The numbers of surviving neurons were counted after a further 48-h incubation and expressed as a percentage of the number counted after NGF deprivation. Control cultures received no reagents following NGF deprivation. The means and standard errors of six separate experiments are shown.

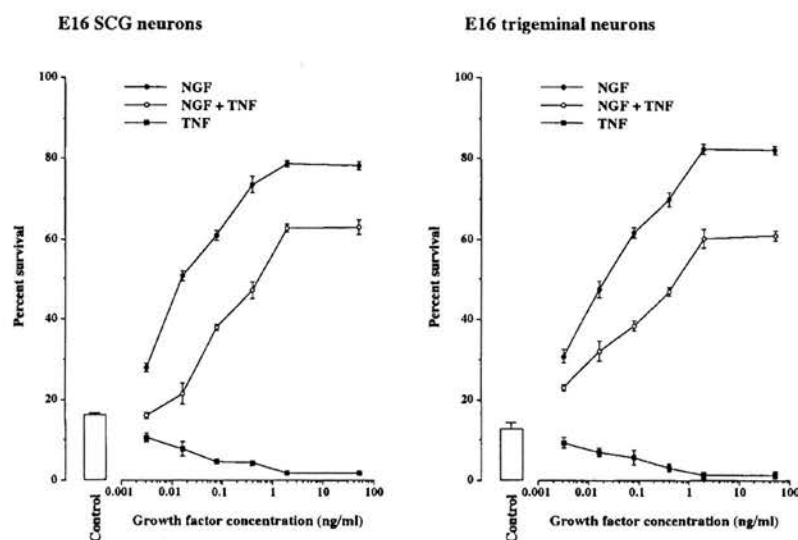
neuronal survival because there was no significant difference between the number of neurons surviving in control cultures and cultures treated with antibodies against an intracellular antigen ( $\beta$ III tubulin) even at concentrations as high as 50 ng/ml. Anti-TNF $\alpha$  and anti-TNFR1 rescued a similar proportion of sympathetic and sensory neurons that were plated in medium containing these antibodies from the outset (data not shown). These results suggest that TNF $\alpha$  synthesized by cells in dissociated cultures of embryonic sympathetic and sensory ganglia is involved in bringing about neuronal death following NGF deprivation, and that this action of TNF $\alpha$  is mediated at least in part by TNFR1.

TNF $\alpha$  treatment also reduced the survival of sympathetic and sensory neurons grown either with or without NGF (Fig. 2). Dose-response analysis revealed that as little as 3.2 pg/ml TNF $\alpha$  caused significant reductions in the survival of neurons grown in either the presence or absence of NGF ( $p < 0.005$ ,  $t$ -tests). This cytotoxic effect of TNF $\alpha$  reached a plateau at a concentration of 2 ng/ml, and no additional death was observed at a 25-fold higher concentration. At these concentrations of TNF $\alpha$ , less than 2% of the neurons were remaining in NGF-free cultures (compared to approximately 15% survival in NGF-free cultures without TNF $\alpha$ ) and there was an approximately 20% drop in

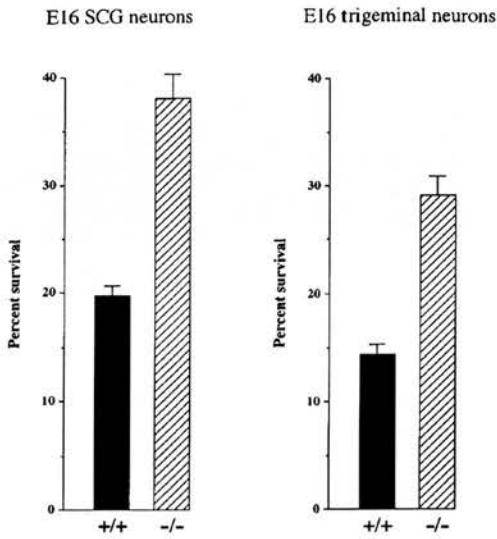
survival of neurons grown with TNF $\alpha$  plus NGF relative to neurons grown with NGF alone. These results suggest that endogenously produced TNF $\alpha$  is not functioning to maximum effect in promoting neuronal death and that only a proportion of the neurons are susceptible to the cytotoxic actions of TNF $\alpha$  at this stage of development. Also, TNF $\alpha$  receptors are active in sympathetic neurons, as TNF $\alpha$  treatment promotes nuclear translocation of the RelA subunit of NF $\kappa$ B (data not shown), although we do not know if this signaling pathway is involved in mediating the cytotoxic action of TNF $\alpha$  on these neurons.

#### Enhanced survival of TNF-deficient neurons in culture

To provide additional evidence for the involvement of TNF $\alpha$  in bringing about neuronal death following NGF deprivation, we compared the survival of embryonic SCG and trigeminal neurons cultured from wild-type mice and mice that are homozygous for a null mutation in the *TNF $\alpha$*  gene. After 48 hours incubation, there were approximately twice as many sympathetic and sensory neurons surviving in cultures established from E16 *TNF $\alpha$* <sup>-/-</sup> mice compared with neurons from wild-type embryos in the same litters (Fig. 3). These results further support the idea that endogenously produced TNF $\alpha$  is involved in bringing about the death of embryonic sympathetic and sensory neurons in the absence of NGF.



**Fig. 2.** E16 SCG and trigeminal neuron dose responses to NGF and TNF $\alpha$ . The neurons were grown for 12 h after plating in medium containing 2 ng/ml NGF. After washing, the cultures were supplemented with NGF, TNF $\alpha$  or NGF plus TNF $\alpha$  over a range of concentrations. The numbers of surviving neurons were counted after a further 48-h incubation and expressed as a percentage of the number counted after NGF deprivation. Control cultures received no reagents following NGF deprivation. The means and standard errors of six separate experiments are shown.



**Fig. 3.** *In vitro* survival of SCG and trigeminal neurons from TNF-deficient and wild-type embryos. Cultures were established from E16  $TNF^{-/-}$  and  $TNF^{+/+}$  embryos. After 48 h incubation in defined, serum-free medium, the number of surviving neurons was counted, and is expressed here as a percentage of the number counted 6 h after plating. The means and standard errors of three separate experiments are shown.

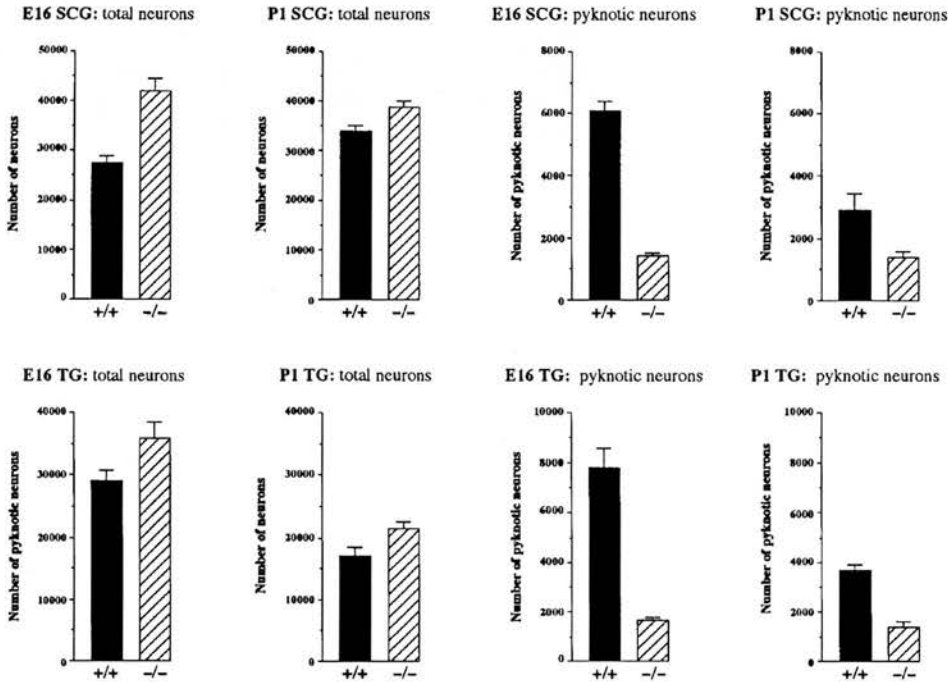
**Reduced neuronal death in TNF-deficient embryos**

To ascertain the physiological relevance of our *in vitro* observations, we estimated the number of surviving neurons and the extent of neuronal death in the SCG and trigeminal ganglia of wild-type and  $TNF\alpha^{-/-}$  mice at two stages during the period of naturally occurring neuronal death by counting the total number of neurons and the number of pyknotic neurons in serial sections (Fig. 4). At E16, the SCG and trigeminal ganglia of  $TNF\alpha^{-/-}$  embryos contained only a quarter as many dying neurons as in wild-type embryos, and there were significantly more neurons

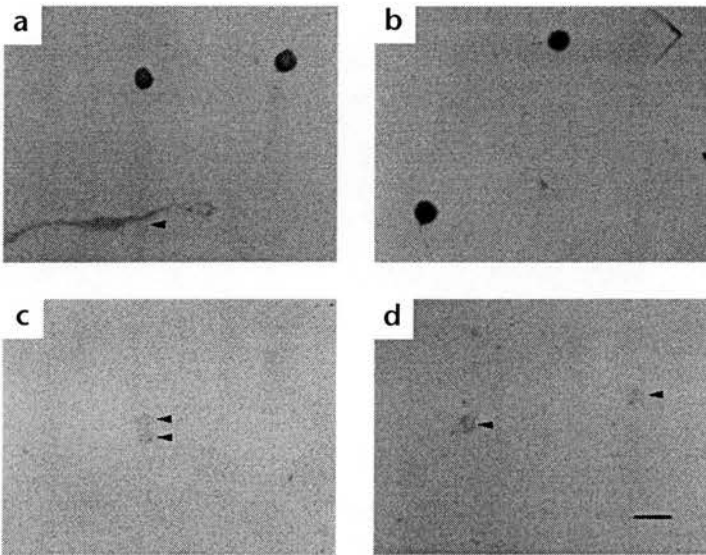
in the SCG (52% increase,  $p < 0.0001$ , *t*-test) and trigeminal ganglia (23% increase,  $p < 0.0001$ , *t*-test). By the first postnatal day (P1), the SCG and trigeminal ganglia of  $TNF\alpha^{-/-}$  embryos contained less than half as many dying neurons as in wild-type embryos, and neurons were still significantly more abundant in the SCG (14% increase,  $p < 0.001$ , *t*-test) and trigeminal ganglia (26% increase,  $p < 0.0001$ , *t*-test) of these  $TNF\alpha$ -deficient embryos compared with wild-type embryos. These results demonstrate that  $TNF\alpha$  is required to stimulate the death of a proportion of neurons in the developing SCG and trigeminal ganglia during the period of naturally occurring neuronal death. Analysis of neuronal death in the nodose ganglia also revealed significantly reduced neuronal death and increased numbers of neurons in  $TNF\alpha$ -deficient mice at these stages of development (data not shown), suggesting that  $TNF\alpha$  is also involved in promoting the death of sensory neurons that depend on BDNF and NT4 for survival during the period of naturally occurring neuronal death.

**Developing neurons express  $TNF\alpha$  and TNFR1**

To further substantiate the role of  $TNF\alpha$  in embryonic sensory and sympathetic neurons and investigate its mode of action, we used immunocytochemistry to identify which cells express  $TNF\alpha$  and TNFR1 in dissociated cultures. Because the



**Fig. 4.** Number of pyknotic neurons and total numbers of neurons in the SCG and trigeminal of E16 and P1  $TNF\alpha^{+/+}$  and  $TNF\alpha^{-/-}$  mice. The means and standard errors of the data obtained from both sets of ganglia from 3 E16 and 4 P1  $TNF\alpha^{+/+}$  mice and from 4 E16 and 5 P1  $TNF\alpha^{-/-}$  mice are shown.



**Fig. 5.** Expression of TNF $\alpha$  and TNFR1 by cultured SCG neurons. E16 SCG neurons from wild-type embryos were stained for TNF $\alpha$  (a) and TNFR1 (b); E16 SCG neurons (arrowheads) from TNF $\alpha$ <sup>-/-</sup> embryos did not stain for TNF $\alpha$  (c), and no neuronal staining was observed in wild-type neurons (arrows) if primary antibodies were omitted (d). The few non-neuronal cells in these cultures showed a low level of TNF $\alpha$  immunoreactivity (arrowhead, a). Scale bar, 20  $\mu$ M.

serum-free medium used in our cultures is not conducive to the survival of non-neuronal cells, most cells remaining in E16 cultures after 48 hours of incubation were neurons (>90%). Virtually all of these neurons were immunoreactive for TNF $\alpha$  protein and for TNFR1 (Fig. 5). The small number of non-neuronal cells in these cultures displayed only a low level of immunoreactivity for TNF $\alpha$  and TNFR1. Neurons in cultures established from TNF $\alpha$ <sup>-/-</sup> embryos showed no immunoreactivity for TNF $\alpha$  and no staining was observed in cultures of wild-type neurons if the primary antibodies were omitted, demonstrating specific staining for TNF $\alpha$  and its receptor. These findings indicate that most if not all neurons synthesize TNF $\alpha$  and express the TNFR1 receptor during the stage of NGF dependence, and suggest that TNF $\alpha$  exerts its actions by an autocrine mechanism.

## DISCUSSION

We have used several complementary experimental approaches to demonstrate that TNF $\alpha$  is involved in bringing about the death of sympathetic and sensory neurons during the phase of programmed cell death in the developing peripheral nervous system. Function-blocking antibodies to either TNF $\alpha$  or TNFR1 but not control antibodies to an unrelated protein prevented the death of a significant proportion of these neurons following NGF deprivation *in vitro*. TNF $\alpha$  treatment promoted the death of cultured sympathetic and sensory neurons in a dose-dependent manner. The sympathetic and sensory ganglia of TNF $\alpha$ -deficient mice contained substantially fewer dying neurons and significantly greater numbers of neurons than the ganglia of wild-type littermates during the phase of programmed cell death *in vivo*. Sympathetic and sensory neurons from TNF $\alpha$ -deficient embryos survived better in culture than neurons from wild-type embryos.

There are some parallels between our findings and recent work on Fas, a member of the TNF receptor family, and its ligand Fas-L, which have a well-established role in triggering apoptosis in lymphocytes<sup>17</sup>. Blocking interaction between Fas and Fas-L reduces cell death induced by neurotrophic factor deprivation in pheochromocytoma cells, cerebellar granule cells and spinal motoneurons<sup>18–20</sup>. Fas is constitutively expressed in

these cells and Fas-L is induced following neurotrophic factor deprivation. Treatment with Fas-L causes the death of many neurons even in the presence of optimal concentrations of neurotrophic factors. Thus, Fas signaling is triggered by neurotrophic factor withdrawal and this in turn brings about the subsequent demise of many of the neurons. Similarly, TNF $\alpha$ -mediated neuronal death following brain injury and ischemia in adult rats is associated with rapid upregulation in the expression of TNF $\alpha$ <sup>21,22</sup>.

Immunocytochemical analysis of TNF $\alpha$  and TNFR1 expression on cultured embryonic sympathetic neurons suggests that virtually all of these neurons express this ligand/receptor combination. This observation, together with our demonstration that inhibition of neuronal death by function-blocking anti-TNF $\alpha$  and anti-TNFR1 antibodies was observed in low dissociated density cultures that contained only a very small percentage of non-neuronal cells, suggest that TNF $\alpha$  contributes to the death of NGF-deprived neurons by an autocrine mechanism. However, the low level of TNF $\alpha$  immunoreactivity observed in non-neuronal cells raises the possibility that the neurons may additionally obtain some TNF $\alpha$  from other cells.

In summary, our findings add a twist to the molecular mechanisms that bring about the death of developing NGF-dependent neurons that fail to obtain adequate NGF to support their survival. The demise of these neurons seems to be caused in part by the cytotoxic actions of TNF $\alpha$  that they produce themselves. In future work, it will be important to ascertain the extent to which TNF $\alpha$ , FasL and possibly other cytotoxic cytokines are involved in bringing about naturally occurring neuronal death in these and other populations of neurons in the developing vertebrate nervous system.

## METHODS

**Neuron culture and survival assays.** Dissociated cultures were established from the SCG and trigeminal ganglia of E16 CD1 embryos. The dissected ganglia were trypsinised and dissociated by trituration and were grown in defined, serum-free medium on a poly-ornithine/laminin substratum<sup>23</sup> in the 11-mm diameter wells of 4-well dishes (Greiner, Stonehouse, UK) at a density of 200–400 neurons per well. After an initial 12-h incubation period with 2 ng/ml NGF, the neurons were washed extensively to remove this factor and were subsequently incubated in medium with either no added factors (control), NGF (gift of A. Rosenthal, Rinat Neuroscience Corporation, Palo Alto, California), TNF $\alpha$  (Calbiochem, Nottingham, UK), NGF plus TNF $\alpha$ , function-blocking anti-TNF $\alpha$  antibody (Upstate Biotechnology, Buckingham, UK), function-blocking anti-TNFR1 antibody (Oncogene Research Products, Nottingham, UK) or anti- $\beta$ -tubulin antibody (Promega, Southampton, UK). After 48 h, the numbers of surviving neurons in each well were counted and expressed as a percentage of the number of attached neurons after the 12-h wash. Very similar results were obtained if the

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cultures were additionally treated with anti-NGF antibody to functionally inactivate any residual NGF after washing.

To further investigate the role of TNF $\alpha$  in promoting neuronal death, we established cultures from mouse embryos that have a null mutation in the TNF $\alpha$  gene. TNF $\alpha$ <sup>-/-</sup> mice (Jackson Laboratories, Maine) were crossed to produce TNF $\alpha$ <sup>-/-</sup>, TNF $\alpha$ <sup>+/-</sup> and TNF $\alpha$ <sup>+/+</sup> embryos. After 16 days gestation, the pregnant females were killed and the embryos were genotyped by a PCR-based method. The SCG and trigeminal ganglia from wild-type and homozygous embryos were separately pooled, and low-density, dissociated cultures were established. The neurons were grown in defined medium for 48 h and the number surviving after this time is expressed as a percentage of the number of attached neurons counted 6 h after plating. All animal work was approved by our institutional animal use committee and by the Home Office.

**Immunocytochemistry.** Immunocytochemistry was used to visualize expression of TNF $\alpha$  and TNFR1 in cultured neurons. The cultures were fixed with methanol at -20°C followed by 1% H<sub>2</sub>O<sub>2</sub> to quench endogenous peroxidase activity and permeabilization in 0.5% Triton X-100 in PBS. After incubating at 4°C overnight with 1:200 dilutions of either anti-mouse TNF $\alpha$  goat polyclonal antibody (L-19, Santa Cruz Biotechnology, Wembley, UK) or anti-mouse TNFR1 goat polyclonal antibody (Calbiochem), bound primary antibody was detected using the Vector Elite ABC kit (Vector Labs, Orton Southgate, UK) according to the manufacturer's instructions. The stained neurons were viewed with a Nikon Diaphot microscope (Nikon, Kingston on Thames, UK).

**Quantification of neurons in the SCG and trigeminal ganglia.** The heads from E16 and P1 TNF $\alpha$ <sup>-/-</sup> and wild-type embryos were fixed for 1 h in Carnoy's fluid (60% ethanol, 30% chloroform, and 10% glacial acetic acid). Following dehydration through a graded alcohol series, the tissue was paraffin-wax-embedded. Serial sections of the heads were cut at 8  $\mu$ m and were mounted onto polylysine-coated slides (BDH) or Gold Seal Ultrastick Slides (Eric Scientific, Loughborough, UK).

To identify all neurons in these sections, the sections were stained for  $\beta$ -tubulin class III. Sections were cleared in xylene and rehydrated before quenching in 3% hydrogen peroxide in methanol for 20 min. Non-specific antibody binding was blocked in 10% horse serum, 0.5% Triton X-100 in PBS before incubation with mouse anti- $\beta$ III tubulin antibody (Promega) diluted 1:10,000 in PBS overnight at 4°C. The cells were then labeled using biotinylated secondary antibody (1:200), avidin, biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC Kit, Vector Labs). The substrate used for the reaction was 1 mg/ml diaminobenzidine tetrachloride (FastDAB, Sigma, St. Louis, Missouri). The sections were then counterstained with Gill's hematoxylin before dehydration and mounting. Neuronal number was quantified using a digital stereology system that employs a combination of the optical disector and volume fraction/Cavalieri methods (Kinetics Imaging, Bromborough, UK). For quantification of the number of pyknotic nuclei in the ganglia, the sections were stained with cresyl fast violet acetate. Pyknotic nuclei were then counted using the digital stereology system. All sections were coded prior to estimating the number of neurons and pyknotic nuclei to avoid any observer bias.

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