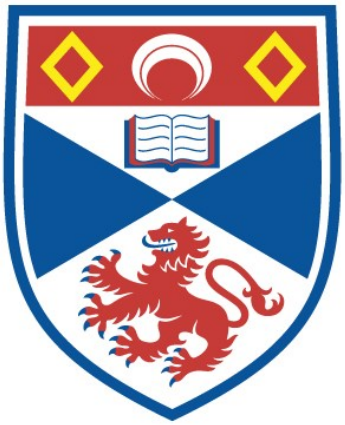
THE ROLE OF BCL-2 PROTEIN FAMILY IN THE DEVELOPMENT OF THE PERIPHERAL NERVOUS SYSTEM

Gayle Helane Middleton

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1998

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A thesis submitted to the University of St. Andrews
for the degree of
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by

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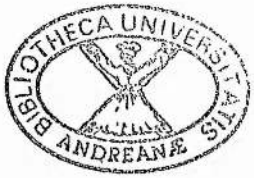

ABSTRACT

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The aim of this study was to ascertain the roles of selected members of the bcl-2 protein family of proteins in the developing peripheral nervous system. Two experimental approaches were used: microinjection of expression plasmids encoding these proteins into cultured neurons and studying the *in vitro* and *in vivo* development of neurons in mouse embryos with targeted null mutations in the genes encoding these proteins. Overexpression of bcl-2, bcl-xL, and bcl-xβ in cultured embryonic sensory neurons prevented the death of these neurons following neurotrophin withdrawal. Accordingly, sensory neurons from *bcl-2*$^{-/-}$ embryos survived less well in culture with neurotrophins than neurons from wild type embryos and there were fewer neurons and more dying cells in the cranial sensory ganglia of *bcl-2*$^{-/-}$ embryos during the phase of naturally occurring neuronal death. In addition to demonstrating a role for bcl-2 in regulating the survival of embyronic sensory neurons, studies of neurons from of *bcl-2*$^{-/-}$ embryos revealed new roles for bcl-2 in neuronal development. bcl-2-deficient sensory neurons extended axons more slowly and underwent an early maturational change more slowly than wild type neurons, indicating that bcl-2 may regulate aspects of early neuronal development independently of its effects on survival. Overexpression of bax in chicken sensory neurons also rescued some of these neurons following neurotrophin deprivation, contradicting the view that bax is exclusively a pro-apoptotic member of the bcl-2 family. However, sensory neurons from *bax-2*$^{-/-}$ embryos survived better in culture than neurons from wild type embryos and there were more neurons and fewer dying cells in the cranial sensory ganglia of *bax-2*$^{-/-}$ embryos, suggesting that bax may play different roles in regulating the survival of certain neurons in these two classes of vertebrates.

DECLARATIONS

I, Gayle Helane Middleton, hereby certify that this thesis, which is approximately 56000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date 7/7/98 Signature of candidate

I was admitted as a research student in October 1994 and as a candidate for the degree of PhD in October 1994; the higher study for which this is a record was carried out in the University of St. Andrews between 1994 and 1998.

Date 7/7/98 Signature of candidate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of PhD in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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TABLE OF CONTENTS

TABLE OF CONTENTS

ABBREVIATIONS

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aFGF - acidic fibroblast growth factor

Bcl-2- B-cell lymphoma/leukaemia-2

BDNF - brain-derived neurotrophic factor

bFGF - basic fibroblast growth factor

BH - Bcl-2 homology region

bp - base pair

cDNA- Complementary decxyribonucleic acid

cm - centimetre

CNS - Central Nervous System

CNTF - ciliary neurotrophic factor

CNTFRα - CNTF receptor alpha

^{0}C - degrees Celsius

CsCl - caesium chloride

DMTG- Dorsomedial portion of the trigeminal ganglion

DNA - deoxyribonucleic acid

dNTP - deoxynucleotide triphosphate

DRG - dorsal root ganglion

E - embryonic day

E.coli - Escherichia coli

EBV - epstein Barr virus

EC_{50} - half-maximally effective concentration

EDTA - ethylenediamine tetra acetic acid

F12 - Ham nutrient mixture F-12

F14 - Ham nutrient mixture F-14

FCS - fetal calf serum

FGF - fibroldast growth factor

G protein - GTP -binding regulatory protein

g - gramme

G1 - first growth phase of the cell cycle

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GDNF- glial cell line - derived neurotrophic factor

GDP - guanosine diphosphate

GPA - growth promoting activity

GPARα - GPA receptor alpha

GTP - guanosine triphosphate

GTPase - guanosine triphosphatase

HBSS - Hank's balanced salt solution

HIHS - Heat inactivated horse serum

ICE - Interleukin - 1 β - converting enzyme

IL-3 - interleukin-3

IMS - industrial methylated spirits

kDa - kilodalton

L15 - Leibovitz's L15 nutrient mixture

LB - Luria broth

LIF - leukaemia inhibitory factor

LIFRβ - LIF receptor beta

M - molar

mg - milligramme

ml - millilitre

mM - millimolar

mm - millimetre

MRNA - messanger ribonucleic acid

NGF - nerve growth factor

NT-3 - neurotrophin-3

NT-4/5 - neurotrophin-4/5

NT-6 - neurotrophin-6

OD - optical density

P - postnatal day

PBS - phosphate buffered saline

PCR - polymerase chain reaction

RNA - ribonucleic acid

rpm - revolutions per minute

RT-PCR - reverse transcription - polymerase chain reaction

S - synthesis phase of the cell cycle

SDS - sodium dodecyl sulphate

TAE - tris-acetate-EDTA

TE - tris - EDTA

TGF-β - transforming growth factor beta

TMN - trigeminal mesencephalic nucleus

UV - ultraviolet

V - volts

vol. - volume

LIST OF FIGURES

LIST OF FIGURES

Chapter 4 - Bcl-2 Transgenic Mice: Neuronal Survival Studies

Chapter 5 - Bcl-2 Transgenic Mice: Neuronal Maturation and Neurite Outgrowth

Chapter 6 - Bax Transgenic Mice: Neuronal Survival Studies

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Finally, I would like to thank the BBSRC for funding this project.

This thesis is dedicated to my mother for all her hard work and dedication.

I. INTRODUCTION

CHAPTER ONE

INTRODUCTION

PROJECT AIMS

The aim of this project was to elucidate further the role that members of the *bcl-2* gene family play in regulating the survival of developing peripheral neurons. The studies were carried out in two species, the chicken, *Gallus domesticus*, and the mouse, *Mus musculus*. Two experimental approaches were taken in this study. First, *bcl-2* family members were overexpressed in cultured chick neurons using microinjection of appropriate cDNA expression constructs to achieve supraphysiological expression. Second, mice carrying null mutations in the genes encoding bcl-2 and bax were analyzed to determine the effect that the lack of these proteins has on neuronal survival both *in vitro* and *in vivo*.

CELL DEATH

There are two distinct forms of death that cells undergo. The first form is physiological cell death, which is widespread during the ontogeny of both vertebrates and invertebrates, and thereafter in postnatal and adult life as part of normal tissue homeostasis, and the second form is necrotic cell death, which occurs in response to pathogenic stimuli (Clarke, 1990; Ellis & Horvitz, 1991). Physiological cell death is an active process which in many cases requires gene transcription and protein synthesis (Martin et al., 1988; Scott & Davies, 1990; Ellis & Horvitz, 1991) and can be blocked pharmacologically. In contrast, necrosis is a passive process, not requiring transcription or protein synthesis and is characterized by clumping of chromatin, swelling of organelles, floculent mitochondria, and membrane disruption (Martin et al., 1988; Scott & Davies, 1990; Ellis & Horvitz,

1991). Three main morphologically distinct forms of physiological cell death occur during development (Schweichel & Merker, 1973).

Autophagic Degeneration

This form of programmed cell death is characterized by the formation of autophagic vacuoles. Mitochondria and endoplasmic reticulum dilate and the Golgi apparatus enlarges. Primary lysosomes discharge their hydrolytic enzymes into autophagic vacuoles which, in turn, spill their contents into the cytoplasm. The cell debris is cleared away by heterophagy.

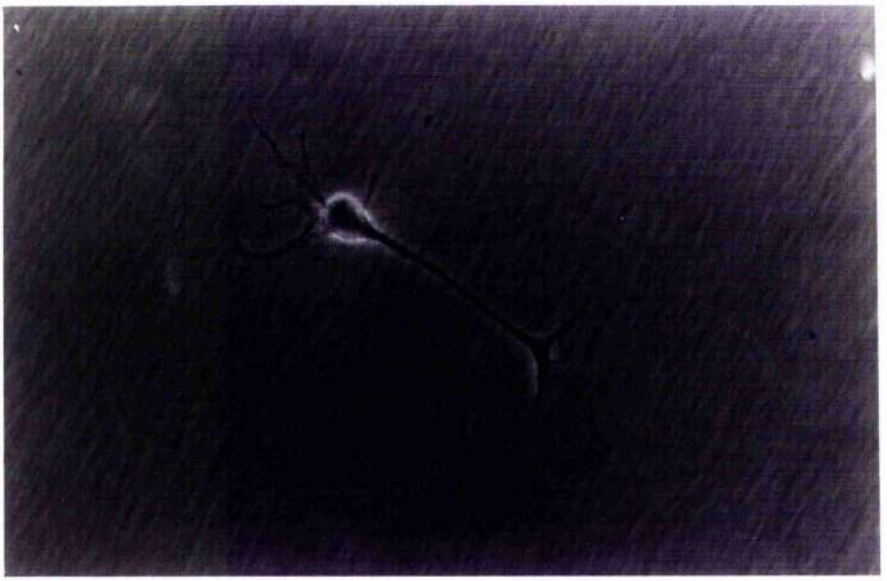
Non-lysosomal Degradation

In this process the intracellular organelles swell and organelle-free "spaces" appear in the cytoplasm. These "spaces" fuse together and connect with the extracellular cavity. The cell is destroyed by the breakdown of the cellular structure into smaller and smaller pieces. The cell's own lysosomes seem not to be involved and there is no detectable phagocytosis by neighboring cells.

Apoptosis

The term apoptosis comes from the Greek and means falling of leaves (Altman, 1992). This form of cell death begins with the condensation of cytoplasm and nuclear DNA. As a result of the latter, the nucleus appears condensed and is described as pyknotic. As the process continues, the nuclear and cellular membranes become convoluted, giving the cell an irregular appearance. Nuclear DNA becomes fragmented into oligonucleosomal lengths, although mitochondrial DNA remains intact. Finally, the cell surface develops blebs that are phagocytosed by adjacent cells. Apoptosis is the process by which cell death occurs in the developing peripheral nervous system (Garcia et al., 1992; Allsopp et al., 1993).

A



B

Figure 1.1: Living and apoptotic neurons from the E10 chick ciliary ganglion.

Scale bar represents 50 μm.

WHY DOES CELL DEATH OCCUR?

The theories of neo-Darwinism state that only individuals possessing characteristics that confer a selective advantage will out-compete other members of their species. Their 'good' characteristics will be maintained within the population whilst disadvantageous characteristics are selected against and will not be maintained in the gene pool. With this in mind, it initially seems counterintuitive that cell death might confer a selective advantage when energy is used to create cells that are then destroyed. It is highly unlikely, however, that a wasteful mechanism would be maintained throughout evolution from Volvox (Pommerville & Kochert, 1982) to the nematode *Caenorhabditis elegans* (Ellis et al., 1991) and mammals such as *Homo sapiens* (Clarke, 1990). There are several hypotheses as to why cell death occurs and how it produces a selective advantage.

Cell death During Morphogenesis

Extensive cell death occurs to form the interdigital spaces in the foot of most amniotes (Saunders & Fallon, 1966). If this cell death process stops early, interdigital webbing occurs (Pautou, 1974) as occurs in the webbed feet of water fowl. The advantage of using cell death to form the individual digits therefore appears to generate scope for adaptability.

Harmful Cells

Cell death can eliminate cells that pose a danger to an organism. For example, some thymocytes carry T-cell receptors capable of recognising and attacking self (Dwyer, 1993). If these cells were to mature and leave the thymus, an autoimmune reaction would occur that could be fatal. Under normal circumstances, self-reactive thymocytes undergo apoptotic cell death whilst still in the thymus.

Cells with a Transient Function

Some cells die at a time when they have completed their function within the developing organism. One of the best examples of this is the tail-spike of *Caenorhabditis elegans* (Sulston et al., 1983). The tail-spike cells generate filaments that shape the long tapering tail. Once these filaments are formed, the cells have completed their function and die.

Cells that Develop Improperly

In the developing visual system, cell death preferentially eliminates neurons that have formed improper connections (Cowan et al., 1984). Early in the development of the isthmo-optic nucleus, a small number of neurons project their axons to the ipsilateral, rather than the contralateral eye. During the phase of naturally occurring cell death, essentially all the ipsilaterally projecting cells die as compared to less than 60% death in the neuronal population as a whole.

Cells Generated in Excess

During development, some cell types are produced in excess and only some of these cells will survive. For example, in the developing dorsal root ganglia (DRG), neurons require trophic support from target-derived neurotrophic factors to survive. Removal of the peripheral target field (limb bud) prior to innervation substantially increases the magnitude of neuronal death (Hamburger & Levi-Montalcini, 1949; Prestige, 1967; Carr & Simpson, 1978; Hamburger & Yip, 1984). Conversely, increasing the size of the target field by grafting in an additional limb bud results in decreased cell death in the DRG. Thus, the innervation is adjusted to suit the size of the target field. This size matching facilitates evolution such that if there is a mutation that changes the size of a body part the density of innervation can be adjusted accordingly. Again, cell death is conferring adaptability.

THE REGULATION OF APOPTOSIS DURING DEVELOPMENT OF THE VERTEBRATE PERIPHERAL NERVOUS SYSTEM

Neurodevelopment

The development of the vertebrate nervous system is a multistage process beginning with neural induction in which the cells of the dorsal ectoderm are induced by the underlying precaudal mesoderm (Spemann, 1938) to form the neural plate (Lillie, 1908). A midline furrow subsequently forms and the neural folds lying on either side of this round up to meet each other in the dorsal midline (Copp et al, 1990; Schoenwolf & Smith, 1990). Cell surface protrusions and lamellipodia are extended just prior to fusion and these interdigitate as cells come into contact to form the intact neural tube. Finally, the neural tube sinks beneath the surface of the ectoderm and the overlying surface ectoderm is repaired. The neural tube gives rise to the central nervous system, i.e. the brain and spinal cord.

The peripheral nervous system is composed of the ganglia of the autonomic and sensory systems and the peripheral nerves which lie outside the brain and spinal cord. The cell bodies of peripheral sensory neurons lie within the dorsal root and cranial sensory ganglia. The cranial sensory neurons are segmented into ganglia on five of the twelve pairs of cranial nerves. The exception to this is the trigeminal mesencephalic nucleus which is a sub-population of sensory neurons located in the midbrain.

Most of the peripheral nervous system is derived from the neural crest. This is a transient structure comprised of cells that detach from the margin of the neural ectoderm at the time of neural tube closure. The cells migrate out into the periphery along well defined pathways and differentiate into most of the neurons and glia of the peripheral nervous system, as well as craniofacial mesenchyme, chromaffin cells of the adrenal medulla, and melanocytes in the skin (Weston, 1962). Some populations of cranial sensory neurons are also derived from placodes

that form as thickenings in the ectoderm of the cephalic region. The anterior pituitary gland, the olfactory sensory epithelium and the lens of the eye are also derived from placodes. The origins of the neurons of the avian cranial sensory neurons have been determined by a series of mapping experiments involving the transplantation of neural crest and placode derived cells (Weston, 1962; Le Douarin, 1973; D'Amico-Martel, 1982). Table 1.1 shows the origins of avian cranial sensory neurons.

Neuronal Population	Cranial Nerve	Embryonic origin
Trigminal Mesencephalic Nucleus	V	Neural Crest
Trigeminal Ganglion	V	
dorsomedial part		Neural crest
ventrolateral part		Trigeminal Placode
Geniculate Ganglion	VII	First Epibrachial Placode
Vestibular Ganglion	VIII	Otic placode
Acoustic Ganglion	VIII	Otic placode
Jugular-superior Ganglion	IX/X	Neural Crest
Petrosal Ganglion	IX	Second Epibrachial Placode
Nodose Ganglion	X	Third Epibrachial Placode

Table 1.1: Origins of avian cranial sensory neurons

Neurons are generated in excess during the development of the peripheral nervous system and shortly after they begin to innervate their target tissues, large numbers degenerate by a process bearing the histological features of apoptosis (Oppenheim, 1991; Allsopp et al., 1993; Garcia et al., 1992) and which requires RNA and protein

synthesis (Martin et al., 1988; Scott & Davies, 1990). In the case of sensory neurons, the magnitude of this cell death ranges from 25% in the vestibular and cochlear ganglia (Ard & Morest, 1984) to over 73% in the trigeminal mesencephalic nucleus (Rogers & Cowan, 1974).

According to the neurotrophic hypothesis, neuronal target fields play an important role in regulating the number of neurons that survive the period of cell death by producing limited quantities of neurotrophic factors (Cowan et al., 1984; Davies 1988b and c; Purves ,1988; Barde, 1989). Neurons that procure an adequate supply of these factors survive whilst those that do not die. The neurotrophic theory was initially proposed on the basis of results of studies investigating the effects of nerve growth factor (NGF), the first neurotrophic factor to be identified (Cohen, 1960). The more recent discovery and characterisation of other members of the NGF family, and other unrelated molecules that also support neuronal survival, have provided further evidence to support the theory.

The Neurotrophins

NGF (Cohen, 1960), BDNF (Barde et al., 1982), NT-3 (Ernfors et al., 1990; Hohn et al., 1990; Jones & Reichardt, 1990; Maisonpierre et al., 1990a,b), NT-4/5 (Berkemeier et al., 1991; Ip et al., 1992) and NT-6 (Gotz et al., 1994) are all members of the neurotrophin family of structurally-related polypeptide neurotrophic factors. Elucidation of the primary structures of these molecules has revealed that their basic conformations are determined by strictly conserved cysteine-rich domains. Distinct regions of variation between the molecules are responsible for their binding to specific high-affinity receptors. All the neurotrophins also bind a low-affinity receptor that is common to all the factors.

These factors have many other effects distinct from promoting neuronal survival such as stimulating neurite outgrowth and promoting maturation and differentiation of neurons (Kalcheim & Gendreau, 1988; Wright et al., 1992).

Nerve Growth Factor

Nerve growth factor (NGF) was initially isolated from a fraction of snake venom (Cohen, 1960). Thereafter, the murine submandibular salivary gland was discovered to be an extremely rich source of this protein (Cohen, 1960). Determination of the amino acid sequence of murine NGF (Angeletti & Bradshaw, 1971) led to the cloning of the murine NGF cDNA (Scott et al., 1983). Subsequently, human, bovine and chick NGF genes were cloned (Ullrich et al., 1983; Meier et al., 1986; Ebendal et al., 1986).

A 307 amino acid precursor polypeptide is encoded by the murine NGF gene and this is further processed to give the mature 118 amino acid glycosylated protein (Berger & Shooter, 1977; Scott et al., 1983; Edwards et al., 1988). Two of these 118 amino acid proteins interact to form a homodimer which is the biologically active form of NGF. Detailed analysis of the tertiary structure of NGF using X-ray diffraction has revealed that mature NGF contains a "cysteine knot" of three interchain disulphide bonds and two antiparallel β strands. Together these form a flat surface where the two subunits associate (McDonald et al., 1991). Four loop regions contain many of the amino acids that vary between neurotrophins, and this suggests that it is these regions which confer receptor binding specificity (Ebendal, 1992).

There is much *in vitro* and *in vivo* evidence that NGF can promote the survival of sympathetic neurons, certain types of sensory neurons and basal forebrain cholinergic neurons (Chun & Patterson, 1977; Hamburger et al., 1981; Davies & Lindsay, 1985; Levi-Montalcini, 1987; Hartikka & Hefti, 1988; Barde, 1989; Davies, 1994c). Furthermore, many tissues that synthesise NGF have been found to be the target tissues of NGF responsive neurons (Korsching & Thoenen, 1983; Heumann et al., 1984; Shelton & Reichardt, 1984; Korsching et al., 1985). A detailed study of the developing murine whisker pad first elucidated the exact identity of NGF secreting target cells (Davies et al., 1987a). The developing whisker pad was

enzymatically dissociated into its main components: the presumptive epidermis and dermis. Subsequent analysis of NGF expression demonstrated that the highest levels of NGF are expressed in the epithelium (Davies et al., 1987a). Furthermore, the concentration of NGF mRNA in the epithelium of the opthalmic, maxillary and mandibular territories of the trigeminal ganglion of the mouse has been found to be related to the innervation density of those territories (Harper and Davies, 1990). The timing of NGF synthesis in target tissues has been shown to coincide with the onset of target field innervation (Davies et al., 1987a; Korsching & Thoenen, 1988; Harper and Davies, 1990).

Mice with a targeted null mutation of the NGF gene have facilitated *in vivo* studies of NGF in the nervous system. Mice homozygous for such a mutation fail to respond to noxious mechanical stimuli due to an impaired nociceptive system (Crowley et al., 1994). Neuronal loss in the trigeminal, dorsal root and sympathetic ganglia of these mice has been demonstrated using histological techniques. The cell loss within the dorsal root ganglia is restricted to a subset of neurons that convey nociceptive and thermoceptive information (Crowley et al., 1994). In the central nervous system, NGF null mutant mice do not suffer significant loss of basal forebrain neurons but do display lighter immunostaining for cholinacetyltransferase as compared to wild type mice. This suggests that NGF is required for regulation of the functional status rather than the survival of these cells (Crowley et al., 1994). In contrast, overexpression of NGF in the murine epithelium results in decreased neuronal loss in certain sensory and sympathetic ganglia (Albers et al., 1994). These studies provide *in vivo* evidence for the dependence of certain sensory and sympathetic neurons on NGF.

Brain-derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) was the second member of the NGF family to be characterised. It was first isolated from porcine brain and is a 12kDa basic protein (Barde et al., 1982). Like NGF, BDNF is synthesised as a precursor polypeptide that is further processed to give a mature 119 amino acid glycosylated protein. BDNF has approximately 50% sequence identity with NGF, including conservation of the six cysteine residues involved in the formation of the "cysteine knot" (Leibrock et al., 1989). The biologically active form of BDNF exists as a homodimer linked by three disulphide bonds (Radziejewski et al., 1992). BDNF has subsequently been cloned in other species including human, mouse and rat (Hofer et al., 1990; Jones & Reichardt, 1990; Maisonpierre et al., 1991).

BDNF has been shown to enhance the survival and differentiation of certain central nervous system neurons *in vitro* including embryonic basal forebrain cholinergic neurons, motoneurons, and retinal ganglion cells (Alderson et al., 1990; Knusel et al., 1991; Oppenheim et al., 1992; Korsching, 1993). In the peripheral nervous system, BDNF has been shown to promote the survival of certain sensory neurons (Lindsay et al., 1985; Davies et al., 1986b; Barde et al., 1987; Davies et al., 1987b; Hofer & Barde, 1988). Northern analysis of BDNF mRNA expression has revealed that the central nervous system is the predominant site of expression (Leibrock et al., 1989; Hofer et al., 1990; Maisonpierre et al., 1990a). The highest levels of BDNF mRNA are present in the hippocampus, cerebral cortex and cerebellum with appreciable levels in the spinal cord and hindbrain which are the central target fields of BDNF-responsive sensory neurons. In the peripheral nervous system, BDNF mRNA is found in neurons of sympathetic, dorsal root and trigeminal ganglia where it is expressed by NGF-dependent neurons suggesting a possible autocrine and/or paracrine mechanism (Schecterson & Bothwell, 1992; Robinson et al., 1996). Outwith the nervous system, BDNF mRNA is found at low levels in muscle, heart, skin and lung (Maisonpierre et al., 1990a; Schecterson & Bothwell, 1992).

The *in vivo* role of BDNF has been further elucidated utilising mice that are homozygous for a null mutation in the BDNF gene. These mice develop symptoms of nervous system dysfunction including ataxia and also display substantially reduced numbers of cranial and spinal sensory neurons. In particular, deficiencies are observed in trigeminal, geniculate, vestibular and nodose ganglia (Ernfors et al., 1994; Jones et al., 1994). Although no gross structural abnormalities are observed in the central nervous system, the expression of neuropeptide Y and calcium binding proteins is altered in many neurons perhaps indicating abnormal functioning (Ernfors et al., 1994; Jones et al., 1994). Also, as these mice show early postnatal lethality, they may simply not survive long enough for a wider phenotype to be observed. This suggests that BDNF is an essential factor for the survival of several populations of sensory neurons during ontogeny, yet it does not appear to be essential for central nervous system development (Ernfors et al., 1994; Jones et al., 1994).

Neurotrophin-3

Neurotrophin-3 (NT-3), the third member of the NGF gene family to be identified, was isolated using the polymerase chain reaction taking advantage of the nucleotide sequence homology between the members of the emerging gene family (Hohn et al., 1990; Maisonpierre et al., 1990b; Rosenthal et al., 1990). Mature NT-3 is cleaved from a 258 amino acid precursor polypeptide (Hohn et al., 1990; Maisonpierre et al., 1990b). NT-3 is related structurally to both NGF and BDNF sharing around 50-60% amino acid identity with these two proteins including all six cysteine residues involved in formation of the "cysteine knot" (Hohn et al., 1990). Like NGF and BDNF, the biologically active form of NT-3 is a homodimer (Radziejewski et al., 1992).

NT-3 mRNA is expressed in heart, liver, kidney, spleen, lung and intestine and also in several regions of the central nervous system. In the fetal rat, NT-3 mRNA is detectable in the tissues of the inner ear, iris, and the target fields of the trigeminal ganglion, especially the epithelium of the whisker follicles (Hallbook et al., 1993;

Pirvola et al., 1992; Ernfors et al., 1992). Confirmation of the expression of NT-3 mRNA in the developing trigeminal target territory has been shown in the mouse using quantitative Northern Blotting. NT-3 mRNA is expressed at the highest levels in the epithelium of the most densely innervated target field at E13, the age at which naturally occurring cell death is occurring in the trigeminal ganglion (Buchman & Davies, 1993).

NT-3-deficient mice exhibit substantial neuronal loss in the dorsal root, nodose and trigeminal sensory ganglia, the trigeminal mesencephalic nucleus, spiral ganglion of the cochlea and the sympathetic superior cervical ganglion (Ernfors et al., 1994; Farinias et al., 1994; Tessarollo et al., 1994). Also, the main components of the proprioceptive system, that is the muscle spindles and Golgi tendon organs, are missing. These are the peripheral sense organs of the large-diameter proprioceptive neurons of the DRG. These neurons are absent in NT-3-deficient mice (Ernfors et al., 1994). No effects have been observed in the central nervous system. However, like BDNF-deficient mice, this may be due to early postnatal lethality.

Neurotrophin-4/5

Neurotrophin 4/5 (NT-4/5) was identified taking advantage of the homology between NGF, BDNF and NT-3 (Hallbook et al., 1991). The NT-4 gene of *Xenopus laevis* encodes a 236 amino acid precursor protein that is processed to give the mature 123 amino acid protein. The mature protein has 50-60% identity with NGF, BDNF, and NT-3 including conservation of all six of the structurally important cysteine residues (Hallbook et al., 1991). A mammalian homolog, designated Neurotrophin-5 was cloned in both human and rat (Berkmeier et al., 1991; Ip et al., 1992). As mammalian NT-5 is believed to be the same molecule as Xenopus NT-4, the protein is now referred to as NT-4/5.

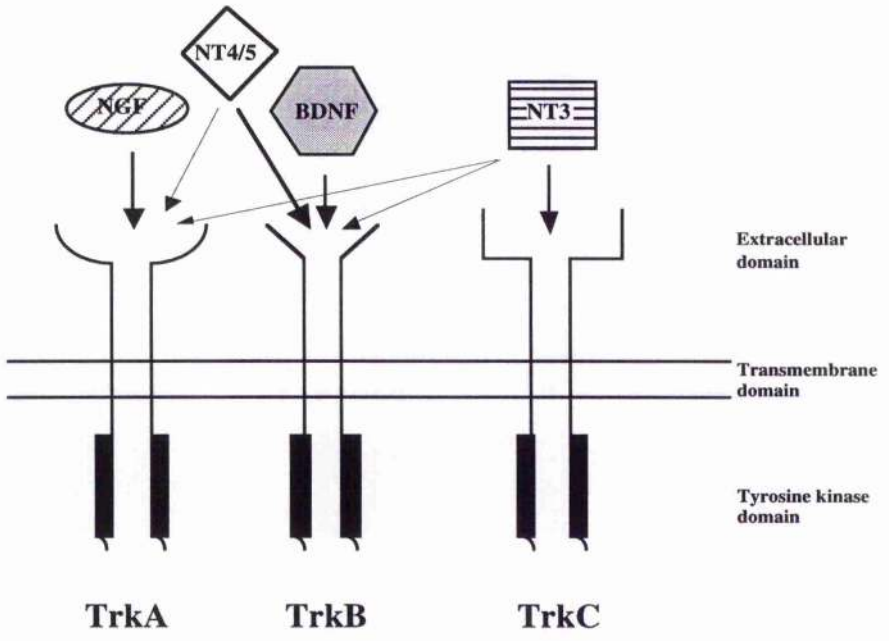
NT-4/5 has been shown to be a mammalian-specific survival factor for distinct populations of sensory neurons. For example, NT-4/5 supports the survival of NGF-

dependent neurons of the murine trigeminal and jugular ganglia during the earliest stages of target field innervation in addition to supporting BDNF-dependent nodose neurons during the period of naturally occurring neuronal death. Mammalian NT-4/5 has negligible survival promoting activity on chick neurons. Since *Xenopus laevis* NT-4 has greater activity on chick neurons than mammalian NT-4/5, this lack of activity of mammalian NT-4/5 on chick neurons may reflect sequence divergence between mammals and chick (Davies et al., 1993). In the central nervous system, NT-4/5 supports the survival of embryonic rat motoneurons and cholinergic forebrain neurons in vitro (Henderson et al., 1993; Friedman et al., 1993).

NT-4/5-deficient mice show severe neuronal loss in nodose, petrosal and geniculate ganglia, whereas sympathetic neurons of the superior cervical ganglion, facial motoneurons and midbrain dopaminergic neurons are not affected (Conover et al., 1995; Liu et al., 1995; Erickson et al., 1996).

Neurotrophin-6

To date, NT-6 has only been identified in the teleost *Xiphophorus maculatus* (Gotz et al., 1994). The predicted structure of NT-6 suggests that the precursor polypeptide is comprised of 186 amino acids and has a molecular weight of 31.5 kDa. This is proteolytically cleaved to give the mature 143 amino acid protein. All six of the structurally important cysteine residues are conserved. NT-6 mRNA is expressed in the developing and mature brain, the gill, liver, eye, skin, spleen, heart and skeletal muscle of adult fish (Gotz et al., 1994). Like NGF, NT-6 can support the survival of embryonic chick dorsal root ganglion neurons and sympathetic neurons but not nodose or ciliary neurons (Gotz et al., 1994), suggesting a spectrum of biological activity that is similar to that of NGF.



Adapted From Davies, 1994b

Figure 1.2: Schematic diagram summarising the interaction of neurotrophins with members of the Trk family of tyrosine kinase receptors.

Neurotrophic Factor Receptors

The members of the NGF family recognise two type of receptors that can be distinguished according to their pharmacological properties (Meakin & Shooter, 1992). One of these types binds all the factors with equal low affinity, having a dissociation constant of 10^{-9}M. In many cells, this receptor corresponds to a cell surface protein with a molecular weight of 75 kDa, and it is referred to as p75 (Johnson et al., 1986; Radeke et al., 1987; Large et al., 1989). The precise role of p75 is as yet poorly understood, but it may act in some way to form part of a complex with, or interact in some other way with the second class of receptors (Bothwell, 1991; Thoenen, 1991). These are products of the *trk* oncogenes, a family of related tyrosine kinase-bearing receptors, Figure 1.2. They show specific, high affinity binding for the neurotrophins and are capable of internalising the bound factor, unlike p75 (Chao, 1992; Meakin et al., 1992). The binding of neurotrophins

to the *trk* receptors induces phosphorylation of tyrosine residues in the kinase domain which in turn activate a number of intracellular secondary messenger systems (Kaplan et al., 1991a, b; Klein et al., 1991a; Jing et al., 1992).

The p75 Receptor

The human p75 gene was discovered using gene transfer assays with subsequent immunological detection of the transfected NGF receptor-expressing cells (Chao et al., 1986). The gene encodes a glycoprotein consisting of a single transmembrane domain polypeptide of 427 amino acid residues, 399 of which make up the mature protein (Johnson et al., 1986; Radeke et al., 1987; Large et al., 1989). The extracellular domain bears four conserved cysteine repeats which have been demonstrated to be responsible for ligand recognition. The cysteine repeats are common to members of a family of cell surface receptors that includes the Fas antigen and Type I and Type II receptors for tumor necrosis factor (Nagata & Golstein, 1995). The intracellular domain of p75 contains a small segment that is homologous to the death domain of Fas and Type I receptor for tumor necrosis factor (Nagata & Golstein, 1995; Chapman, 1995). A soluble, truncated form of p75 has been isolated from conditioned media of p75-producing cells and in various biological fluids (DiStefano & Johnson, 1988). This comprises the extracellular domain only and is thought to be produced by post-translational processing as opposed to differential splicing. The biological significance of this truncated form is unclear. It binds NGF, BDNF and NT-3 with equal affinity to the full length receptor. Truncated p75 may limit the activity of neurotrophins by competing for these factors with the full-length receptor or act as a neurotrophin transporter.

The initial characterisation of p75 indicated that it is insufficient for high-affinity binding of neurotrophins and NGF responsiveness (Chao et al., 1986; Radeke et al., 1987; Hempstead et al., 1989). The finding that the trk tyrosine kinases are essential for high-affinity binding and biological function of the NGF family of

neurotrophins and can mediate the effects of neurotrophins in the absence of p75 has cast questions as to the relevance of p75 for neurotrophic function (Chao, 1990; Meakin et al., 1992). The p75 neurotrophin receptor belongs to a family of transmembrane molecules including Fas and CD40 (Chao, 1994) which also serve as receptors for the tumor necrosis factor family of cytokines. Each receptor shares a common extracellular structure containing conserved cysteine-rich repeats. Because all the members of the NGF family bind p75 with similar affinity, p75 may either act as a common subunit in a neurotrophin receptor complex with the trk family members or it may mediate the actions of the neurotrophins by independent mechanisms. The role of the intracellular domain still remains to be fully elucidated. However, it has been shown that deletions in this region interfere with the ability of p75 to form functional high affinity NGF receptors (Hempstead et al., 1989). Recent evidence suggests that the intracellular domain has sphingomyelinase activity and activates ceramide intracellular second messenger pathways (Dobrowsky et al., 1994).

Studies of p75-deficient murine neurons *in vitro* have shown that p75 is not required for the survival response of neurons to neurotrophins. However, the response of embryonic sensory and sympathetic postnatal neurons which are p75-deficient to NGF requires a higher NGF concentration that wild type cells. This suggests that p75 specifically enhances the sensitivity of NGF-responsive neurons to NGF (Davies et al., 1993; Lee et al., 1994a). The sensitivity of neurons to other neurotrophins is not reduced in the absence of p75. This is in keeping with the phenotype of p75 deficient mice which exhibit a partial loss of NGF-dependent sensory and sympathetic neurons with no major effects on neurons of the central nervous system (Lee et al., 1992; Lee et al., 1994b).

p75 has been shown to selectively modulate the actions of neurotrophins in cells expressing different Trk receptors. The ability of NT-3 to activate TrkA in PC12 cells

is enhanced when p75 is prevented from binding to TrkA using antibodies or when p75 expression is very low. (Clary & Reichardt, 1994; Benedetti et al., 1993).

There is some experimental evidence to suggest that p75 is able to promote apoptosis in the absence of a ligand. In PC12 cells grown without NGF, p75 expression appears to accelerate apoptosis (Rabizadeh et al., 1993). Likewise, antisense p75 oligonucleotides can promote the survival of postnatal rat dorsal root ganglion cells grown without NGF (Barrett & Bartlett, 1994). In contrast, antisense p75 oligonucleotides reduce the survival-promoting effects of NGF in embryonic rat DRG neurons suggesting that there is a developmental switch in the role that p75 plays in regulating apoptosis (Barrett & Bartlett, 1994). p75 cytotoxicity has also been shown to be triggered by NGF binding (Farde et al., 1996; Casaccia-Bonnefil et al., 1996). Anti-NGF antibodies or antibodies that prevent NGF binding to p75 cause a significant reduction in apoptosis of chick embryo retina prior to innervation from the tectum (Farde et al., 1996). Mature oligodendrocytes grown *in vitro* rapidly undergo apoptosis when NGF is added to the culture medium (Casaccia-Bonnefil et al., 1996). In both cases, the cells express p75 but not TrkA. This raises the possibility that in the absence of TrkA, NGF-bound p75 conveys a death-promoting signal.

Trk Tyrosine Kinase Receptors

The human *trk* (tropomysosin kinase) proto-oncogene locus was first identified when it became malignantly active in a colon carcinoma patient (Martin-Zanca et al., 1986; Martin-Zanca et al., 1989). This proto-oncogene was found to encode two tyrosine protein kinase isoforms of 790 and 796 amino acid residues known as TrkA (Martin-Zanca et al., 1989; Meakin et al., 1992; Barker et al., 1993; Horigome et al., 1993) which exhibits the structural characteristics of a growth factor receptor (Chao, 1992). The structure includes a signal peptide followed by an extracellular domain responsible for interaction with NGF, a single transmembrane domain and a

cytoplasmic region that encompasses the tyrosine kinase catalytic domain (Martin-Zanca et al., 1989).

The trkA gene was found, by in situ hybridisation, to be highly expressed in NGF dependent neurons in the mouse embryo (Martin-Zanca et al., 1990). A large body of evidence has accumulated showing that TrkA is a component of the NGF high affinity receptor. For example, NGF stimulates tyrosine autophosphorylation of TrkA in PC12 cells, in sensory neurons of spinal ganglia and in NIH 3T3 cells transfected with trkA cDNA (Kaplan et al., 1991a,b). Furthermore, ectopic expression of trkA in NGF non-responsive cells renders them responsive to NGF (Allsopp et al., 1993).

Low stringency hybridisation of cDNA libraries using trkA probes led to the subsequent isolation of two related receptors, trkB and trkC. The intracellular tyrosine kinase domains of TrkA, TrkB and TrkC share 85% sequence homology. trkB has been shown to be the signalling receptor for BDNF (Klein et al., 1991a; Glass et al., 1991; Soppet et al., 1991; Squinto et al., 1991) and NT-4 (Berkemeier et al., 1991; Ip et al., 1992; Klein et al., 1992), whereas TrkC has been shown to be the signaling receptor for NT-3 (Lambelle et al., 1991). There appears to be some cross-talk between NT-3 and TrkA and TrkB (Klein et al., 1991b; Soppet et al., 1991; Squinto et al., 1991).

Several isoforms of the receptor tyrosine kinases have been discovered. However, their physiological significance is not fully understood. The trkA gene encodes two proteins that differ due to the presence of six amino acids in the extracellular domain (Barker et al., 1993). The trkB gene generates several different transcripts that, apart from the full length receptor, encode two truncated receptors lacking the entire intracellular catalytic domain (Klein et al., 1990a,b; Middlemas et al., 1991). TrkB also has several other isoforms that contain deletions in the leucine rich motifs of the extracellular domain (Ninkina et al., 1997). The trkC gene encodes at least four isoforms: the full length receptor, two isoforms with different

insertions in the tyrosine kinase domain, and a truncated receptor that lacks the intracellular tyrosine kinase domain (Valenzuela et al., 1993; Tsoulfas et al., 1993).

Studies on mice that have null mutations in either TrkA, TrkB or TrkC have further elucidated the role of these receptors in the nervous system. TrkA-deficient mice are insensitive to pain and show loss of neurons in distinct populations. They exhibit more than 90% neuronal cell loss in sympathetic ganglia at P0 and at the same age show 70% loss of neurons in the trigeminal and dorsal root ganglia (Smeyne et al., 1994). Other deficiencies in the central nervous system have also been noted (Smeyne et al., 1994). Likewise, TrkB-deficient mice display neuronal loss in distinct populations. These include more than 80% neuronal loss in the nodose ganglia and 30% neuronal loss in the dorsal root ganglia at P0 (Klein et al., 1993). TrkC-deficient mice exhibit 20% neuronal loss in the dorsal root ganglia and a range of deficiencies in the central nervous system at P0 (Klein et al., 1994). These findings reaffirm the role of the Trk receptors in modulating neurotrophin function. The TrkA-deficient mice exhibit deficiencies in neurons that are NGF-dependent such as those of the trigeminal ganglia, and the phenotype is highly similar to that of NGF deficient mice. This is consistent with the role of TrkA as the high-affinity neurotrophin receptor for NGF. Likewise, TrkB-deficient and BDNF-deficient mice exhibit similar phenotypes. The phenotypes of TrkC and NT-3 Knockouts are not identical suggesting that there may be signalling of NT-3 via TrkA and TrkC during development. No gross phenotype is observed in structures of the central nervous system that express *trkC* transcripts, although TrkC null mutant mice generally do not survive until adulthood (Klein et al., 1994). Detailed neuroanatomical analysis of these mice has revealed specific defects in the cochlear ganglion and in the innervation of hair cells within the inner ear (Schimmang et al., 1995). In addition, the vestibular ganglia of TrkC null mutant mice also have significantly reduced numbers of neurons (Schimmang et al., 1995). These detailed studies have also revealed differences between the phenotypes of TrkC and NT-3 deficient mice. For example, there is a 66% reduction in the complement of neurons within the lumbar

DRG of neonatal NT-3 null mutant animals (Ernfors et al., 1994; Fariñas et al., 1994) In contrast, in the lumbar DRG of trkC$^{-/-}$ animals, there is only an 18% reduction (Klein et al., 1994 ; Minichiello et al., 1995). Similarly, the number of neurons in the spinal ganglia of NT-3$^{-/-}$ mice is reduced by around 86%, whereas the decrease seen in TrkC$^{-/-}$ neonates is 51% (Schimmang et al., 1995). These differences may be due to the ability of NT-3 to signal via the TrkA or TrkB receptors, since NT-3 has been demonstrated to promote the in vitro survival of mid-gestation trigeminal and nodose sensory neurons from TrkC$^{-/-}$ embryos, but not from embryos that also lack functional TrkA or TrkB receptors (Davies et al., 1995).

Neurotrophic Factors not Related to NGF

Other molecules not related to NGF have been shown to promote the survival of neurons of the peripheral and central nervous systems in vitro.

Ciliary Neurotrophic Factor

Ciliary neurotrophic factor (CNTF) was initially identified as a result of its ability to promote the survival of the parasympathetic neurons of the chick ciliary ganglion in vitro (Adler et al., 1979). CNTF was purified from extracts of embryonic chick intraocular tissue (Barbin et al., 1984) and subsequently from adult rat sciatic nerve (Manthorpe et al., 1986). Partial amino acid sequence data gave the basis for the cloning of the cDNAs for rat and rabbit CNTF (Stockli et al., 1989; Lin et al., 1989). Subsequently, mouse and human clones were identified (Kaupmann et al., 1991; Lam et al., 1991). CNTF is a 200 amino acid protein with a molecular weight of 22.7 kDa (Stockli et al., 1989; Lin et al., 1989). Although, no high degree of sequence homology has been found between CNTF and other known proteins, structural similarities have been identified between CNTF and other cytokines such leukemia inhibitory factor and interleukin-6 based on the tertiary structure of these proteins (Bazan, 1991).

CNTF supports the survival of several population of Peripheral and Central Nervous System neurons in vitro. These include the sensory neurons of the chick ciliary, nodose, trigeminal and dorsal root ganglia and the sympathetic neurons of the lumbar sympathetic ganglia (Barbin et al., 1984; Manthorpe & Varon, 1985). In the CNS, CNTF provides in vitro trophic support for spinal motoneurons (Arakawa et al., 1990), hippocampal neurons (Ip et al., 1991) and Purkinje cells (Larkfors et al., 1994).

CNTF binds to a multi-component receptor. The receptor complex is believed to have two sub-units in common with the receptor complex for IL-6 (gp130) and one in common with the LIF receptor (LIFR β)(Davis & Yancopoulos, 1993). A third receptor component, CNTFR α, has been identified as being essential for receptor functioning (Ip et al., 1993). In CNTF-responsive cells, the binding of CNTF to its receptor induces tyrosine phosphorylation of gp130 and LIFRβ. The phenotype on CNTFRα Knockout mice is much more severe than that of CNTF Knockout mice. This suggests that a second ligand for the CNTFRα exists which regulates embryonic motoneuron survival.

Fibroblast Growth Factor

The founder members of this growth factor family are basic and acidic fibroblast growth factors (aFGF and bFGF). These are cytosolic proteins of approximately 16kDa, sharing about 55% amino acid sequence homology (Esch et al., 1985). They are both found in the embryonic brain (Risau et al., 1988) and in adult nervous tissues (Gospodarowicz, 1990) and were initially identified as mitogens for fibroblasts and myoblasts (Gospodarowicz, 1990). They were shown to induce the division of endothelial cells, indicating that FGFs may play a role in blood vessel formation (Folkman & Klagsburn, 1987).

A number of studies have also shown that FGF (mostly aFGF and bFGF) can promote the survival of embryonic neurons *in vitro*. These include neurons from the

hippocampus (Walicke et al., 1986), the cerebral cortex, late embryonic rat striatum, septum, and thalamus (Walicke, 1988), and the chick ciliary ganglion and spinal cord (Unsicker et al., 1987). Both aFGF and bFGF have been shown to induce fibre outgrowth from several cell types, including PC12 cells, newborn rat chromaffin cells (Claude et al., 1988), and postnatal rat retinal ganglion cells (Lipton et al., 1988).

The exact role of aFGF and bFGF in the developing nervous system remains unclear. More recently, however, studies have been carried out using FGF-5 (Hughes et al., 1993). This was originally isolated as the product of a human oncogene and later identified to be a member of the FGF family (Zhan et al., 1988) although unlike aFGF and bFGF it is thought to be a secretory protein. Recombinant human FGF-5 promotes the survival of chick embryonic spinal motoneurons *in vitro* and both FGF-5 mRNA and protein can be detected in embryonic rat skeletal muscle during the time of naturally occurring motoneuron death as well as in adult muscle (Hughes et al., 1993). This indicated a potential role for FGF-5 as a survival factor during embryonic development for motoneurons and a putative role in maintenance in the adult.

Growth Promoting Activity

GPA is a chick growth factor which shows 57% amino acid sequence homology with rat CNTF and which can support the survival of ciliary neurons *in vitro* (Eckenstein et al., 1990; Leung et al., 1992), suggesting that GPA is the chick homologue of mammalian CNTF (Eckenstein et al., 1990). The mRNA transcript for this 21.5kDa protein is most highly expressed in the uvea of the eye which contains tissues innervated by the ciliary ganglion. GPA mRNA is also expressed in the E18 sciatic nerve of the chick (Leung et al., 1992). Expression of GPA in the chick eye increases between E11 and E19 which is the period when neurons of the ciliary ganglion undergo naturally occurring neuronal death (Leung et al., 1992). *In vitro* GPA can support neurons of the ciliary ganglion and lumbar sympathetic chain. In

contrast to CNTF, GPA also promotes the survival of E8 DRG neurons (Eckenstein et al., 1990; Manthorpe et al., 1982). GPA has a signal sequence in contrast to CNTF. Therefore it could be secreted and may be a target derived factor. The timing and localisation of its expression in the eye supports the notion that GPA is the target derived survival factor for chick ciliary neurons in vivo (Leung et al., 1992). GPA signals through a GPI-anchored protein, GPAR α (Heller et al., 1993). This receptor is expressed in several neuronal populations prior to the period of neuronal death and on cells not previously shown to be targets of either CNTF or GPA.

Glial Cell Line-Derived Neurotrophic Factor

Glial cell line-derived neurotrophic Factor (GDNF) was initially purified and cloned (Lin et al., 1993) from the rat B49 glial cell line and identified as a distantly related member of the transforming growth factor-β (TGF-β) family (Massague et al., 1994).

GDNF protects adult dopaminergic neurons from axotomy-induced degeneration in vivo (Tomac et al., 1995; Beck et al., 1995). It also promotes the survival of embryonic motoneurons in vitro being more potent than any of the members of the NGF family or CNTF. In vivo, GDNF rescues facial motoneurons from axotomy-induced cell death (Henderson et al., 1994). GDNF mRNA has been detected in the vicinity of motoneurons during the period of naturally occurring neuronal cell death. More recent but similar studies on facial motoneurons have verified GDNF's importance in motoneuron survival (Yan et al., 1995; Oppenheim et al., 1995). Likewise, GDNF has been shown to have survival promoting effects on the certain chick sensory and sympathetic neurons in vitro (Buj-Bello et al., 1995).

More recently two further members of the GDNF family have been identified, these are neurturin and persephin (Kotzbauer et al., 1996). Neurturin has been shown to promote the survival of embryonic chick sensory neurons in vitro but persephin has

not shown any neurotrophic effects to date (A. Forgie and A.M. Davies, unpublished observations).

Intracellular Mechanisms Regulating Neuronal Survival

Until recently, little was known about the intracellular mechanisms regulating neuronal survival whilst much was known about the role of extrinsic factors. To date, the intracellular mechanisms involved in the suppression of apoptosis in neurons by neurotrophic factors are still unknown. The recent discovery of the bcl-2 protein family has enabled some progress to be made in the understanding of the regulation of apoptosis in this system.

The bcl-2 Protein Family

In 1985, the human bcl-2 (B-cell lymphoma/leukaemia -2) gene was identified at the breakpoint of the translocation between chromosomes 14 and 18 found in a high proportion of human follicular B-cell lymphomas (Tsujimoto & Croce, 1986). This translocation moves bcl-2 from its normal position at 18q21 into juxtaposition with the immunoglobulin heavy chain locus at 14q32 which results in its transcriptional activation and the overproduction of the 26kDa bcl-2 protein in lymphoma cells.

Traditionally, oncogenes were thought to control tumourigenesis by increasing cell proliferation, however, evidence has accumulated that bcl-2 overexpression leads to tumorogenesis by suppressing the signals that would otherwise lead to apoptotic cell death. For example, overexpression of bcl-2 can prevent apoptosis after growth factor withdrawal in certain haematopoitic cell lines (Nunez et al., 1990; Vaux et al., 1992; Cuende et al., 1993; Otani et al., 1993) and in primary cultures of neuronal cells (Allsopp et al., 1993; Garcia et al., 1992).

The regulation of cell death by the bcl-2 protein family has been conserved through evolution. The nematode *Caenorhabditis elegans* undergoes developmental cell

death which is regulated by several proteins, one of which, ced-9, shows considerable amino acid homology with bcl-2, particularly in the BH1 and BH2 domains. These are the regions of highest homology between bcl-2 family members within a given species as well as between species (Oltvai et al., 1993; Yin et al., 1994). Overexpression of human bcl-2 in *Caenorhabditis elegans* prevents cell death in cells which rely on the actions of ced-3 and ced-4 to induce death (Hengartner & Horvitz, 1994b). ced-3 is related to the mammalian caspase proteins indicating further evolutionary conservation in the death pathway. The bcl-2 gene itself has been isolated in mammals and chick (Eguchi et al., 1992), and two homologues have been discovered in the anuran *Xenopus laevis* (Cruz-Reyes & Tata, 1995), both of which contain BH1 and BH2.

bcl-2 is the founder member of a growing family of genes that regulate cell survival (Steller et al., 1995; Thompson, 1995; Vaux, 1993) (Table1.2) including bcl-x (Boise et al., 1993; Fang et al., 1994), bad (Yang et al., 1995), bak (Kiefer et al., 1995; Chittenden et al., 1995), bax (Oltvai et al., 1993), bik (Boyd et al., 1995), bfl-1 (Choi et al., 1997), BRAG-1 (Thompson, 1996), mcl-1 (Kozopas et al., 1995) and A1(Lin et al., 1993). Homology between family members is greatest in two regions, the BH1 and BH2 domains. Recent work suggests another two homology regions: BH3 found in all family members and BH4 which is thought to be important for the action of the death-inducing family members. In addition to this, the α or full-length forms of the bcl-2 family proteins contain a hydrophobic stretch of amino acids at their C-termini which appears to be important for membrane attachment.

Pro-apoptotic	Anti-apoptotic
bax	bcl-2
bad	bcl-xL
bak	Mcl-1
bik	A1
bcl-x_S	BRAG-1
	Bfl-1

Table 1.2: Members of the bcl-2 protein family

bcl-2

The murine bcl-2 gene is located on Chromosome 1 and contains two exons separated by more than 15kb. There are two mRNAs produced by alternative splicing. A 7.5kb transcript encodes the 236 amino acids that encodes bcl-2α which has a molecular mass of 26kDa. A second smaller 2.4kb transcript encodes the 199 amino acids of bcl-2β. This smaller protein has a molecular mass of 22kDa and lacks the C-terminal hydrophic domain (Negrini et al., 1987). bcl-2β does not prevent apoptosis, suggesting that the carboxy terminal hydrophobic domain has functional significance (Tanaka et al., 1993).

Gene transfer experiments have shown that bcl-2 expression is able to prevent apoptosis in certain haematopoietic cell lines following cytokine deprivation (Nunez et al., 1990; Vaux et al., 1992; Cuende et al., 1993; Otani et al., 1993). Also, microinjection of a bcl-2 expression vector into embryonic neurons that are dependent on one or more members of the NGF family of neurotrophic factors for their survival has shown that these neurons can be rescued by bcl-2 overexpression after neurotrophic factor deprivation (Allsopp et al., 1993; Garcia et al., 1992). CNTF-dependent ciliary neurons cannot be rescued by bcl-2

following removal of CNTF from the culture medium. By growing neurons of the trigeminal mesencephalic nucleus in CNTF instead of BDNF these cells also become refractory to the effects of bcl-2, indicating that there at least two death pathways operating in neurons that can be distinguished by their susceptibility to bcl-2. Moreover, neurons can die by either pathway depending on which factors they have been exposed to.

Further evidence for a role for bcl-2 in regulating neuronal survival comes from studies of dorsal root ganglion neurons from mice constituitively overexpressing bcl 2. These show extended survival in vitro without trophic support as compared to wild type neurons (Farlie et al., 1995). In addition, overexpression of bcl-2 can prevent motor neuron death induced by facial nerve axotomy (Dubois-Dauphin et al., 1994) and sciatic nerve axotomy (Farlie et al., 1995). Not only are motor neurons preserved by increased expression of bcl-2, but also the surviving neurons retain functional electrophysiological properties including a functional second messenger system for at least 20 days after injury (Alberi et al., 1996).

bcl-2 overexpression can also rescue cells following various other apoptotic stimuli (Zhong et al., 1993a) such as baculovirus infection (Alnemri et al., 1992), glutamate toxicity (Behl et al., 1993; Zhong et al., 1993b), c-myc induced apoptosis (Fanidi et al., 1992; Vaux et al., 1988), etoposide-induced apoptosis (Kamesaki et al., 1993), adenovirus infection (Rao et al., 1992a,b), chemotherapeutic drugs (Miyashita & Reed, 1993; Dole et al., 1994), and retinoic acid (Okazawa et al., 1996).

The bcl-2 protein is found in the adult mouse within various cell types and tissues characterised by high cell turnover, such as the spleen and thymus (Negrini et al., 1987; Hockenbery et al., 1991). Within these areas it is topographically restricted to long-lived progenitor cells that renew cell lineages such as the germinal centres of lymphoid follicles. In the adult, bcl-2 is also found in several postmitotic cells that

require an extended life span such as neurons (Abe et al., 1993). In the peripheral nervous system, Bcl-2 expression is maintained throughout life but in the central nervous system it declines with age (Merry et al., 1994).

Immunohistochemical techniques have shown that Bcl-2 protein is found in a wide variety of tissues other than neurons and lymphocytes in the human embryo (Lu et al., 1993) such as breast duct epithelium, liver and the lower crypts of the small and large intestines. Interestingly, in the adult, long-lived cells of mesodermal origin such as muscle, frequently do not express *bcl-2* (Hockenbery et al., 1991). In the chick embryo, there is expression in muscle and the bursa as well as in the other tissues described for mammalian embryos (Lu et al., 1993).

The localisation of the Bcl-2 protein within cells has also been closely studied. In cell lines, Bcl-2 has been found to be integrated into the lipid bilayer of microsomal membranes, in particular those of the perinuclear endoplasmic reticulum (Chen-Levy et al., 1990). A 19 amino acid COOH-terminal hydrophobic signal anchor sequence is responsible for membrane localisation (Nguyen et al., 1993). Protease protection assays have shown that Bcl-2 is predominantly localised to the cytoplasmic face of membranes (Chen-Levy et al., 1990). This localisation is consistent with the theory that Bcl-2 may control apoptosis by regulating calcium fluxes through the endoplasmic reticulum (Lam et al., 1994). Direct evidence of ion channel activity of Bcl-2 and Bcl-x_L has been obtained from recent *in vitro* studies (Minn et al., 1997, Schendel et al., 1997). The structure of Bcl-x_L reveals a compact seven α–helical bundle, at the centre of which lies a hairpin comprised of two long hydrophobic core α-helices surrounded by five amphipathic α-helices (Muchmore et al., 1996). The two core α-helices are presumed to insert perpendicularly across the lipid bilayer with the surrounding amphipathic helices folding upwards and resting on top of the membrane (Schendel et al., 1997). Further *in vitro* data indicates that Bax, a pro-apoptotic member of the Bcl-2 family, also has channel activity (Reed, 1997). As Bax can heteodimerise with either Bcl-2 or Bcl-x_L, when they are in their compact α-

helical bundles in aqueous environments, these proteins may produce heterodimeric channels when integrated into membranes.

Other work has shown that Bcl-2 is a mitochondrial membrane protein (Hockenbery et al., 1990; Krajewski et al., 1993; Nakai et al., 1993; Nguyen et al., 1993; Tanaka et al., 1993; Tsujimoto & Croce, 1986), suggesting that Bcl-2 may protect cells from apoptosis by altering mitochondrial function. However, in human mutant cell lines lacking mitochondrial DNA, Bcl-2 still prevents apoptosis (Jacobson et al., 1994). The location of Bcl-2α at the inner surface of the cell membrane could imply a putative role in signal transduction. Indeed Bcl-2α has GTP-binding activity in cellular extracts from both human and murine pre-B cells. Sequence analysis has indicated some weak similarities between the Bcl-2α sequence and the sequences of several small G proteins, particularly in regions that are important for GTP/GDP binding. Six proteins with homology to Bcl-2 include Rho (31%), Ral (33%), Arf2B (31%), Smg-21(36%), Smg-25A(31%) and Ha-Ras (35%) (Halder et al., 1989).

There is evidence to suggest that Bcl-2 may exert its anti-apoptotic effect by protecting cells from oxidative stress. Oxidative stress can lead to apoptosis and certain stimuli of apoptotic death are known to lead to oxidative stress within cells due to excess production of reactive oxygen species (Hockenbery et al., 1993; Buttke & Sandstrom, 1994). Furthermore, antioxidants have been shown to inhibit cell death not suspected to be associated with oxidative stress. For instance, N-acetylcysteine, a weak antioxidant, inhibits apoptosis in hematopoetic cell lines following IL-3 withdrawal (Hockenbery et al., 1993). Cellular antioxidant enzymes protect against several types of apoptotic cell death. Overexpression of manganous superoxide dismutase has been shown to block cell deaths induced by tumour necrosis factor, several chemotherapeutic agents and ionising radiation (Wong et al., 1989; Hirose et al., 1993). Bcl-2 has been shown to localise to the mitochondrial membrane, endoplasmic reticulum and the nuclear envelope all of which are sites implicated in the production of reactive oxygen species (Hockenbery

et al., 1990; Krajewski et al., 1993; Nakai et al., 1993; Nguyen et al., 1993; Tanaka et al., 1993). Bcl-2 can block cell death that results from exposure to hydrogen peroxide, *t*-butyl peroxide, menadione or the glutathione synthetase inhibitor buthionine sulfoxide (Hockenberry et al., 1993; Zhong et al., 1993b). Furthermore, direct oxidative damage to lipid membranes which can occur during apoptosis can be blocked by Bcl-2 (Hockenbery et al., 1993; Hockenbery, 1995). The enhanced resistance of cells overexpressing Bcl-2 to oxidative stress could be due to a mechanism that limits free radical production or it may function downstream to limit the effects of free radicals. Evidence exists to support both scenarios. Bcl-2 has been shown to inhibit lipid peroxidation, a downstream event in oxidative stress (Hockenbery et al., 1993), suggesting that Bcl-2 functions to limit the deleterious effects of free radicals. However, Bcl-2 overexpression has also been shown to directly reduce the generation of reactive oxygen species (Shimizu et al., 1995; Jacobson et al., 1995). Of course, it is possible that Bcl-2 has no direct effects on the oxidant pathway but that the observations made are due to indirect effects of Bcl-2 on this system.

A further putative role for Bcl-2 during cellular morphogenesis has recently been reported. Overexpression of Bcl-2 in a dopaminergic cell line has been shown to induce robust neurite outgrowth without a cessation of cell division (Oh et al., 1996). Most molecular markers of neuronal and or dopaminergic maturation remain unchanged, although levels of the synaptosomal associated protein SNAP-25, which has previously been implicated in playing a role in neuite extension (Osen-Sand et al., 1993) were increased 3 fold (Oh et al., 1996). Further to this, cells of a human tumorogenic neural crest cell line show extensive neurite outgrowth at low serum concentration when transfected with Bcl-2 whilst there is no appreciable effect on survival (Zhang et al., 1996). Expression of neuron-specific enolase was also increased by overexpression of Bcl-2 (Zhang et al., 1996). It is possible that Bcl-2 plays a role in both neurite outgrowth and neuronal differentiation. In accordance

with this, Bcl-2 expression is enhanced during differentiation of neuroblastoma cells (Hanada et al., 1993; Lasorella et al., 1995) and PC12 cells undergo differentiation after Bcl-2 overexpression in serum-free medium (Sato et al., 1994), but not in the presence of serum (Batistatou et al., 1993). The effect of Bcl-2 on differentiation is also observed in other ectodermal derivatives. Cells of the mammary epithelium undergo a morphogenetic, but not a tumorogenic change following Bcl-2 overexpression. Furthermore, there is elevated expression of markers indicative of epithelial-mesenchyme conversion (Lu et al., 1995). In support of a role for Bcl-2 in differentiation, Bcl-2 is expressed during embryogenesis in cells undergoing a morphological transition from undifferentiated stem cells to committed precursor cells, including skin, cartilage and kidney (Lu et al., 1993).

A number of studies have provided data suggesting that Bcl-2 can influence the cell cycle (Willingham & Bhalla, 1994). In human epithelial cell lines, Bcl-2 localises to mitotic nuclei, in particular to the chromosomes. There is strong Bcl-2 expression during mitosis which is most intense during prophase and metaphase and declines during telophase (Lu et al., 1994). However, other workers have found that Bcl-2 is not a cell cycle regulated protein (Reed et al., 1992). Bcl-2 overexpression results in the inhibition of cell division and cell cycle progression. The effects of Bcl-2 seem to be focused at the G_1 to S phase transition giving between 30 and 60% increase in the length of G_1. This is a critical point in the decision between cell cycle progression or induction of physiological cell death (Mazel et al., 1996). Induced Bcl-2 expression results in a retardation of cell proliferation and protection from apoptosis. Both effects can be reversed by Bax (Borner et al., 1994). Furthermore, there is some evidence that Bcl-2 may mediate its effects on the cell cycle through interaction with the tumour suppressor protein p53. A non-conserved region of Bcl-2 (residues 51 to 85) has been found to permit p53-induced apoptosis and inhibit cell proliferation. Deletion of this region reverses these effects (Uhlmann et al., 1996).

Bcl-2-deficient mice appear normal at birth (Nakayama et al., 1993, 1994; Veis et al., 1993; Kamada et al., 1995) and are at first indistinguishable from their litter mates. By 1 week *post partum* they are smaller, have small external ears and immature facial features. The size of mutant mice varies from between 30 - 90% of the weight of their litter mates. The mutation is lethal and there is a wide range in the onset of morbidity from 10 days to 10 weeks, with a clustering at 2-3 weeks. The mice develop polycystic kidney disease and have blood urea and serum creatinine levels significantly higher than their wild type and heterozygous litter mates. The number of lymphocytes markedly decreases within a few weeks of birth while other haematopoietic lineages remain unaffected. Mutant mice initially have black hair but this turns grey at 4 - 5 weeks of age. There are decreased numbers of oocytes and primordial follicles established in postnatal females (Ratts et al., 1995), suggesting a role for Bcl-2 in preventing follicular atresia. The problems with the hair and kidneys suggest that Bcl-2 may have a role where interactions between epithelium and mesenchyme are important. Furthermore, E12 Bcl-2-deficient kidneys grow and develop less well in culture than wild type kidneys, suggesting that the problems observed during kidney development are cell autonomous (Sorenson et al., 1995). Results so far show that the nervous system, intestines, and skin appear normal despite the fact that these organs show high levels of endogenous Bcl-2 expression in wild type mice.

In summary, since the discovery of the Bcl-2 protein, numerous examples of its role in preventing apoptosis have been published (Korsmeyer, 1995). More recent reports have implicated Bcl-2 in other cellular events including differentiation (Hanada et al., 1993; Lasorella et al., 1995) and the cell cycle (Lu et al., 1994). However, the biochemical basis of the actions of Bcl-2 remains elusive.

Bax

Bax (Oltvai et al., 1993) is a Bcl-2-associated protein showing 21% homology with Bcl-2 at the amino acid level. It is a 21kDa protein initially identified by

coimmunoprecipitation with Bcl-2. The human *Bax* gene is located on chromosome 19 and has four possible transcripts. The full transcript is Baxα whilst Baxβ lacks the transmembrane anchor domain. BaxT lacks the transmembrane anchor domain, BH1 and BH2 whilst Baxγ lacks exon 3 about which little is known (Apte et al., 1995). Bax can heterodimerise with Bcl-2, Bcl-x_L, Mcl-1 and A1 (Sato et al., 1994; Sedlak et al., 1995), and can also form homodimers. Bax is widely expressed in the neonatal rat central nervous system but the levels are 20 to 140 fold lower in the adult with the decrease occurring after the period of naturally occurring neuronal death (Vekrellis et al., 1997). Bax overexpression in IL-3 dependent hematopoetic cell lines accelerates apoptosis following factor withdrawal and inhibits the death repressor action of Bcl-2 in these cells (Oltvai et al., 1993). Analysis of point mutations in Bcl-2 suggests that direct binding of Bcl-2 to Bax is essential for the death-repressor activity of Bcl-2 (Yin et al., 1994). A model has been proposed in which the ratio of Bcl-2 to Bax determines whether cells survive or die following exposure to an apoptotic stimulus (Korsmeyer et al., 1993). Bax-Bax and Bax-Bcl-2 dimer formation require the presence of a conserved domain distinct from BH1 and BH2, termed BH3 (Zha et al., 1996). All members of the Bcl-2 protein family possess this domain except Bad. Overexpression of Bcl-2 increases the half-life of Bax protein, by a process involving post-translational modification (Miyashita & Reed, 1995). Bax also interacts with and antagonises the survival promoting effects of Bcl-x_L. However, mutational analysis has shown that Bcl-x_L can suppress apoptosis by a mechanism that does not require binding to Bax (Cheng et al., 1996).

The glutamate analogue, kainic acid, causes seizures and neuron damage in rodents. In the murine brain, *bax* is upregulated and *bcl-2* is down-regulated in response to kainic acid treatment (Gillardon et al., 1995), suggesting that Bax is involved in the apoptosis that occurs in the central nervous system in response to this stimulus. Likewise, Bax protein is up-regulated by p53 in M1 myeloid leukaemia cells as an

immediate response p53 expression whilst Bcl-2 is down-regulated. Furthermore, TGFβ1 down-regulates Bcl-2 but does not up-regulate Bax. In this case, the cells still undergo apoptosis even when Bax is not upregulated. (Selvakumaron et al., 1994; Zhan et al., 1994).

Transgenic mice overexpressing Baxα, specifically in T cells, show accelerated apoptosis in response to a wide variety of apoptotic stimuli (Brady et al., 1996). Mice with a null mutation in the *bax* gene (Knudson et al., 1995) are viable but exhibit lineage-specific aberrations in cell death. For example, superior cervical ganglia and facial nuclei displayed an increased neuron number. In addition, neonatal sympathetic neurons and facial motor neurons from Bax-deficient mice survive NGF deprivation and axotomy (Deckwerth et al., 1996). Thymocytes and B cells from Bax null mutants display hyperplasia and ovaries display unusual atretic follicles with excess granulosa cells. Males are infertile as a result of disordered seminiferous tubules with an accumulation of atypical premeiotic germ cells but no mature haploid sperm. Multinucleated giant cells and dysplastic cells are accompanied by massive cell death in the testis (Knudson et al., 1995). Thus lack of *bax* expression can result in either hyperplasia or hypoplasia depending upon the cellular context.

Bcl-X

Bcl-X was discovered due to its high sequence homology with Bcl-2 (Boise et al., 1993; Fang et al., 1994). There are three Bcl-x variants , Bcl-x_L, Bcl-x_β, and Bcl-x_s (Boise et al., 1993) which arise due to alternative splicing. Bcl-x_L is a 233 amino acid protein that has the highest homology to Bcl-2 and acts in a similar manner rescuing cells from apoptosis. Bcl-x_β lacks a C-terminal hydrophobic domain required for membrane attachment. Bcl-x_S is a 170 amino acid protein that lacks BH1 and BH2 which are the regions of greatest homology between the family members and which have been implicated in Bcl-2 function (Yin et al., 1994). In contrast to the other Bcl-x variants, Bcl-x_s has consistently been shown to promote apoptosis. It

can sensitise MCF-7 human breast carcinoma cells, which highly express Bcl-x_L, to chemotherapy-induced apoptosis (Sumantran et al., 1995).

The structure of Bcl-x_L, as determined using both X-ray crystallography and nuclear magnetic resonance, consists of two central primarily hydrophobic α-helices which are surrounded by amphipathic helices (Muchmore et al., 1996). The three conserved region, BH1, BH2, and BH3 are in close spatial proximity and form an elongated hydrophobic cleft which may represent the binding site for other family members.

Bcl-x_L and Bcl-x_β are expressed in both embryonic and postnatal tissues of the central nervous system (Gonzalez-Garcia et al., 1995). As well as expression in the brain, *bcl-x* expression has been observed in the dorsal root ganglia, various cells of the immune system, reproductive tissues and a variety of epithelial cells (Krajewski et al., 1994). Intracellularly, *bcl-x* gene products appear to be cytosolic and closely associated with intracellular organelles (Krajewski et al., 1994). In particular the Bcl-x_L protein has been found to be localised to mitochondria (Gonzalez-Garcia et al., 1994).

Microinjection of both Bcl-x_L and Bcl-x_β into primary sympathetic neurons inhibits apoptosis following growth factor withdrawal. This suggests a role for Bcl-x proteins in regulating neuronal survival (Gonzlez-Garcia et al., 1995). Bcl-x_L has also been shown to support the survival of thymocytes (Ma et al., 1995), to protect cells of a cancer cell line, T47D, from p53-mediated apoptosis (Schott et al., 1995), and to prevent apoptosis in murine myeloma cells (Gauthier et al., 1996). When human leukaemia cells differentiate they die by apoptosis. However, Bcl-x_L can prevent this apoptosis indicating that down-regulation of Bcl-x_L may be a component of the apoptotic response that is coupled to cell differentiation in leukaemia cells (Benito et al., 1996). Contrary to these findings, rat Bcl-x_β has been shown to promote apoptosis in promyeloid cells (Shiraiwa et al., 1996)

Studies on Bcl-x-deficient mice (lacking all isoforms) have helped to elucidate its function (Motoyama et al., 1995). The Bcl-x-deficient mice die around E13, exhibiting extensive apoptotic cell death in postmitotic immature neurons of the brain, spinal cord and dorsal root ganglia. Haematopoietic cells in the liver also show increased apoptosis. In addition, Bcl-x null mutant immature lymphocytes have a shortened lifespan in Bcl-x chimeric mice. This suggests a role for Bcl-x promoting the viability of immature cells during the development of the nervous and haematopoietic systems.

Bad

Bad is a 204 amino acid, 22kDa protein containing several PEST motifs (Yang et al., 1995). These are amino acid sequences commonly found in rapidly degraded proteins, and therefore suggest that Bad has a short half life (Rogers et al., 1986). Bad shows limited homology to Bcl-2 and other family members. Homology is restricted to BH1 and BH2 domains where only amino acids that are universally conserved within the family are present in Bad. Bad can dimerise with Bcl-2 and Bcl-x_L but not with Bax, Bcl-x_S, Mcl-1, A1, or itself. It inhibits the death repressor activity of Bcl-x_L but not Bcl-2 in mammalian cells and acts by displacing and replacing Bax in Bcl-x_L: Bax dimers. Since, Bad lacks the C-terminal anchor domain which other Bcl-2 proteins possess. It exists free in the cytosol. *bad* mRNA has been found in the central and peripheral nervous systems, but how this expression changes during development is not known (Yang et al., 1995; Merry & Korsmeyer, 1997).

Bad is a possible candidate for linking signal transduction events to the Bcl-2 protein family and so to the regulation of apoptosis. Phosphorylated Bad complexes with the protein 14-3-3 in the cytosol, whilst non-phosphorylated Bad heterodimerises with Bcl-x_L at intracellular membrane sites. When IL-3 is withdrawn from hematopoetic cell lines that are dependent upon it for their survival, Bad is de-

phosphorylated and binds to Bcl-x_L. It may be that in so doing Bad inactivates the survival promoting effects of Bcl-x_L (Zha et al., 1996).

Bak

Bak was identified using degenerate primers to clone cDNA fragments encoding Bcl-2 homologues (Kiefer et al., 1995; Chittenden et al., 1995). The new homologous sequence was used to isolate full-length cDNA sequences form human heart and Epstein Barr (EBV)- transformed human B-cell libraries. Bak has 53% amino acid sequence homology with Bcl-2 and shows marked similarity to other family members particularly in BH1 and BH2. Expression of Bak reverses the protective effects of E1B 19K in primary cultures of rat sympathetic neurons deprived of NGF (Farrow et al., 1995). It also reverses the survival promoting effects of Bcl-2 in a pro-B cell line (Chittenden et al., 1995). In cell lines derived from neoplastic colon, apoptosis is accompanied by an increase in *bak* mRNA without consistent changes in other Bcl-2 family members, suggesting a role for Bak in intestinal cell apoptosis (Moss et al., 1996). However, Bak is also able to prevent some cell deaths (Kiefer et al., 1995). Bak interacts with Bcl-2 and more strongly with Bcl-x_L. The pro-apoptotic action of Bak is dependent on it binding to Bcl-x_L indicating that it may act by binding to Bcl-x_L and inhibiting its death repressor function. A screen of Bak deletion mutants has shown that the BH3 domain is required both for interaction with Bcl-x_L and promotion of cell death. This has lead to the identification of BH3 as the important domain for this interaction (Chittenden et al., 1995). *bak* mRNA expression is widespread in many tissues (Kiefer et al., 1995). Although there is widespread *bak* mRNA expression in the adult brain (Kiefer et al., 1995) there is little or no Bak protein detected (Krajewski et al., 1996).

Other Bcl-2 Related Proteins

There are other proteins that have been found to be homologous to Bcl-2 but about which only a small amount of information is available. These include A1, BRAG-1,

Mcl-1, Bik and Bfl-1. A1 is a Bcl-2-related haematopoietic tissue specific gene that is induced during myeloid cell differentiation (Lin et al., 1993). It has been shown to prolong cell survival and permit myeloid differentiation (Lin et al., 1996). A1 has a restricted pattern of expression compared to other members of this protein family. It is expressed in murine thymus and spleen, but not in a variety of non-hematopoietic mouse tissues (Lin et al., 1993).

BRAG-1 was isolated from a human glioma cell line and is a 31kDa protein sharing significant homology with Bcl-2 in the BH1 and BH2 regions. BRAG-1 mRNA is predominantly expressed in brain. The transcript expressed in brain is larger than that expressed in glioma cells suggesting that a truncated variant of the BRAG-1 protein may play a role in oncogenesis in glial cells (Thompson, 1996).

Mcl-1 is a 37kDa protein that was initially isolated from the ML-1 human myeloid leukaemia cell line that had been induced to differentiate along the monocyte/macrophage pathway. Mcl-1 can function as a survival promoting factor (Reynolds et al., 1994). It is localised to the mitochondrial and light membrane fraction in ML-1 cells. In the same cells, Bcl-2 is found predominantly in mitochondrial and nuclear membranes (Yang et al., 1995). Mcl-1 protein has also been detected in the soma of sympathetic and dorsal root ganglion neurons (Krajewski et al., 1995). However, little or no protein has been detected in the central nervous system.

Bik is a 160 amino acid, 18kDa protein that can heterodimerise with the anti-apoptotic members of the Bcl-2 protein family promoting cell death. It displays very little homology to other Bcl-2 protein family members lacking both BH1 and BH2 regions. However, Bik does contain the C-terminal hydrophobic domain and the BH3 region, suggesting that BH3 may be critical in modulating death promoter activity (Boyd et al., 1995; Chittenden et al., 1995).

Bfl-1 was initially isolated from the liver of human embryos. It has 72% amino acid identity with murine A1, with BH1, BH2, BH3 and BH4 all highly conserved although the C-terminal anchor domain is not highly conserved. Bfl-1 is highly expressed in bone marrow and also at low levels in the lung, spleen and oesophagus (Choi et al., 1995). Bfl-1 is able to suppress p53-induced apoptosis (D'Sa-Eipper et al., 1996).

Other Proteins that Regulate Cell Survival

In addition to the bcl-2 family members, a number of proteins have been found to play a role in the regulation of apoptosis. One such protein is E2F-1 which is a member of a family of transcription factors that are believed to be regulators of cell cycle progression. Mice which are E2F-1-deficient have excessive T cells due to a lack of apoptosis, yet show testicular atrophy and exocrine gland dysplasia. They also exhibit aberrant cell proliferation (Field et al., 1996; Yamasaki et al., 1996; DeGregori et al., 1995) This phenotype suggests a function both in regulating apoptosis and in regulating cell proliferation. There is no suggestion of an interaction between E2F-1 and any of the genes already characterised as having a role in apoptosis. However, it is likely that as the roles of this protein are further elucidated, some activation of other genes involved in apoptosis will be discovered.

A protein that appears to play a key role in regulating cell survival is p53 (Holstein et al., 1991). In normal cells, p53 acts as a tumour suppressor protein and loss of p53 function appears to play a role in the majority of human tumours. There is some evidence for interaction between bcl-2 family members and p53. The bcl-2 gene contains a 195bp segment in the 5' untranslated region that is necessary for p53 mediated down-regulation of bcl-2 expression (Miyashita et al., 1994). In addition, bcl-2 overexpression is able to prevent p53-dependent apoptosis (Chiou et al., 1994). This suggests that p53 may function upstream of bcl-2 and is critical in determining whether a cell undergoes physiological cell death by apoptosis or enters the cell cycle.

Many proteins can interact with bcl-2 to regulate cell survival. For example, BAG-1 (Takayama et al., 1995) is a bcl-2-binding protein with no significant homology to any family members. Co-expression of bcl-2 and BAG-1 increases protection from apoptosis compared to expression of either protein alone. bcl-2 also interacts with a member of the Ras superfamily of small GTPases, p23-R-Ras (Wang et al., 1995; Fernandez-Sarabia & Bischoff, 1993). A correlation has been found between increased levels of R-Ras protein and faster rates of cell death following growth factor deprivation. bcl-2 can counteract this effect implying that bcl-2 acts downstream of Ras to promote cell survival.

c-myc has been shown to induce apoptosis under certain conditions (Vaux et al., 1988). Inappropriate *c-myc* expression under conditions such as heat shock or serum-deprivation leads to rapid onset of apoptosis (Bissonnette et al., 1992). Constitutive expression of bcl-2 is able to prevent Myc-induced apoptosis (Bissonnette et al., 1992; Fanidi et al., 1992).

Caspases are the mammalian homologues of the nematode *Caenorhabdidits elegans* gene ced-3 and are likely to act downstream of the bcl-2 protein family in the cell death pathway. The first of these proteins identified was ICE (Yuan et al., 1993). Apart from the structural correlation with Ced-3, overexpression of ICE in Rat-1 fibroblasts induces apoptosis suggesting a functional correlation with the nematode protein (Miura et al., 1993). Eight homologues of Ced-3 and ICE have been characterised to date: ICH-1(Kumar et al., 1994; Wang et al., 1994); CPP-32 (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Tewari et al., 1995); ICH-2 (Faucheu et al., 1995; Kamens et al., 1995; Munday et al., 1995); ICErel-III (Munday et al., 1995); Mch-2 (Fernandes-Alnemri et al., 1995a); Mch-3 (Duan et al., 1996; Fernandes-Alnemri et al., 1995b; Lipke et al., 1996); FLICE (Muzio et al., 1996) and ICE-LAP6 (Duan et al., 1996). Ectopic expression of these proteins in a variety of cells promotes apoptosis. There is no evidence for a direct link between the bcl-2 protein family and the caspases. However, in the nematode *Caenorhabditis elegans*

where the cell death pathway is well understood, the bcl-2 homolog, Ced-9, influences the action of the caspase homologue Ced-3 via Ced-4. Very recently the mammalian homolog of ced-4, Apf-1 has been reported.

In light of the recent developments in elucidating the intracellular pathways involved in the control and execution of apoptosis, this project considers the role of one particular family of proteins, the bcl-2 protein family, during the development of the peripheral nervous system. Here, using DNA microinjection, bcl-2, bcl-xL, and bcl-xβ were found to support neuronal survival following growth factor withdrawal. Surprisingly, this was also true for bax. bcl-xs did not show any survival promoting effects when overexpressed in this way. bcl-2 Knockout mice were also used to investigate the effects of the absence of bcl-2 on murine neurons both *in vitro* and *in vivo*. Cells derived from *bcl-2* knockout mice were able to respond as well as their wild type counterparts to neurotrophins but showed poor long-term survival in culture at specific stages in their development. Counts of neuron number *in vivo* using histology showed a clear difference in neuron number later in development after the period of neuronal death. Furthermore, the neurons of the knockout mice are morphologically less mature than those of the wild type mice. In addition, bcl-2-deficient neurons were shown to have shorter neurites than wild type neurons. Therefore, bcl-2 seems to have more than one role in the developing peripheral nervous system with effects reported here on survival, neurite outgrowth and maturation. A further study using *bax* Knockout mice showed that neurons derived from animals homozygous for a null mutation in the *bax* gene survive better than their wild type counterparts long-term *in vitro* at E18 and P1. Also, the cells from the mutant animals were able to survive without neurotrophic factors and indeed after a few days in culture were surviving better without factors than with factors. Furthermore, a proportion of these cells had very short neurites which extended considerably if factors were added to the dish. Although the precise nature of the role played by bax during the development of sensory neurons has not been fully elucidated, the evidence presented here clearly does indicate a role for bax. The role of the bcl-2 protein family in regulating the survival

of developing neurons is complex and, these proteins may play more than one role in the developing nervous system. They not only regulate survival but also may have a role in neuronal maturation and stimulation of neurite outgrowth.

II. MATERIALS AND METHODS

CHAPTER TWO

MATERIALS AND METHODS

TISSUE CULTURE

Dissection Techniques

Instruments

A number of instruments were used in the course of each dissection, including toothed forceps, straight and curved watchmakers forceps, and fine scissors. Instruments were sterilised by flaming after soaking in 100% industrial methylated spirits (IMS). 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 0.5 mm diameter tungsten wire which was cut to 3 to 5 cm lengths and the terminal 1 cm was bent at an angle of approximately 60°. This end was immersed for a few minutes in 0.5 M 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. 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.

Chick Dissections

All procedures were carried out in a laminar flow cabinet using standard sterile technique. Embryos for chick dissections were obtained from fertile White Leghorn eggs incubated at 38°C in a forced-draft incubator (Western C-100). Prior to opening, the eggs were washed twice in 70% IMS to sterilise the surface. To open the eggs, the shell was cracked by tapping with toothed forceps in a line around the

airspace, which is located at the blunt end of the egg if the egg is incubated with this end uppermost. The shell above the cracked line, along with the inner shell membrane, was carefully removed and the embryo extracted by placing a pair of curved forceps beneath the neck. The embryo was then decapitated and 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.

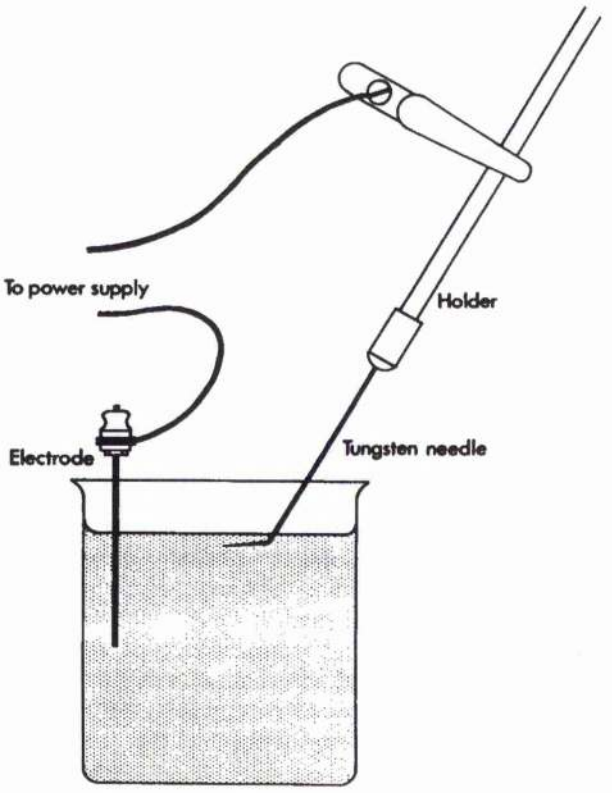


Figure 2.1:Making Tungsten Needles (from Davies, 1988)

Dorsal Root Ganglia (DRG)

The thoracic and abdominal viscera were removed using curved watchmakers forceps, and the posterior abdominal and thoracic walls washed with Hanks Balanced Salt Solution (HBSS) (Gibco) using a Pasteur pipette. The DRG were removed from the lumbosacral region. This was done by passing the points of a pair of straight watchmakers forceps between the ganglia and the spinal cord to sever the spinal roots. The ganglia were then collected by holding the nerve distal to each ganglion with forceps and transferring to a small petri dish containing sterile HBSS. Finally, attached nerves were removed using tungsten needles.

Trigeminal Mesencephalic Nucleus (TMN)

The TMN was dissected by firstly removing the cranial vault, with a pair of watchmakers forceps, to expose the brain. This was then removed by carefully passing a small spatula between it and the cranial base. The midbrain was isolated by two coronal incisions (figure 2.2) and the pia mater, which is the membrane surrounding the brain and spinal cord, was peeled off initially from the ventral aspect. Next, the roof of the cerebral aqueduct was dissected from the midbrain. Finally, the median portion of the nucleus which contains all the TMN neurons, was dissected out using tungsten needles and transferred to sterile HBSS.

Ciliary Ganglion

To dissect the ciliary ganglion, the head was anchored firmly with a pair of watchmakers forceps through the beak and the skin covering of the developing eye carefully removed by scoring around the edge of the eyeball with watchmakers forceps. The eyeball was then extracted as a whole with watchmakers forceps, taking care not to damage it, and the ciliary ganglion simply plucked from it. The spherical ganglion can be easily recognised as distinct from the flattened stalk of the optic nerve, which can also be seen coming out of the rear of the eye. Finally,

the ganglia were collected in sterile HBSS and any attached nerves removed with tungsten needles.

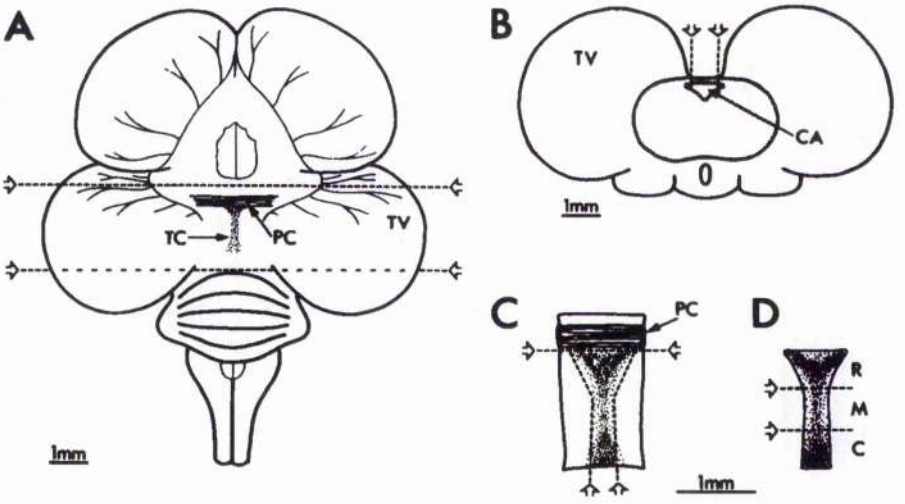


Figure 2.2: The Successive Stages in the Dissection of the Median Part of the TMN

(a) dorsal aspect of the brain showing location of the two coronal incisions (interrupted lines) for isolating the midbrain.

(b) caudal aspect of the two parasaggital incisions for removal of the roof of the cerebral aqueduct after stripping of the pia mater.

(c) dorsal aspect of the roof of the cerebral aqueduct showing the location of the incisions for cutting out the median portion of the TMN. R= rostral; M= medial; C=caudal; TV= tectal vesicle; TC= tectal commissure; PC= posterior commissure; CA= cerebral aqueduct (from Davies, 1988).

Dorso-medial Portion of the Trigeminal Ganglion (DMTG)

The small-diameter cutaneous sensory neurons of the dorsomedial portion of the ganglion (DMTG) were dissected by first removing the brain, as described for the TMN dissection, to reveal the cranial sensory ganglia (figure 2.3). The trigeminal ganglia were removed with a pair of watchmakers forceps, and the ophthalmic lobe removed using tungsten needles, figure 2.3. The dosomedial portion of the

remaining maxillomandibular lobe contains the NGF-responsive cells, whilst the cells of the ventrolateral portion are BDNF-responsive. The two parts were separated as shown in figure 2.3 using tungsten needles and the DMTGs collected in HBSS.

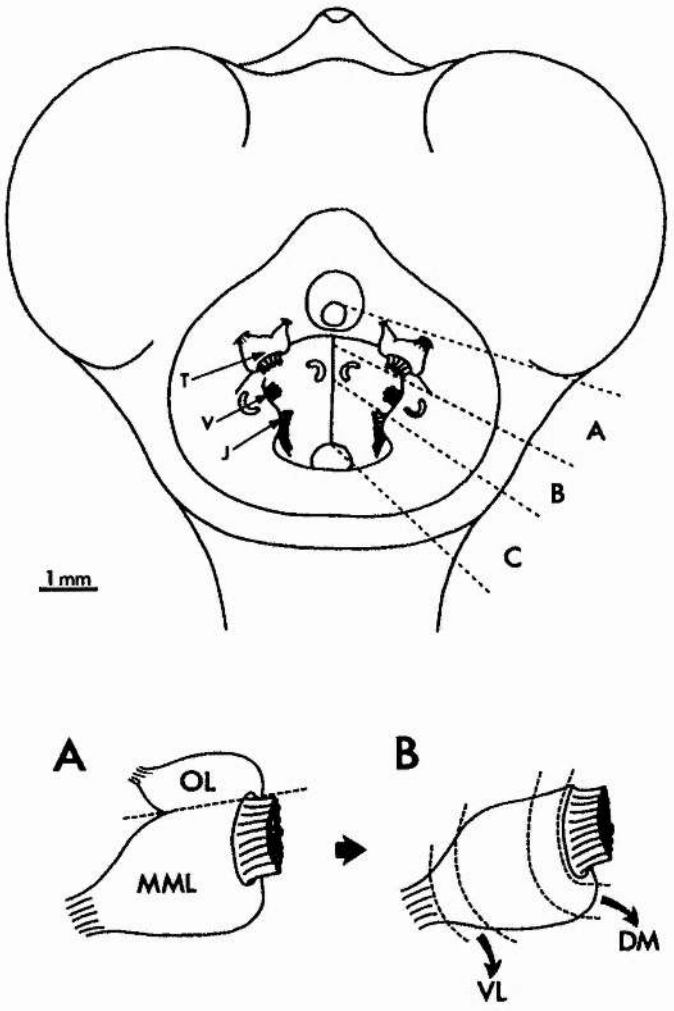


Figure 2.3: Dorsal aspect of the cranial base after removal of the brain showing the lines for subdissecting the tissue into blocks that contain the trigeminal ganglion (T) (block A), the vestibulo-acoustic ganglion (V) and geniculate ganglion (block B), and the Jugular (J) and petrosal ganglia (block C). Subdissection of the trigeminal ganglion is achieved by separating the opthalmic lobe (OL) from the maxillomandibular lobe (MML) along the interrupted line (A). The dorsomedial portion (DM) can be separated from the ventrolateral as shown (B).

Mouse Dissections

Isolation of Mouse Embryos

The abdomen of the pregnant female was washed with 70% IMS and a small incision made in the skin at the front of the abdomen. The skin just above and below the incision was grasped between the index finger and thumb of each hand and the skin pulled away from the incision exposing the abdominal muscles. Using fine scissors, a small hole was made in the anterior abdominal wall allowing air to enter the peritoneal cavity, and so enabling the incision was able to be extended without the risk of cutting the intestines and contaminating the dissection with bacteria. Next, each uterine horn was carefully removed and placed in a sterile petri dish containing L-15. The embryos were removed from the horn and transferred to fresh L-15. For E13 and older embryos this was done by making a single continuous incision along the anti-mesometrial border of each uterine horn exposing the embryos enclosed within their membranes, which were then removed with watchmaker's forceps. Younger embryos were removed by making a small hole in the musculature of the anti-mesometrial border of the uterine horn. The embryo, enclosed within its membranes, was then extruded through this hole due to contraction of the remaining uterine muscles.

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. 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.

For younger embryos, tungsten needles were used to make two coronal incisions through the head, one just above the eye and the other between the maxillary and mandibular processes of the first branchial arch (Figure 2.4). 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.

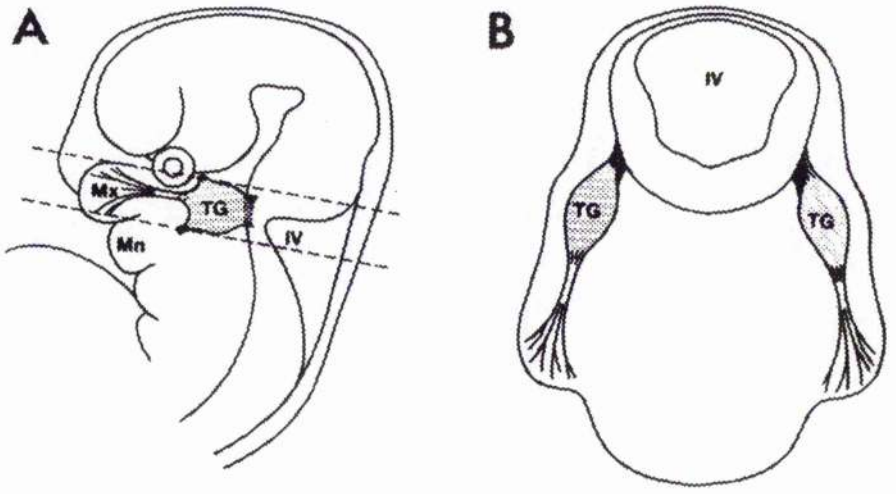


Figure 2.4: Dissection of the mouse trigeminal ganglion. Transverse incisions are represented by interupted lines. Mx= maxillary process; Mn= mandibular process; IV= 4th ventricle; TG= trigeminal ganglion.

Nodose Ganglion

The top of the skull and underlying forebrain were removed using the same plane of section as described for the first incision of the trigeminal dissection. The embryo was decapitated and the head cut in half along the saggital plane using tungsten needles up to E12 and fine scissors thereafter (figure 2.5). 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. The ganglion was then dissected out and cleaned of any attached nerves using the tungsten needles, before being transferred to HBSS.

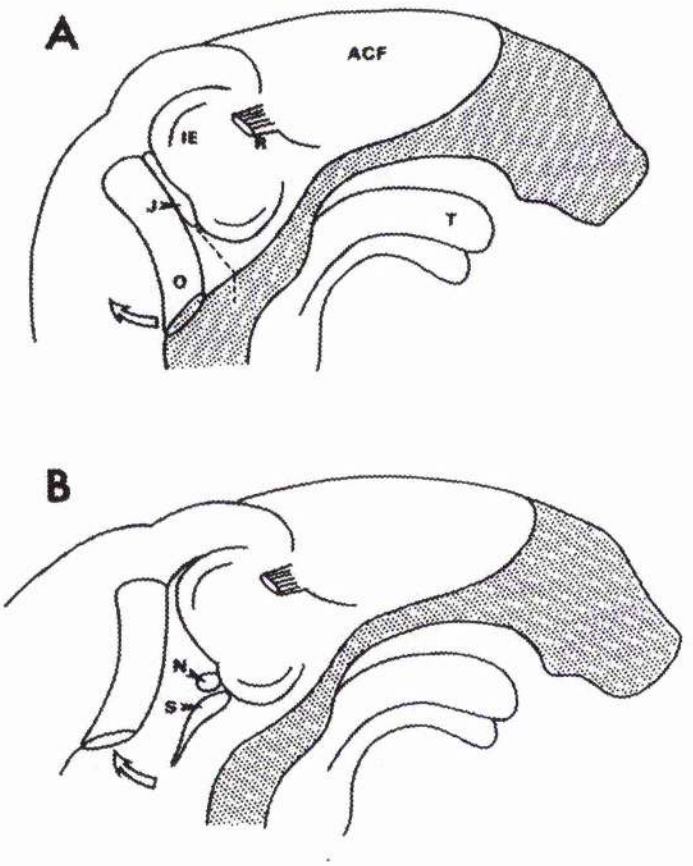


Figure 2.5: Dissection of the Mouse Nodose Ganglion at E14 . A. The incision passing from the jugular foramen (J) to the midline is shown by an interrupted line. The direction in which the large ossified part of the occipital bone (O) should be reflected to open up the jugular foramen after making the previous incision is shown by an arrow. T= tongue; ACF= anterior cranial fossa; R= root of the trigeminal nerve; IE= inner ear. B. The nodose ganglion (N) and the superior cervical ganglion (S) are revealed.

Sympathetic Chain Ganglia

To dissect the sympathetic chain ganglia, the abdominal and thoracic viscera were removed from embryos as described previously for the chick DRG dissection revealing the sympathetic chain lying ventral to the DRG. This was then carefully removed with sharp watchmakers forceps and cleaned of attached nerves and

adherent tissue using tungsten needles, before being transferred to HBSS as described previously.

Tissue Dissociation

For the purpose of this project, it was important that the tissue be dissociated to single cells prior to plating. This ensures that any observed effects are not due to signals passed through intercellular contacts but by direct effects of factors added to the dish or injected into the cells.

Using a Pasteur pipette, the dissected tissue was transferred to a sterile 10 ml conical tube containing 1 to 2 ml of calcium- and magnesium- free HBSS. Balanced salt solutions, such as Hanks contain a combination of salts and glucose which control osmotic pressure and pH whilst providing an energy source. Calcium and magnesium free Hanks was used because calcium and magnesium ions can prevent the trypsin from working. The tissue was centrifuged briefly at 2000 rpm, to pellet the ganglia, and the supernatant removed. 900 μl of fresh HBSS and 100 μl 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 ganglia. For instance for chick embryo ciliary ganglia, 15 minutes trypsinisation was required at E10, 12 minutes is required at E8 and only 10 minutes at E6. The tube was then centrifuged briefly, and as much trypsin solution as possible removed prior to the addition of approximately 10 ml 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 the washing procedures, ganglia were gently pelleted by 2 min centrifugations at 2000 rpm. The ganglia were triturated in 1 ml 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.

Differential Sedimentation

Differential sedimentation was used to separate neurons from non-neuronal cells prior to plating. This technique depends on the differences in sedimentation rates of cells in a liquid medium such that larger cells, such as neurons sediment more quickly than smaller ones (Davies, 1988a). Differential sedimentation was only used for chick neurons as mouse neurons are too small to give good separation until postnatal ages. Also, for transgenic experiments the use of columns would be impractical as each embryo was dissected and the tissue from it dissociated, separately. Because this particular separation technique allows the cells to sediment through a culture medium it gives high neuronal viability at the end of the procedure. It is also very efficient, giving recovery of around 60% of the neurons in the starting tissue, mostly uncontaminated by other cells.

Sedimentation was carried out in a 100ml cylindrical, siliconised, glass dropping funnel with a ground glass outlet tap. This was autoclaved prior to use and assembled under sterile conditions before being clamped vertically to a stand. The funnel was filled with sterile F14 (Gibco) + 10% heat inactivated horse serum to a height of approximately 10 cm and placed on a vibration-free surface, at 2°C, where it was left overnight. The next day, dissociated tissue, prepared as described above, was layered onto the culture medium in the funnel by taking the cell suspension up in a siliconised, flame-polished Pasteur pipette and carefully running it down the inside of the funnel. After one hour, 5ml aliquots were run off into sterile tubes and 0.5ml samples of these fractions placed in a 24 multi-well dish for examination with phase contrast microscopy to determine which fractions contain only neurons. The neuronal fractions were then pooled for plating.

Plating the cells

Neurons were grown on a laminin/polyornithine substratum in 35mm or 60mm diameter plastic petri dishes (Nunc). To prepare these plates, 1ml or 2ml, 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 10ug/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_2 incubator at 37°C for at least 4 hours. Thereafter, the dishes were washed twice with F12 + 10% HIHS and finally 1ml of the appropriate culture medium added to each dish. For embryonic chick neurons, this medium was F14 + 10% HIHS and for mouse neurons, 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 used. At this point the neurotrophins were also added to the culture dish at their required concentrations. 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. Then, 1ml was added to each culture dish using a circular movement. The culture medium was not touched by the tip of the pipette as this could transfer small amounts of neurotrophic factors between the dishes.

Preparation of Neurotrophins

Neurotrophins were received as concentrated stocks from Genentech Inc. 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.

DNA MICROINJECTION

Preparation of DNA

cDNAs for microinjection experiments, in appropriate expression vectors, were kindly donated by Gabriel Nunez, University of Michigan Medical School. In order to obtain sufficient quantities of DNA for microinjection, cDNAs were transformed into competent E. Coli and amplified in 500ml cultures. Alkaline lysis was then used to extract cDNA from the bacterial cultures.

Preparation of Competent E. Coli

This method gave a transformation frequency of 10^6 transformant colonies per μg supercoiled DNA. As far as possible, all procedures were carried out in a laminar flow cabinet to maintain sterility. The E. Coli strain used was XL1 Blue and stocks of these cells were kept at -80°C in Luria broth (LB broth) (Gibco) with 15% glycerol. In order to produce single colonies, cells were scraped from the surface of a frozen stock aliquot, streaked across a Luria agar (LB agar) (Gibco) plate using a flamed inoculating loop and incubated at 37°C, in an inverted position, for 12 hours.

A single colony was used to innoculate 25ml of LB broth which was subsequently incubated overnight at 37°C in a rotary incubator. The 25ml overnight culture was diluted into 250ml sterile LB broth and incubated at 37°C, in a rotary incubator until the OD_{550} was 0.48. The 250ml culture was then chilled on ice for 5 minutes before pelleting the bacteria by centrifuging in sterile, ice-cold tubes at 6000 rpm, for 10 minutes, at 4°C. From this point on the cells had to be kept as cold as possible and so were put on ice whenever possible. The supernatant broth was discarded and the cells resuspended in 100ml of filter-sterilised ice-cold buffer I (30mM potassium acetate, 100mM rubidium chloride, 50mM manganese chloride,

and 15% glycerol, pH5.8). The resuspended cells were left on ice for 3 minutes before being pelleted by centrifugation for 5 minutes, at 6000 rpm at 4°C. Following this, the supernatant was discarded and the cells resuspended in 10ml of filter-sterilised, ice-cold buffer II (10mM MOPS, 75mM calcium chloride, 10mM rubidium chloride and 15% glycerol, pH6.5). After incubation the cells were dispensed in 200μl aliquots and placed on a mixture of dry ice and IMS to snap freeze them prior to storage at -80°C.

The competent cells were transformed with a known concentration of plasmid DNA, typically 1-2ng, to test their efficiency.

Transformation of Competent Cells

A 200μl aliquot of competent cells was split to give 100μl for transformation and 100μl of control cells and these aliquots placed on ice. 1μl of the required plasmid DNA, at 10 ng/ml, was added to the cells to be transformed and these were placed back on ice for 30mins. Following this, the cells were heatshocked at 42°C for 2 minutes and immediately returned to ice. 500μl of LB broth was added to each tube before transferring to the shaking incubator at 37°C. After a 30 minutes incubation at 37°C, to allow time for the plasmid protein giving ampicillin resistance to be expressed, the cells were plated onto LB agar plates containing 100μg/ml ampicillin. 100μl of non-transformed control cells were spread on one plate, to check for contamination of the stock by any bacteria possessing innate antibiotic resistance, and the remaining plates spread with either: 100μl of neat transformed cells, a 1:10 dilution in sterile distilled water, a 1:100 dilution, or a 1:200 dilution. The plates were incubated, upside down, at 37°C overnight. There should be no colonies on the control plate, as the cells did not contain the plasmid with the ampicillin resistance gene, but colonies should be observed on all the other plates.

Small-scale Isolation of Plasmid DNA by Alkaline Lysis (Miniprep)

This procedure was carried out to ensure that the colonies selected for the large scale isolation of DNA contained the correct plasmid. Each plasmid miniprep was carried out on a single colony selected from one of the plates and for each transformed plasmid, 6 colonies were selected to ensure that a clone containing the correct plasmid was isolated for the subsequent procedure. The small-scale isolation allows for quick, convenient isolation of plasmid DNA without column purification or banding in caesium chloride gradients. The resultant plasmid DNA is suitable for restriction enzyme analysis. Each single colony picked from the agar plate was grown overnight, at 37°C in a rotary incubator in 5ml of LB broth containing 100μg/ml ampicillin. The next day, 1.5ml samples of each overnight culture were dispensed into microfuge tubes and the bacteria were pelleted by centrifuging at 13000 rpm for 3 minutes. The supernatant was discarded and the bacterial pellet resuspended in 300μl of solution I (25mM Tris HCl pH8.0, 10mM EDTA pH8.0, 50mM Glucose). Following this, the resuspended bacteria were placed on ice, and 400μl of solution II (0.2M sodium hydroxide, 10% sodium dodecyl sulphate) was added and the tubes gently mixed. The SDS in solution II denatures bacterial proteins whilst the sodium hydroxide denatures chromosomal but not plasmid DNA. Immediately, 300μl of solution III (3M potassium acetate solution pH 5.5) was added to neutralise solution II, and again the tubes were mixed gently before being placed on ice for 10 minutes.

The samples were centrifuged for 5 minutes, at 13000 rpm and 4°C, to pellet the cell debris and chromosomal DNA. Subsequently, the supernatant was transferred to a fresh microfuge tube and after the addition of 0.5ml of isopropanol, the tubes were placed at -20°C, for 10 minutes, to precipitate the plasmid DNA.

The DNA was pelleted by centrifugation, at 13000 rpm, for 3 minutes and the supernatant was discarded. This pellet was washed in 70% ethanol to dissolve any salts, and allowed to air-dry. After resuspending the pellet in 50μl sterile distilled

water, 100µl of 7.5M sodium acetate were added, the tubes mixed and the tubes left at -20°C for 10 minutes. Protein was pelleted by centrifuging for 5 minutes at 13000 rpm to pellet the protein and the supernatant was transferred to a fresh tube. 80µl of isopropanol was added to the supernatant and the tubes were placed at -20°C for 10 minutes, to precipitate the DNA. Following a 5 minute centrifugation at 13, 000rpm, the supernatant was removed and the pellet was washed in 70% ethanol. After drying the DNA pellet and resuspending it in 25µl sterile distilled water, 2µl was taken for a restriction digest to check the identity of the plasmid. The DNA derived from this procedure is best stored at -20°C or lower as it is prone to degradation by contaminating nucleases. Once the identity of the plasmid was confirmed, the remaining 2ml of overnight culture could be used to innoculate media for the large-scale alkaline lysis.

Restriction Digests and Agarose Gel Electrophoresis

2µl of the DNA obtained from the above mini prep procedure were digested in a reaction containing1µl 10x restriction endonuclease buffer, 5-10 units of the restriction endonuclease and sterile deionised water to give a total volume of 10µl. The reaction was carried out at the recommended optimum temperature for each endonuclease (usually 37°C). The products of the digest were visualised by electrophoresis on a 1% agarose gel. 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, and ethidium bromide was added to a final concentration of 0.5μg/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°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 TAE buffer and the comb removed. The DNA samples were mixed with 6x gel-loading buffer (40mM EDTA; 0.1% Bromophenol Blue; 20% glycerol) and loaded onto the gel. A molecular weight standard was loaded into one well to allow the size of the DNA bands to be measured. A voltage of 1-5v/cm was applied across the tank. After electrophoresis was complete, a permanent record of the gel could be made by photographing it on the UV transilluminator.

Large-scale Preparation of Plasmid DNA by Alkaline Lysis (Maxiprep)

Two 2l flasks, each containing 500mls of LB broth with ampicillin (100μg/ml), were inoculated with the cultures that were not used in the miniprep and incubated overnight at 37°C in a rotary incubator. The next day, the cells were collected by centrifugation, at 10000 rpm for 10 minutes. They were then washed in 50ml Tris-HCl, pH 8.0, to remove traces of LB broth and centrifuged again for a further 10 minutes, at 10000 rpm. Next, the pelleted cells were resuspended in 50ml of Solution I + lysosyme and left on ice for 30 minutes. Following this, 80ml of denaturing Solution II were added and after careful mixing, the tubes were transferred to ice for 10 minutes. The DNA solution was neutralised by the addition of 40ml of cold Solution III. Following gentle mixing, the tubes were placed on ice for 15 minutes. The tubes were then spun at 8000 rpm, for 5 minutes, and the supernatant filtered into a fresh tube through 2 layers of gauze. Approximately 0.6 volumes of isopropanol at -20°C were added to precipitate the DNA. After mixing, precipitated DNA was immediately recovered by centrifuging at 8000 rpm for 5

minutes and aspirating the supernatant. After alkaline lysis, plasmid DNA was purified away from chromosomal DNA, protein and RNA by ultracentrifugation on a Caesium chloride/ ethidium bromide gradient. In this process ethidium bromide intercalates into the DNA becoming inserted between adjacent base pairs in the helix. This results in a slight untwisting of the helix and a decrease in the buoyant density of the DNA in CsCl solution. The physical constraints of a double-stranded supercoiled DNA molecule limit the amount of dye which can be bound per unit length. Consequently, the density decrease is less in a super-coiled molecule than in linear or relaxed circular DNA and so supercoiled forms such as plasmid DNA can be readily separated from other DNA on a caesium chloride gradient. The gradient is formed when a CsCl solution is centrifuged at very high speed. The air-dried pellet was dissolved in 3.81ml of sterile distilled water before adding 4.016g of caesium chloride added and 150µl of ethidium bromide (10mg/ml). The resultant solution was transferred to a Beckman ultracentrifuge tube and mineral oil added to exclude any air which could shatter the tube whilst spinning. The tubes were spun in a Beckman Ultracentrifuge (L-7) at 40000 rpm for at least 16 hours.

After centrifugation, 2 bands could be seen, the lower one representing the covalently closed circular DNA of the plasmid and the other containing the relaxed chromosomal DNA. The plasmid DNA was harvested using a Pasteur pipette. Ethidium bromide was removed by repeated extraction with caesium chloride saturated isopropanol. An equal volume of CsCl saturated isopropanol was added to the plasmid DNA, the tube was mixed, the layers allowed to separate, and the upper layer containing the ethidium bromide was discarded. This process was repeated until no colour remained in the isopropanol layer. Next an equal volume of sterile distilled water was added plus 0.1 volumes of 3M sodium acetate (pH 5) and 0.6 volumes of isopropanol. After mixing, precipitated DNA was recovered by centrifuging at 14000 rpm for 2 minutes. The supernatant was removed and the pellet was washed in 70% ethanol prior to vacuum drying and resuspending in 200µl

of sterile distilled water. Finally, the identity of the plasmid DNA was checked by restriction analysis.

To ascertain the concentration of the DNA obtained, the absorption of diluted plasmid was measured at 260nm in a spectrophotometer. The ratio of OD_{260} to OD_{280} gives a measure purity of the plasmid DNA. If the DNA is pure the ratio will be about 1.8. A higher figure would indicate the presence of too much RNA and a lower figure would indicate protein contamination. To calculate the concentration of the plasmid, the following formula was used

$2 \times 50 \times OD_{260}$

where 2 is the inverse of the light path length through the cuvette

50 is the mathematical constant used for plasmid DNA

This gives a concentration in μg/ml and the plasmid was then diluted to 100μg/ml for microinjection. Plasmids were stored at -80°C prior to microinjection.

Tissue culture for microinjection

Cultures were set up as described previously, but the cells were plated on 60mm diameter Nunc dishes rather than 35mm dishes. Nine square grids were scribed onto the bottom of these dishes with a scalpel blade. The total area of the grid was 144mm^2, each square of the 9 square grid was 4mm x 4mm. Cells were plated about 12 hours before injection in the presence of their appropriate neurotrophic factor. Cells were plated at a density such as to give approximately 50 cells per square of the grid.

DNA microinjection

Needles were made from Narishige 1mm x 90mm glass tubing using a microelectrode puller. 0.7μl of the plasmid to be injected was loaded into the needle using an Eppendorf microloader. Before loading the plasmid DNA solution was spun

at 13000 rpm for three minutes to sediment any particulate matter which could block the microinjection needle. During the injection procedure, the vial containing the plasmid was kept on ice to inhibit the action of any nucleases present. Prior to injection, each loaded needle was checked under a dissection microscope for the presence of any air bubbles which could block the needle. If present, these were easily removed by shaking the needle firmly. Finally, the needle was the placed in the micromanipulator arm (Narishige) which was attached to a Nikon inverted phase contrast microscope.

The dish to be injected was prepared by washing 5 times in 10 ml of F12 + 10% HIHS to remove the neurotrophins. Following this, 8ml of F12 +10% HIHS were added to act as the medium during injection. The dish was then placed on the stage of the microscope and a square of the grid was selected for microinjection. Turning the microscope to dark field, the needle was positioned to lie centrally within the field of view and at a low level near the cells. The microscope was then turned to phase bright x400 and the bottom of the dish was brought into focus. Next, the tip of the needle was broken against the bottom of the dish to allow the plasmid DNA solution an exit from the needle. The cells were then brought into focus, ready for injection.

As the cell membrane is most resilient in the area overlying the nucleus, the DNA was injected directly into the nucleus by pressure from a pressure system. After penetration, the needle was left in the nucleus until the nucleus was seen to swell, and then the needle was withdrawn. When all the cells in a given square were injected, the dish was washed a further 3 times with F12 + 10% HIHS. Finally 8ml of F14 + 10% HIHS + 5% Fetal calf serum (FCS) (Gibco) containing the required neurotrophic factors was added to the culture dish and the culture was returned to the CO_2 incubator at 37°C.

The cells were counted 1 hour post-injection because by this time any cells which were likely to die from the effects of the injection would have done so. Also, at this

time, the number of cells in another uninjected square of the dish was counted to give an internal control for any neurotrophic factors remaining in that particular dish.

TRANSGENIC MICE

Breeding

bcl-2 Knockout Mice

An original small colony comprising 12 male and 9 female mice of C57/BL6 background strain and heterozygous for a null mutation in the bcl-2 gene was received from the Paterson Institute for Cancer Research in Manchester. bcl-2 knockout mice show early postnatal lethality (Nakayama et al., 1993; Veis et al., 1993; Kamada et al., 1995) and so embryos homozygous for the mutation were obtained from heterozygote crosses. Early indications were that the colony showed a male bias in its sex ratio and this was confirmed statistically using a t-test. The sex ratio at birth is approximately 2:1 males : females (n=317). The average gestation period is 20 days(n=77) and the average litter size is 6.8 (n=77). Approximately 15-20 heterozygous females had to be time mated each month to give adequate pregnant animals for experimentation, since the vaginal plugging efficiency (i.e. the percentage of vaginal plugs noticed which resulted in a pregnancy) was 32% (n= 69). Given the above data, quite a large breeding stock was required to provide adequate females for experiments. Owing to the difficulty in telling whether an individual mouse was pregnant post coitus, a pregnancy test was devised whereby the mice were weighed on the day that the vaginal plug was found and thereafter the percentage change in body weight from this original weight could then be calculated (Fig 2.6). This test could reliably indicate whether a mouse was pregnant by 8 days post coitus.

From the above statistics it can be seen that of each 24 individuals born, 8 will be female and of these eight there will be two homozygotes, four heterozygotes, and two wild type individuals. Therefore to get twenty heterozygous females per month,

we require that 120 mice are born, that is approximately 17 litters. To help to build up stock initially, wild type females were also used for stock breeding, crossing them with heterozygous males. As the colony expanded this practice became obsolete.

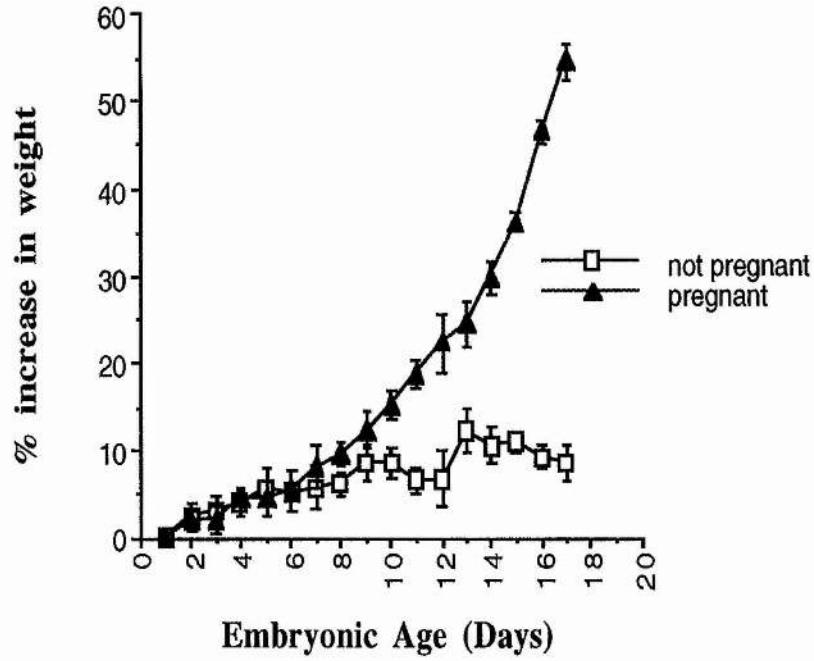


Figure 2.6: Percentage increase in body weight with time for pregnant and non pregnant C57/BL6 bcl-2 +/- mice

Because C57/BL6 mice are poor breeders, the original bcl-2 knockout mice were crossed with CD1 mice which are renowned as good breeders. The resultant mice were seen to be inbred after three generations. The mice used for the survival studies in Chapter Four were C57/BL6 backgroung strain. Those used in Chapter Five were of CD1 background strain.

bax Knockout Mice

Two male and two female mice heterozygous for a null mutation in the bax gene were received from Stanley Korsmeyer, Howard Hughes Medical Institute. The bax colony was derived from a series of heterozygous crosses whilst the mice were in rabies quarantine. The average gestation period for these mice was 21 days (n=54) with an average litter size of 8 (n=54).

Genotyping

DNA Extraction

By the constraints of our animal license, the mice could not be genotyped until they were at least 4 weeks old. At this age, the mice were sedated, using diethyl ether or halothane and 1.5cm was 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. Very few homozygotes survived to genotyping, most were sacrificed prior to this on phenotypic grounds. Heterozygotes were used for matings both for experiments and to supplement breeding stock. Wild type individuals were eliminated.

For embryos, DNA was extracted form any tissue that remained after dissection of the relevant ganglia for tissue culture, or subdissection of the embryos for histology.

The tissue obtained was chopped into small pieces and placed in an Eppendorf tube with 500µl proteinase K solution (50mM Tris HCl pH8.0; 10mM EDTA; 0.1M sodium chloride; 1% sodium dodecyl sulphate and 500µg/ml proteinase K) incubated overnight at 55°C in a water bath.

(i) Phenol Extraction

First, 500μl of basic phenol were added to the tube following Proteinase K digestion and the contents of the tube were thoroughly mixed. The tube was then centrifuged, at 13000 rpm for 10 minutes, to separate the mixture into an upper aqueous phase containing DNA and a lower phenolic phase containing proteins and much of the RNA. 250 μl of the upper aqueous phase was transferred to a fresh tube and 25μl 3M sodium acetate pH 5.4 were added followed by 675μl absolute ethanol. Following thorough mixing, the ethanolic mixture was left at -20°C for 1 hour to allow the DNA to precipitate. Next, the samples were centrifuged, at

13000 rpm for 10 minutes, to pellet the DNA and the supernatant was discarded. The DNA pellet was washed with 70% ethanol, to remove any salts in the pellet, and finally and resuspended in 200μl of sterile dd$H_2$0 or TE buffer. The DNA solution was triturated several times through a 25G needle attached to a 1ml syringe. The purpose of this was to break up the DNA allowing the PCR primers better access to their binding sites.

(ii) High Salt DNA Extraction

Following Proteinase K digestion, 210μl of 5M sodium chloride solution was added to each tube and the contents of the tubes mixed thoroughly by vortexing for 15 seconds. This step was followed by a 10 minute centrifugation, at 13000 rpm, to pellet the proteins. The resultant supernatant was transferred to a fresh microfuge tube and an equal volume of 100% ethanol was added. DNA was pelleted by centrifugation, at 13000 rpm for 10 minutes, and the supernatant was discarded. The DNA pellet was washed in 1ml of 70% ethanol to remove any salts present in the pellet. It was then centrifuged, at 13000 rpm, for 5 minutes. Again the supernatant was discarded and the pellet allowed to air-dry before it was resuspended as described for the phenol extraction method.

Polymerase Chain Reaction

To produce bcl-2 and bax knockout mice, a segment of both genes was replaced by a neomycin resistance gene cassette by homologous recombination in embryonic stem cells. In addition to preventing production of functional bcl-2 or bax, 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 bcl-2 or bax alleles mice carry (i.e. wild type or neomycin casette disrupted). A three primer PCR reaction was used to allow identification of both wild type and mutated alleles in a single PCR reaction. One primer was located in the bcl-2 or bax coding sequence and was common to both alleles. For detection of the wild type allele, a second primer was located in the bcl-2 or bax coding region in the region replaced by the Neo gene in the mutant. To detect the mutant allele, a third primer was designed that would bind to the Neo gene. 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. The PCR products were of different sizes and were easily resolved on an agarose gel.

Oligonucleotide primers were synthesised on an Applied Biosystems DNA synthesiser. The sequence required was typed in and the synthesised oligonucleotide collected on an oligopak column (Applied Biosystems). To cleave oligonucleotides from the column, the column was flushed through with 3ml 40% ammonium hydroxide (Applied Biosystems) many times over the course of 3 hours taking care not to allow the filter to dry out. The ammonium hydroxide containing the eluted primers was transferred to a screw-top glass vial and deprotected by incubating at 55 ^{o}C overnight. The next day the liquid was allowed to cool down and transferred to microfuge tubes for rotary evaporation of the ammonium hydroxide. The primers were resuspended in 200µl sterile ddH_2O and spectrophotometer readings of a 1:100 dilution were used to calculate the concentration:

100 x 2 x 33 x OD_{260} = concentration in µg/ml

where 100 is the dilution factor

2 is the inverse of the light path length through the cuvette

33 is the mathematical constant used for oligonucleotides

$$\frac{(OD260 \times 20)}{xmer} = \text{concentration in nmol}/\mu l.$$

where xmer is the length in bases of the primer

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.

Bcl-2 Knock Out Mice

Each reaction was carried out in a 20μl reaction volume. Each tube contained:

2μl	10x reaction buffer (Buffer B, Promega)
1.2μl	25mM magnesium chloride
1μl	5mM dNTPs (Pharmacia)
1μl	cDNA3
1μl	cDNA5
1μl	PGKNM
0.5μl	Taq thermostable DNA polymerase (Promega)
1μl	template DNA
11.3μl	ddH2O

The primer sequences were:

pGKNM (5') GCC TAC CCG CTT CCA TTG CTC AGC

cDNA5 (5') TAC AGA CCA ACT TAC TCA GAC CCG

cDNA3 (5') CGT CCC GCC TCT TCA CCT TTC AGC

The PCR reaction itself consisted of 33 cycles, following an initial 5 min denaturing step at 94°C. Cycle conditions were: 1 minute at 94°C (denature), followed by 1 minute at 64°C (primer annealing), followed by 1 minute 30 seconds at 72°C (elongation). There was also a final elongation stage of 7 mins at 72°C. The products of the reaction were visualized on a 1% agarose gel containing ethidium bromide. The mutant band, which was amplified by pGKNM and cDNA5, was 400bp and the wild type, which was amplified by primers cDNA3 and cDNA5, was 950bp. Fig 2.7 shows how the three possible genotypes appear on such a gel.

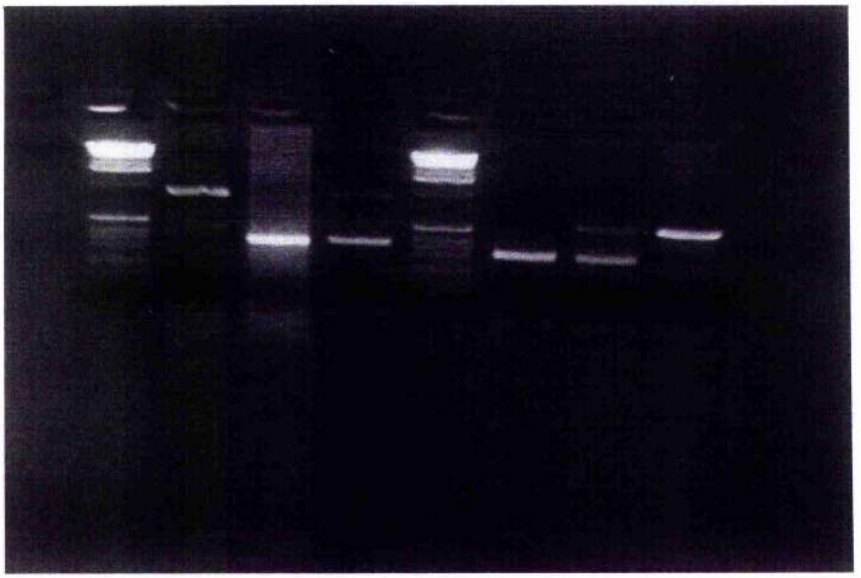


Figure 2.7: Gel showing the results of genotyping reactions

Lane 1: Molecular weight standard
Lane 2: Bcl-2 +/+
Lane 3: Bcl-2 -/-
Lane 4: Bcl-2 +/-
Lane 5: Molecular weight standard
Lane 6: Bax +/+
Lane 7: Bax +/-
Lane 8: Bax -/-

Bax Knock Out Mice

Each reaction for the Bax genotyping was carried out in a 20μl reaction volume, each reaction containing as follows:

1μl 5mM dNTPs (Pharmacia)

2μl 10x reaction buffer (Promega buffer B)

1.2μl 25mM magnesium chloride

0.5μl Taq thermostable DNA Polymerase (Promega)

1μl 16uM BaxEx5F

1μl 4uM NeoR

1μl 16uM BaxIN5F

1ml template DNA

11.3μl ddH_2O

The primers for this reaction were:

Bax IN5R (5')	GTT GAC CAG AGT GGC GTA GG
BaxEX5F (5')	GTA CTA CCA AGA CTA GTC GA
Neo (5')	GGC GAC TCG TTA CCT TCG CC

After and initial denaturing step of 94°C for 5 minutes, 35 cycles were carried out consisting of: 1 minute at 94°C, followed by 1 minute at 55°C, followed by 1 minute at 72°C. A final 7 minute elongation at 72°C was followed by refrigeration at 4°C.

The PCR products were visualized on a 2% agarose gel containing ethidium bromide. The mutant band, which was amplified by Bax IN5R and Neo, was 507bp and the wild type, which was amplified by Bax IN5R and Bax EX5F, was 307bp. All three possible genotypes are seen in Figure 2.7.

Tissue Culture

At the time of setting up the cultures, the genotype of each individual embryo was unknown therefore it was impossible to pool the ganglia for dissociation and trituration as described previously. When the embryos were removed from the mother they were placed in individual numbered 60mm dishes, containing sterile L15 ,for dissection. Once the ganglia were dissected from an individual, they were placed in a labelled 10ml tube with 975μl HBSS and the remains of the embryo was taken for genotyping.

To dissociate the tissue, 25υl of 0.1% Worthington's trypsin was placed in each tube and the tubes were placed in a water bath, at 37°C, for a length of time that was dependent on embryonic age. Thereafter, the HBSS containing the trypsin was aspirated and 10ml F12+10% HIHS was added to each tube to inactivate residual trypsin. Following this, the tube was centrifuged briefly to sediment the ganglia, the F12 was discarded and 1ml F14+Sato was added to each tube. The ganglia of the first embryo were then triturated with a flame polished siliconised pipette. After 2 or 3 passages, a drop was observed under the microscope to check the level of dissociation. The pipette was then rinsed in L15 to flush out any cells from the first embryo remaining in the pipette, allowing the same pipette to be used for all the ganglia. After the ganglia of the other embryos were dissociated, the cells were diluted to an appropriate concentration and volume before being plated and finally placed in the CO_2 incubator at 37°C.

To assess cell survival, the number of neurons were counted at three hours after plating and at regular intervals thereafter. Percent survival was calculated relative to the three 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. Each condition was studied in triplicate in each experiment for each embryo.

Cohort experiments were also carried out to follow the fates of individual cells with regards to proliferation, maturation and survival. This was done by scibing a 6mm by 6mm grid, comprising sixteen 1.5mm by 1.5mm squares, on the bottom of a 60mm Nunc petri dish. The cells were plated within this dish at low density to give a final density of around 20 to 50 cells on the grid. The cells in the grid were drawn at various time intervals and the data evaluated to calculate the percentage of new neurons that mature during the experiment, taking the figures from 18 hours after plating as 100%. In addition the survival of individual neurons, and the state of maturation was assessed. Neurons were classified as immature if they had phase dark, spindle-shaped cell bodies and short neurites, whilst mature neurons had phase-bright cell bodies and long neurites. Intermediary neurons were only classified as mature if they possessed a phase-bright cell body and definite neurites.

Cohort experiments were also used to quantify neurite outgrowth. To examine growth rates, accurate serial camera lucida drawings of the same individual neurons over a set time period were made using a drawing tube. From these drawings, total neurite length was calculated using the NIH Imager graphics package. To exclude any differences in growth rate that could arise as a consequence of differences in neuronal viability, only neurons that survived throughout the culture period were included in the analysis.

Histology

Fixation and Embedding

Embryos for histology were sub-dissected by making a transverse cut rostral to the upper limbs and a second rostral to the eyes. The lower portion of the body was used for genotyping and the upper portion fixed in neutral buffered formalin for 3-4 days. After fixation, the tissue was washed in tap water for 2-4 hours to wash out the fixative. Older tissue (E16 and older) was then transferred to 10% EDTA for 1 week to decalcify. Thereafter, the tissue was dehydrated through a graded alcohol

series before being transferred to an alcohol/chloroform mix which in turn was followed by 2 changes of chloroform. Chloroform was used as a clearing agent as this bleaches the tissue prior to cresyl fast violet staining. If another clearing agent such as xylene was used, the staining criteria were altered. Finally, the tissue was embedded in paraffin after 3 changes in paraffin wax at 56°C.

Cresyl fast Violet Staining

8μm sections were cut using a microtome. These were cleared in xylene before rehydration through a graded alcohol series and staining with 0.025% cresyl fast violet. The length of time that the sections were stained for varied with embryonic age. Younger specimens took up the stain more readily reflecting the increased water content of these cells. After staining the cells were differentiated in 96% alcohol until the nucleoplasm of the neurons was seen to be free of stain. The sections were then dehydrated and cleared in xylene before mounting with DPX.

Estimation of Neuronal Number

To count the number of neurons per ganglion, camera lucida drawings were made of every tenth section and the area of the drawn section was measured using the NIH Imager software package in conjunction with a graphics pad. The average number of cells per 0.01mm^2 was counted at x1000 using an eyepiece graticule. On average five fields were counted per section drawn. Using this and the area measurement, the approximate number of cells in that section could be calculated and all the sections analysed added together to give the approximate number of cells in 10% of the ganglion. By simply multiplying by 10 the approximate number of cells in the ganglion could be obtained.

Neurofilament Staining

The heads of E12 embryos were fixed in Carnoy's fluid (60% ethanol, 30% chloroform, 10% acetic acid) for 20 minutes prior to dehydration and wax

embedding. The bodies were retained for genotyping. Serial sections of the heads were cut at 8μm and mounted on polysine coated microscope slides. The sections were cleared in xylene and dehydrated before quenching (10% methanol, 3% hydrogen peroxide in PBS). Non-specific interactions were blocked using 10% horse serum in PBS with 0.4% Triton X-100. The sections were incubated in primary antibody (Monoclonal anti-Neurofilament 160, Sigma Immunochemicals, used at 1:500) for 1 hour and then labelled using biotinylated secondary antibody (1:200) and avidin and biotinylated horse radish peroxidase macromolecular complex (Vectastain ABC Kit, Vector Laboratories). The substrate used for the peroxidase reaction was 1 mg/ml diaminobenzidine tetrachloride (Sigma). After staining the sections were washed in tap water prior to rehydration and mounting. Estimation of neuronal number was carried out as for cresyl fast violet stained sections. Here, neurons were identified as neurofilament positive cells.

Estimation of Number of Pyknotic Neurons

To evaluate the extent of cell death, all pyknotic nuclei were counted at x400 magnification in every fourth section along the entire rostrocaudal length of the trigeminal ganglion. Estimates of the total number of pyknotic nuclei in the ganglion were obtained by multiplying this number by four.

Immunocytochemistry

Tissue culture

Cells were plated in 4-well dishes, each well being of 10mm diameter. This allows plating at high density in a small area thus minimizing the quantity of reagents used.

Cell Fixation

After growing cells in culture in a 4% CO_2 incubator at 37°C, for 24 hours, the medium was carefully removed from each well with a pipette and 1.5ml of pre-warmed (37°C) 4% paraformaldehyde solution added to each dish. After incubation

at 4°C for 2 hours, the cells were washed twice with PBS/glycine (1xPBS, 0.1M glycine, pH 7.2) at room temperature for 5 minutes each wash. This solution neutralizes the unreacted paraformaldehyde. The two PBS/glycine washes were followed by a single wash in PBS (pH7.2), at room temperature, for 5 minutes.

Permeabilised Staining for Neurofilament

The fixed cells were preincubated in 1.5ml P blocking buffer (1x PBS, 1% bovine serum albumin, 2% goat serum, 0.4% saponin) to permeabilise the cells. After this was removed, the cells were incubated with 70μl of a 1:100 dilution of the primary antibody in P blocking buffer, at room temperature, for 1 hour. At the end of this incubation period, the cells were washed in three changes of PBS/saponin (1x PBS, 0.4% saponin) for 5 minutes each wash. Meanwhile, the secondary antibody was centrifuged at 3000 rpm for 5 minutes, at 4°C, to remove any aggregates before being diluted 1:100 in P blocking buffer. 70μl of secondary antibody solution was added to each well and incubated at room temperature, for 1 hour, in the dark. The cells were washed with 2 changes of PBS/saponin and one of PBS, for 5 minutes each change, before mounting with citifluor (Agar). Cells were visualised using an Axioscope fluorescent microscope.

III. OVEREXPRESSION STUDIES

CHAPTER THREE

OVEREXPRESSION STUDIES

INTRODUCTION

bcl-2, bcl-x, and bax are members of a family of cytoplasmic proteins that influence cell survival. Whereas increased expression of bcl-2 or bcl-x promotes cell survival following withdrawal of survival factors, increased expression of bax is thought to suppress survival. To investigate the potential roles of these proteins in regulating the survival of developing neurons, the effects of overexpressing these proteins in embryonic neurons, deprived of different neurotrophic factors, in vitro, were compared.

bcl-2 is a 26 kDa, intracellular, membrane-associated protein of vertebrates (Nunez & Clarke, 1994; Davies, 1995) that is homologous to the nematode ced-9 protein which functions as a negative regulator of cell death (Hengartner & Horvitz, 1994b). Overexpression of bcl-2 prevents the death of several cytokine-deprived haematopoietic cell lines following cytokine withdrawal (Vaux et al., 1988; Nunez et al., 1990) and rescues embryonic neurons deprived of the neurotrophins: Nerve Growth Factor (NGF) (Garcia et al., 1992), Brain-Derived Neurotrophic Factor (BDNF) and Neurotrophin-3 (NT-3) (Allsopp et al., 1993). Examination of transgenic mice carrying a bcl-2 gene that is over-expressed in the immune system (McDonnell et al., 1989), and mice with bcl-2 null mutations, (Nakayama et al., 1993, 1994; Veis et al., 1993) has shown that bcl-2 plays an important role in promoting and regulating the survival of B and T lymphocytes (Linette & Korsmeyer, 1994). bcl-2 is not, however, universally effective in counteracting signals that induce apoptosis. For example, bcl-2 overexpression does not protect all cytokine-dependent haematopoietic cell lines following cytokine withdrawal (Nunez et al., 1990) and does not protect neurons following withdrawal of Ciliary neurotrophic factor (CNTF) (Allsopp et al., 1993).

Several genes have recently been identified that are homologous with *bcl-2*, including *bcl-x* (Boise et al., 1993), *bax* (Oltvai et al., 1993), *bad* (Yang et al., 1995) and *bak* (Chittenden et al., 1995; Farrow et al., 1995; Kiefer et al., 1995). The Bcl-x variant, Bcl-x_L has the highest homology with Bcl-2, and like Bcl-2 is able to prevent apoptosis of IL3-dependent cells following IL3 withdrawal (Boise et al., 1993). Bcl-x_S lacks BH1 and BH2 which are the regions of highest homology between family members and cannot rescue haematopoietic cells from apoptotic cell death following cytokine withdrawal (Boise et al., 1993). Another Bcl-x variant, Bcl-x_β (Gonzalez-Garcia et al., 1994) lacks a C-terminal hydrophobic domain that is necessary for membrane attachment. Mice with a null mutation in the *bcl-x* gene die *in utero* by excessive apoptosis in the immune and nervous systems (Motoyama et al., 1995). Bax has 21% amino acid sequence homology with Bcl-2 (Oltvai et al., 1993). Bax heterodimerises with Bcl-2 and also forms homodimers. Bax overexpression in IL3-dependent cells accelerates apoptosis following IL3 withdrawal and inhibits the death repressor action of Bcl-2 in these cells (Oltvai et al., 1993). Consequently, it is thought that the ratio of Bcl-2 to Bax determines survival or death following an apoptotic signal; excess Bcl-2 leading to survival, excess Bax causing death (Oltvai et al., 1993; Oltvai & Korsmeyer, 1994; Korsmeyer, 1995). Mice with a null mutation in the *bax* gene display both hyperplasia and hypoplasia depending upon the cellular context. In the immune system, both thymocytes and B cells display hyperplasia, whilst in the male reproductive system there is a disordering of the seminiferous tubules and an accumulation of atypical premeiotic germ cells, but no mature haploid sperm. There are multinucleated and giant cells accompanied by massive cell death (Knudson et al., 1995). Bak was identified by using PCR with degenerate primers to clone cDNA fragments encoding Bcl-2 homologues. The new homologous sequence was subsequently used to isolate full-length cDNA sequences from human heart and Epstein Barr (EBV)- transformed human B-cell libraries. Bak was found to have 53% amino acid sequence homology with Bcl-2 and shows marked similarity to other family members particularly in BH1

and BH2. Bak interacts with Bcl-2 and more strongly with Bcl-x_L. Bak has been shown to promote or inhibit apoptosis, depending on the cellular context (Chittenden et al., 1995; Farrow et al., 1995; Kiefer et al., 1995). The Bad protein (Yang et al., 1994) only shows homology to Bcl-2 in BH1 and BH2. It can dimerise with Bcl-2 and Bcl-x_L but not with Bax, Bcl-x_S, MCL-1, A1, or itself. It inhibits the death repressor activity of Bcl-x_L but not Bcl-2 in mammalian cells, and acts by displacing Bax from Bcl-x_L/ Bax heterodimers and thus inducing apoptosis.

To test the generality of the Bcl-2/Bax rheostat model (Korsmeyer, 1995) and to clarify the role of Bcl-2-related proteins in regulating neuronal survival, microinjection was used to introduce expression vectors for Bcl-2, Bcl-x_L, Bcl-x_S, Bcl-x_β, and Bax into cultures of embryonic chicken neurons that were deprived of different neurotrophic factors. Four populations of neurons were studied at E10 and E12. These were the neurons of the trigeminal mesencephalic nucleus, dorsomedial portion of the trigeminal ganglion, lumbar dorsal root ganglia, and ciliary ganglion.

The Trigeminal Mesencephalic Nucleus (TMN) consists a slender strand of cells that extend from the pontine trigeminal nucleus through the midbrain. The proprioceptive sensory neurons of the TMN are the only primary sensory neurons incorporated into the central nervous system rather than being in the cerebrospinal ganglia. The neurons of the TMN are supported by BDNF (Davies et al., 1986b).

The trigeminal ganglion is the most rostral of the cranial sensory ganglia and is associated with Cranial Nerve V. The neurons of the ganglion are derived from both neural crest and epidermal placodes. The cutaneous sensory neurons of the dorsomedial part of the trigeminal ganglion (DMTG) are supported by NGF (Davies & Lindsay, 1985).

The sensory neurons of the dorsal root ganglia (DRG) are derived from the neural crest and from each cell body, two processes arise. One passes medially into the dorsal horn of the spinal cord and the other outwards to form a sensory fibre which terminates at a sensory ending. The mixed sensory neurons of dorsal root ganglia (DRG) are supported by NGF or BDNF (Lindsay et al., 1985).

The parasympathetic ciliary ganglion is located behind the eye and is the target of the axons of the preganglionic parasympathetic neurons of the Edinger-Westphal nucleus of the oculomotor complex. The cells of the ganglion innervate the constrictor pupillae muscles of the iris and the ciliary muscles. The neurons of the ciliary ganglion are supported by CNTF, glial-cell derived neurotrophic factor (GDNF) (Buj-Bello et al., 1995), growth promoting activity (GPA) and basic fibroblast growth factor (bFGF) (Barbin et al., 1984; Unsicker & Westermann, 1992; Allsopp et al., 1995).

Using these four populations of neurons, those of the ciliary ganglion, DMTG, DRG and TMN, primary neuronal cultures were set up and these neurons microinjected to overexpress a number of proteins at two ages in embryonic development - E10 and E12, that is after 10 or 12 days incubation.

RESULTS

Overexpression of Bcl-2, Bcl-xL, Bcl-xb and Bax rescues neurotrophin deprived sensory neurons

E10 and E12 DRG, E10 and E12 DMTG and E10 TMN neurons were purified free of non-neuronal cells and were grown with either NGF (DRG and DMTG neurons) or BDNF (TMN neurons) for 12 hours. Neurons were then deprived of neurotrophic factors by extensive washing before being microinjected with expression vectors for Bcl-2, Bcl-x_L, Bcl-x_β, Bcl-x_s, or Bax. The survival of injected neurons was compared

at intervals with the survival of neurons injected with the pSFFV expression vector without inserted cDNA.

Confirming previous findings (Allsopp et al., 1993), Bcl-2 overexpression prevented the death of many NGF-deprived E10 and E12 chick DRG and DMTG neurons and BDNF-deprived TMN neurons (Figs. 3.1, and 3.2). Overexpression of Bcl-x_L and Bcl-x_β also rescued neurotrophin-deprived neurons as effectively as Bcl-2 overexpression (Figs. 3.1, and 3.2). Surprisingly, Bax overexpression also prevented the death of neurotrophin-deprived neurons (Figs. 3.1, and 3.2). Although Bax overexpression did not rescue as many neurons as Bcl-2, Bcl-x_L or Bcl-x_β overexpression, there were over 3-fold more Bax-injected neurons surviving compared with vector-injected neurons 24 hours post-injection in cultures of E10 DRG and DMTG neurons deprived of NGF, and the difference between Bax-injected and control-injected neurons increased to between 4 and 6 fold after 72 hours (Figs. 3.1 and 3.2). Although Bax overexpression did not rescue BDNF-deprived TMN neurons as effectively as NGF-deprived DRG and DMTG neurons (Figs. 3.1, and 3.2), there were significantly more Bax-injected TMN neurons surviving 24 hours post-injection compared with control injected neurons ($p < 0.001$, t-test). In contrast to the other Bcl-2 family members, overexpression of Bcl-x_s in the pSFFV expression vector was not able to rescue embryonic sensory neurons deprived of neurotrophic factors at E10 or E12 (Figures 3.1 and 3.2). It is not possible to tell if overexpression of Bcl-x_s is having a negative effect on survival in the absence of factors as the neurotrophin-deprived sensory neurons die so quickly.

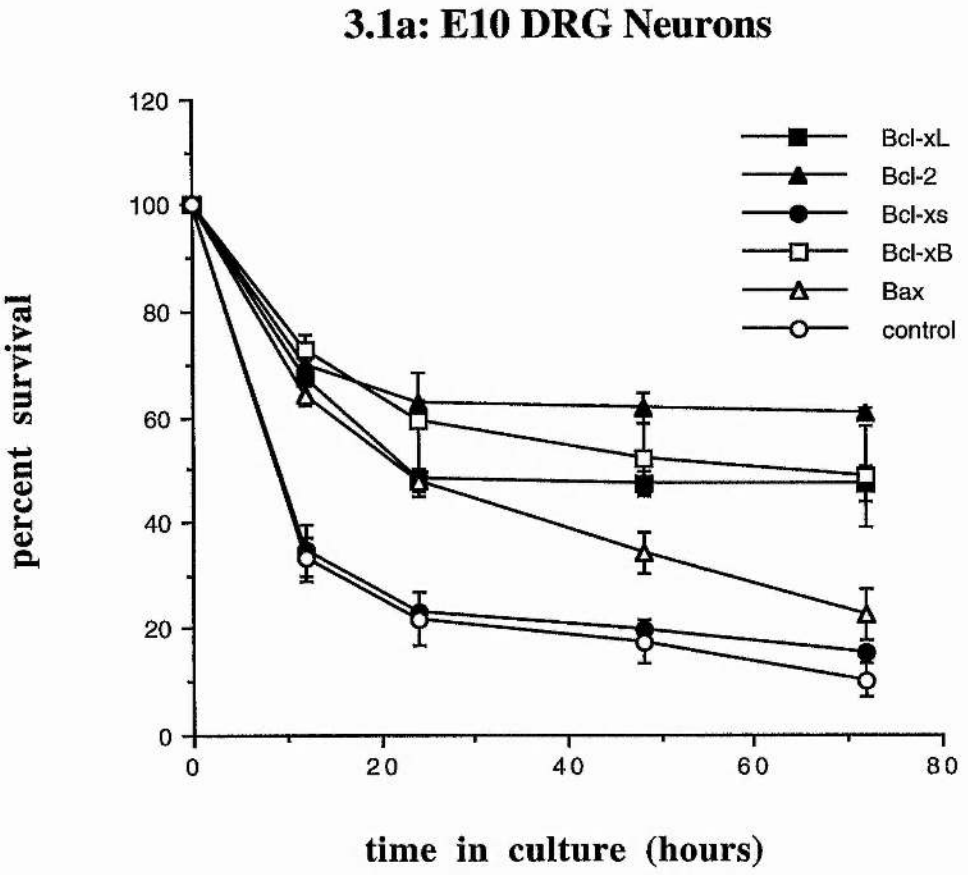


Figure 3.1a: The effects of injecting NGF-deprived E10 DRG neurons with Bcl-2, Bcl-x_L, Bcl-x_β, Bcl-x_S or Bax expression vectors or the pSFFV vector without an insert (control injections). After 12 hours incubation with 5 ng/ml NGF, the neurons were washed extensively to remove these factors before being injected with expression vectors. The number of neurons surviving at intervals after injection is expressed as the percentage of the initial number of neurons injected. Each graph shows the results of three separate experiments. In each experiment, three petri dishes were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

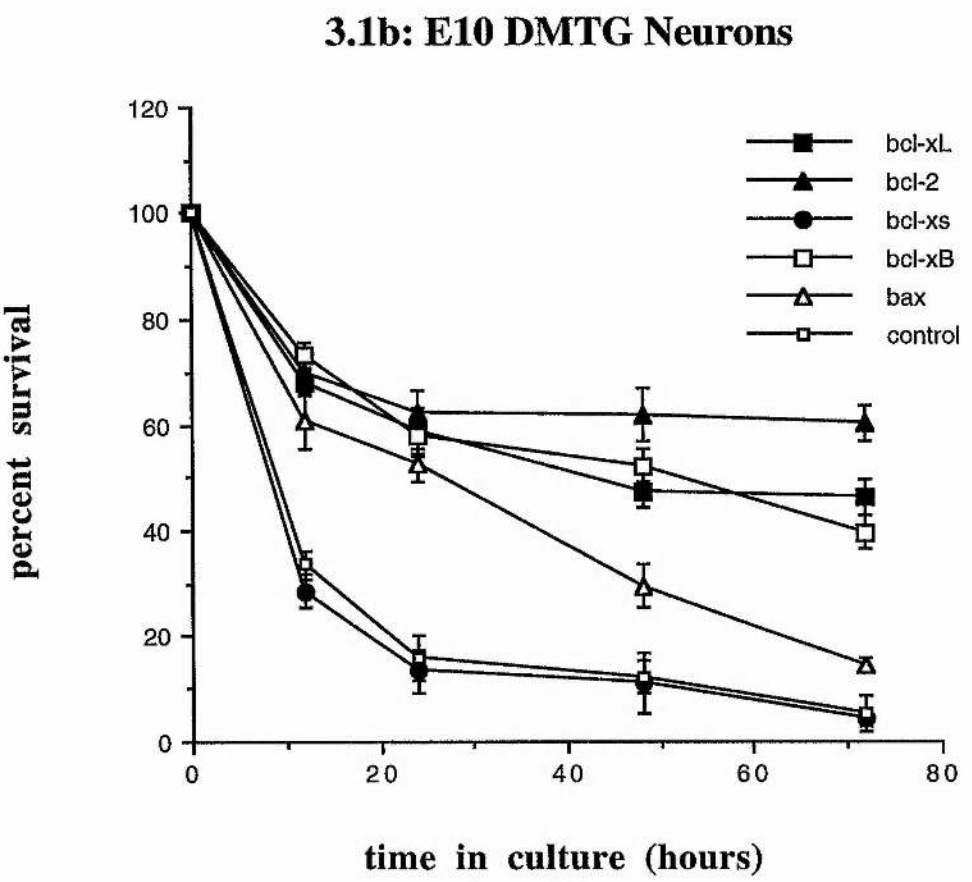


Figure 3.1b: The effects of injecting NGF-deprived E10 DMTG neurons with Bcl-2, Bcl-x_L, Bcl-x_β, Bcl-x_S or Bax expression vectors or the pSFFV vector without an insert (control injections). After 12 hours incubation with 5 ng/ml NGF, the neurons were washed extensively to remove these factors before being injected with expression vectors. The number of neurons surviving at intervals after injection is expressed as the percentage of the initial number of neurons injected. Each graph shows the results of three separate experiments. In each experiment, three petri dishes were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

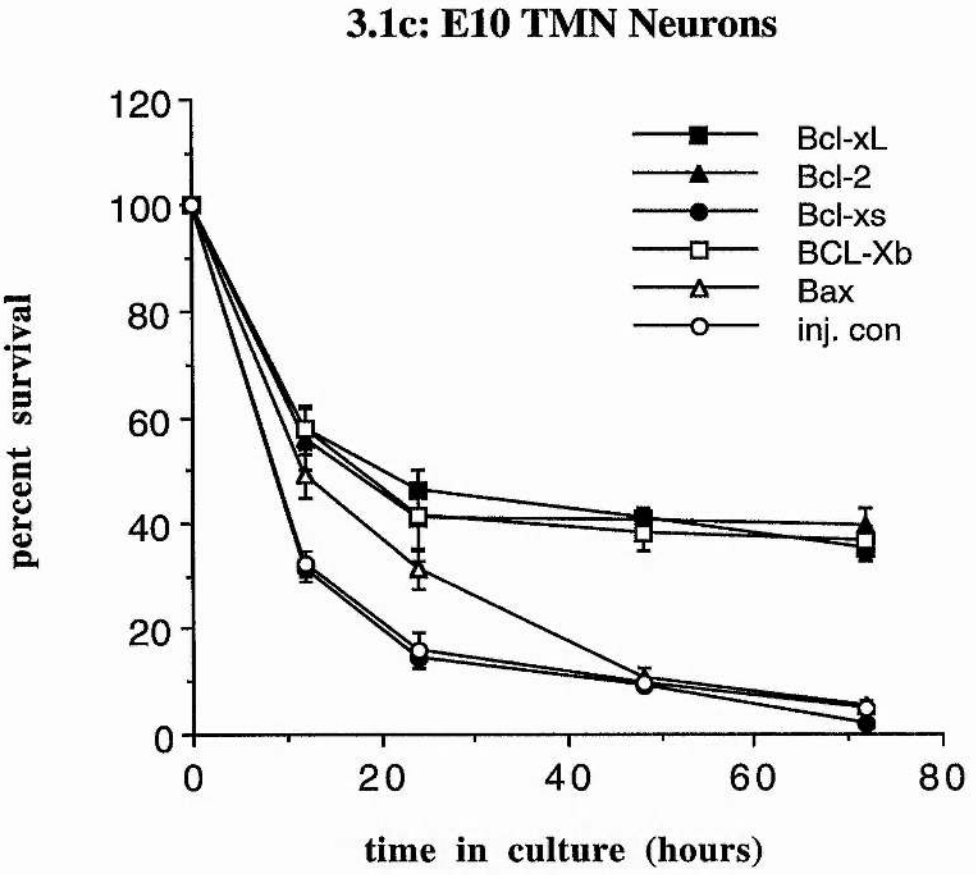


Figure 3.1c: The effects of injecting BDNF-deprived E10 TMN neurons with Bcl-2, Bcl-x_L, Bcl-x_β, Bcl-x_S or Bax expression vectors or the pSFFV vector without an insert (control injections). After 12 hours incubation with 5 ng/ml BDNF, the neurons were washed extensively to remove these factors before being injected with expression vectors. The number of neurons surviving at intervals after injection is expressed as the percentage of the initial number of neurons injected. Each graph shows the results of three separate experiments. In each experiment, three petri dishes were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

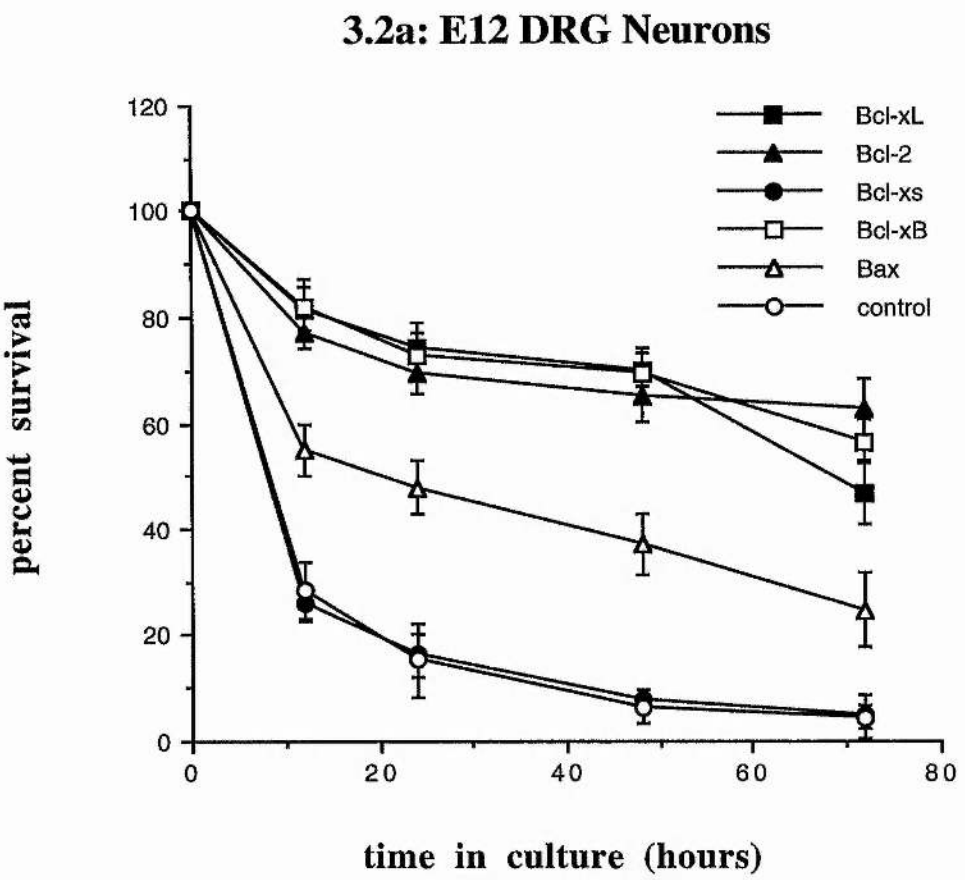


Figure 3.2a: The effects of injecting NGF-deprived E12 DRG neurons with Bcl-2, Bcl-x_L, Bcl-x_β, Bcl-x_S or Bax expression vectors or the pSFFV vector without an insert (control injections). After 12 hours incubation with 5 ng/ml NGF, the neurons were washed extensively to remove these factors before being injected with expression vectors. The number of neurons surviving at intervals after injection is expressed as the percentage of the initial number of neurons injected. Each graph shows the results of three separate experiments. In each experiment, three petri dishes were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

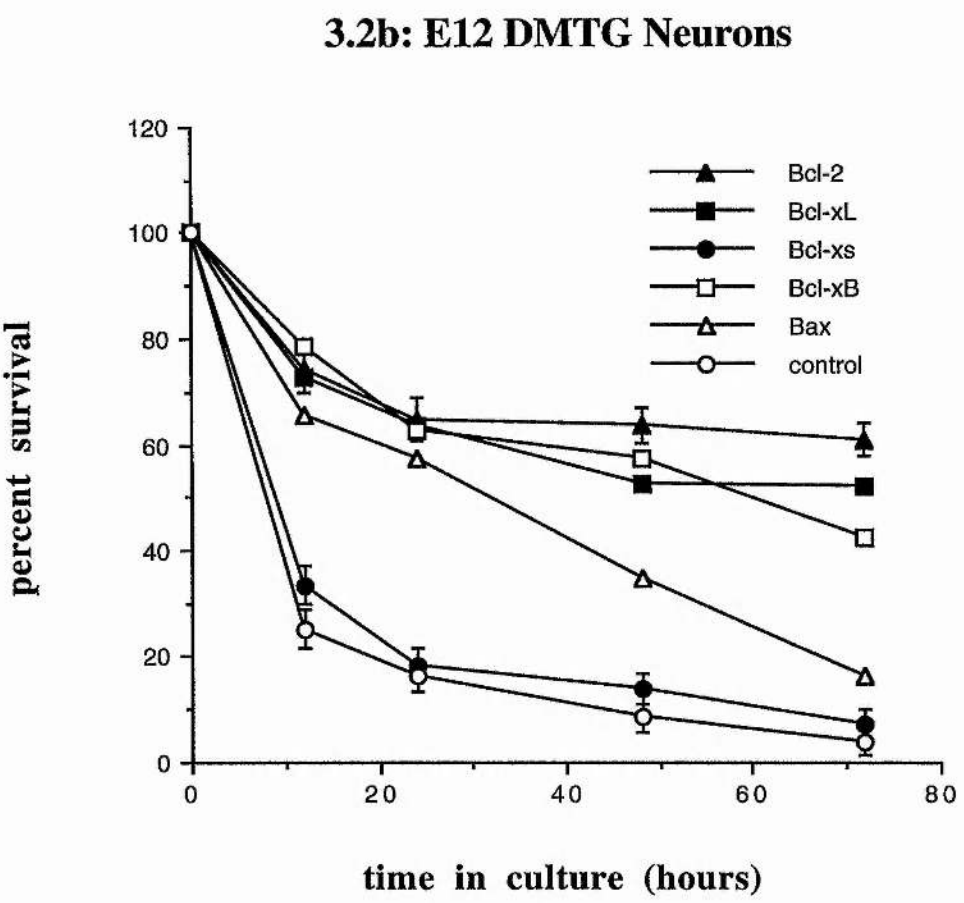


Figure 3.2b: The effects of injecting NGF-deprived E12 DMTG neurons with Bcl-2, Bcl-x_L, Bcl-x_β, Bcl-x_S or Bax expression vectors or the pSFFV vector without an insert (control injections). After 12 hours incubation with 5 ng/ml NGF, the neurons were washed extensively to remove these factors before being injected with expression vectors. The number of neurons surviving at intervals after injection is expressed as the percentage of the initial number of neurons injected. Each graph shows the results of three separate experiments. In each experiment, three petri dishes were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

Overexpression of bax but not bcl-2, bcl-xL, bcl-x_β, or bcl-x_s transiently rescues a subset of CNTF-deprived parasympathetic neurons

It has previously been shown that overexpression of bcl-2 is ineffective in rescuing embryonic chicken ciliary ganglion neurons deprived of CNTF (Allsopp et al., 1993). To determine if overexpression of bcl-xL, bcl-x_β, bcl-x_s, or bax is able to rescue CNTF-deprived ciliary neurons, E10 and E12 ciliary neurons were grown with CNTF for 12 hours before washing and injection with expression vectors containing bcl-2, bcl-xL, bcl-x_β , bcl-x_s, and bax cDNAs. Ciliary neurons injected with bcl-2, bcl-xL, bcl-x_β, or bcl-x_s expression vectors died rapidly and there was no significant difference between the number of neurons injected with these constructs or vector alone (Fig. 3.3) or uninjected CNTF-deprived neurons. These results suggest that bcl-2, bcl-xL and bcl-x_β have similar specificity in rescuing neurons deprived of different neurotrophic factors. In contrast, bax overexpression rescued a small number of ciliary neurons following CNTF withdrawal. Although this effect of bax was smaller and more short-lived than its effect on NGF-deprived neurons, this effect was consistently observed and was statistically significant at 12 and 24 hours post-injection for both E10 and E12 neurons ($p < 0.001$, t-test). These findings clearly demonstrate that bax overexpression does not invariably cause apoptosis, but can rescue neurons following neurotrophic factor deprivation. Moreover, bax seem to have a broader spectrum of activity among neurons in this respect than bcl-2, bcl-xL and bcl-x_β. Again, bcl-x_s had no survival promoting effect and again it is impossible to tell from these data if there is any negative effect on cell survival.

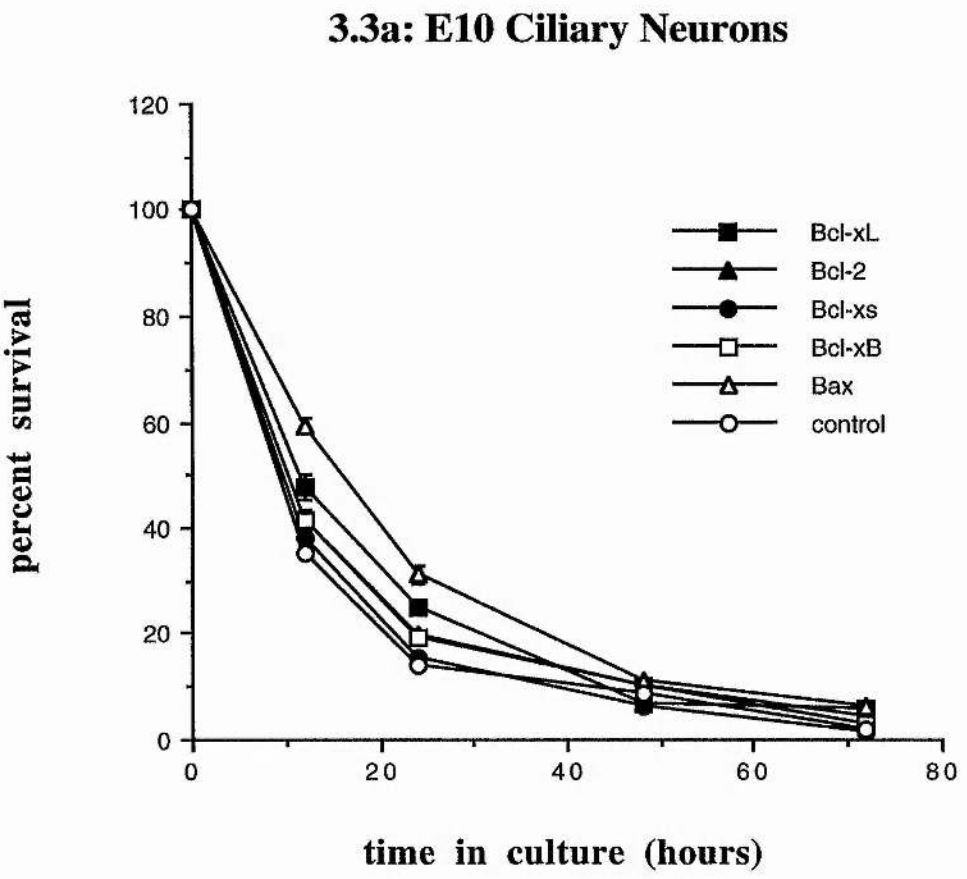


Figure 3.3a: The effects of injecting CNTF-deprived E10 ciliary neurons with Bcl-2, Bcl-x_L, Bcl-x_β, Bcl-x_S, or Bax expression vectors or the pSFFV vector without an insert (control injections). After 12 hours incubation with 5 ng/ml CNTF, the neurons were washed extensively to remove this factor and were injected with expression vectors. The number of neurons surviving at intervals after injection is expressed as the percentage of the initial number of neurons injected. The graph shows the results of three separate experiments. In each experiment, three petri dishes were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

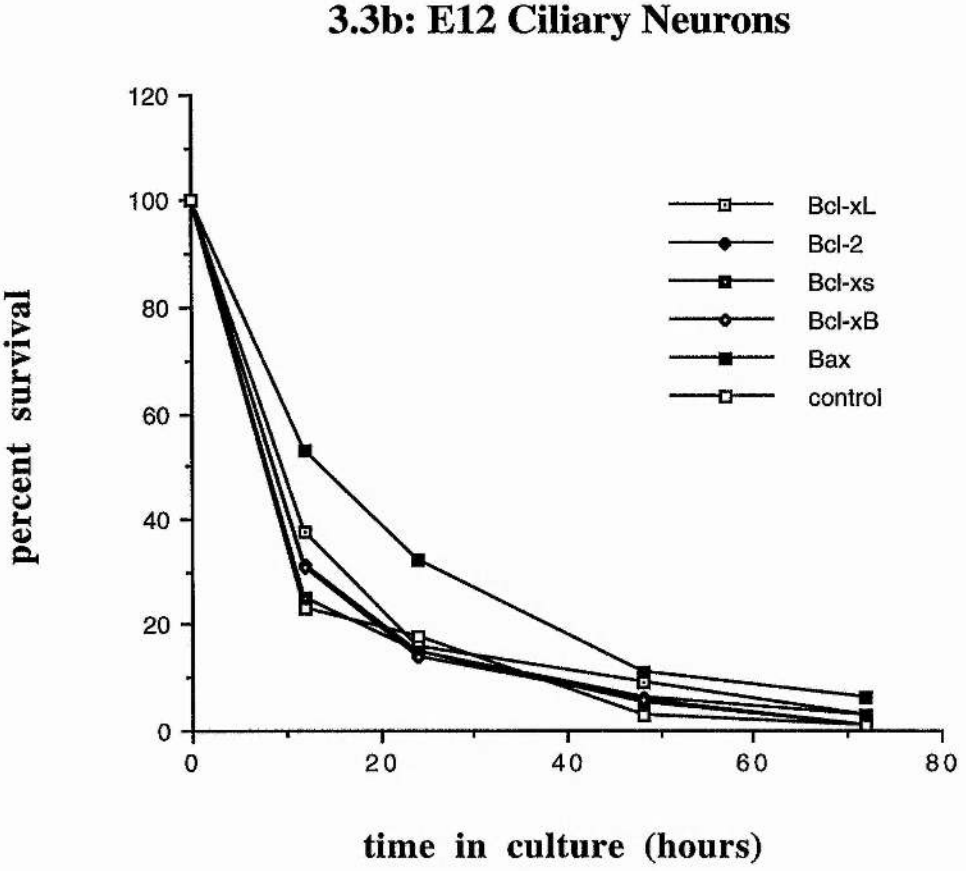


Figure 3.3b: The effects of injecting CNTF-deprived E12 ciliary neurons with Bcl-2, Bcl-x_L, Bcl-x_β, Bcl-x_S, or Bax expression vectors or the pSFFV vector without an insert (control injections). After 12 hours incubation with 5 ng/ml CNTF, the neurons were washed extensively to remove this factor and were injected with expression vectors. The number of neurons surviving at intervals after injection is expressed as the percentage of the initial number of neurons injected. The graph shows the results of three separate experiments. In each experiment, three petri dishes were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

Bax overexpression partially inhibits the survival response of neurons to neurotrophic factors

To investigate whether overexpression of Bax modulates the survival response of neurons to NGF and BDNF, a comparison was made of the survival of neurons injected with the Bax expression vectors grown with and without these neurotrophins. For comparison, neurons were also injected with the Bcl-2 expression vector to determine if this protein affects the neurotrophin survival response differently. DRG, DMTG and TMN neurons were initially grown for 12 hours with either NGF (DRG and DMTG neurons) or BDNF (TMN neurons) before being deprived of these neurotrophins and injected with expression vectors for Bcl-2 or Bax. Following microinjection, neurons were grown with or without the same neurotrophin for a further 3 days. Figure 3.4 shows that Bcl-2 overexpression does not interfere with the neurotrophin survival responses of these neurons. Neurons that were injected with the Bcl-2 expression vector and grown with NGF or BDNF survived slightly better than uninjected neurons grown with these neurotrophins. In marked contrast, injection of the Bax expression vector reduced the number of neurons surviving in neurotrophin-supplemented medium to virtually the same number that is sustained by Bax overexpression in the absence of neurotrophins.

Similar studies were carried out to see if Bax also antagonises the survival response of ciliary neurons to neurotrophic factors. As with sensory neurons, Bax overexpression reduced the number of neurons surviving in medium containing CNTF to the same number that are supported by Bax overexpression in medium that does not contain CNTF (Fig. 3.5). Taken together, the results of this set of experiments clearly show that Bax can partially oppose the response of neurons to neurotrophic factors and that the effects on survival are dominant to that of neurotrophic factors.

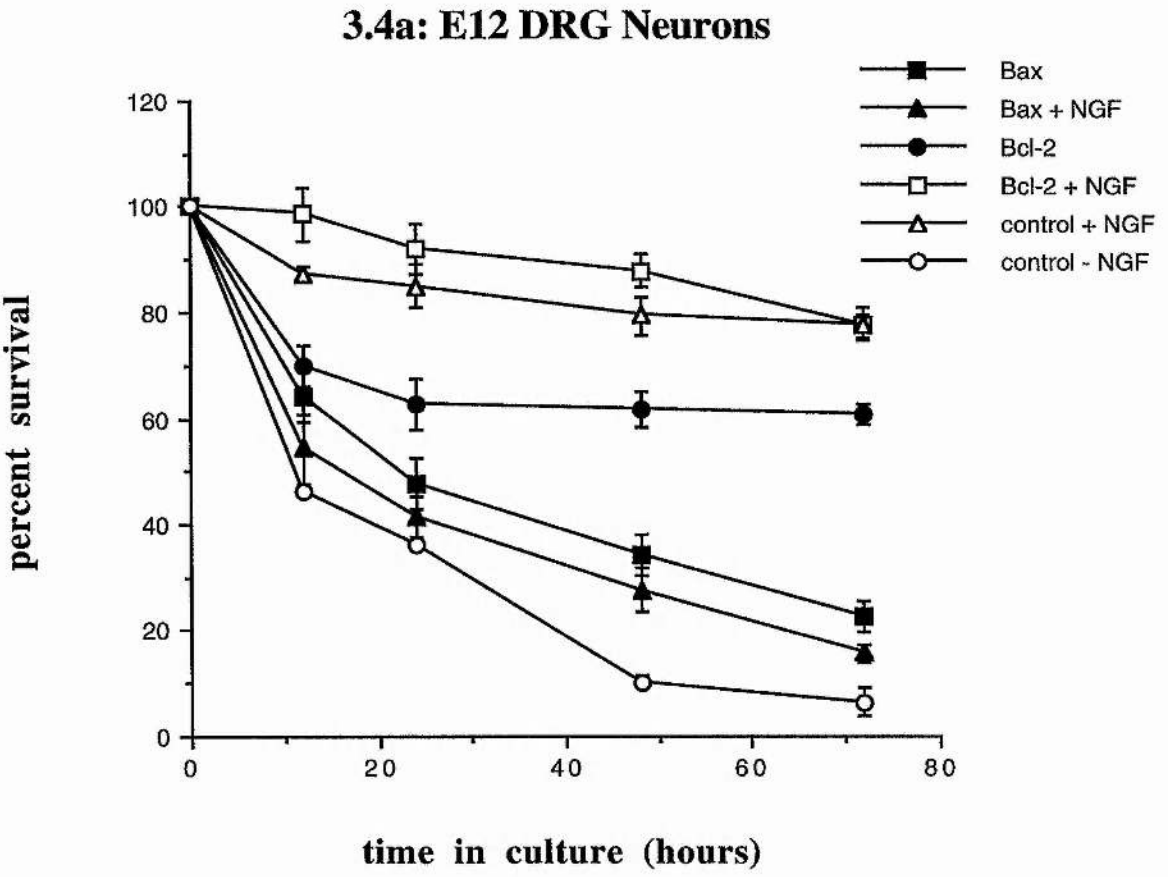


Figure 3.4a: The effects of overexpressing Bcl-2 and Bax on the survival responses of E12 DRG neurons to NGF (5ng/ml). The number of neurons surviving at intervals after washing is expressed as the percentage of the initial number of neurons at the time of washing. Each graph shows the results of three separate experiments. In each experiment, three petri dishes were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

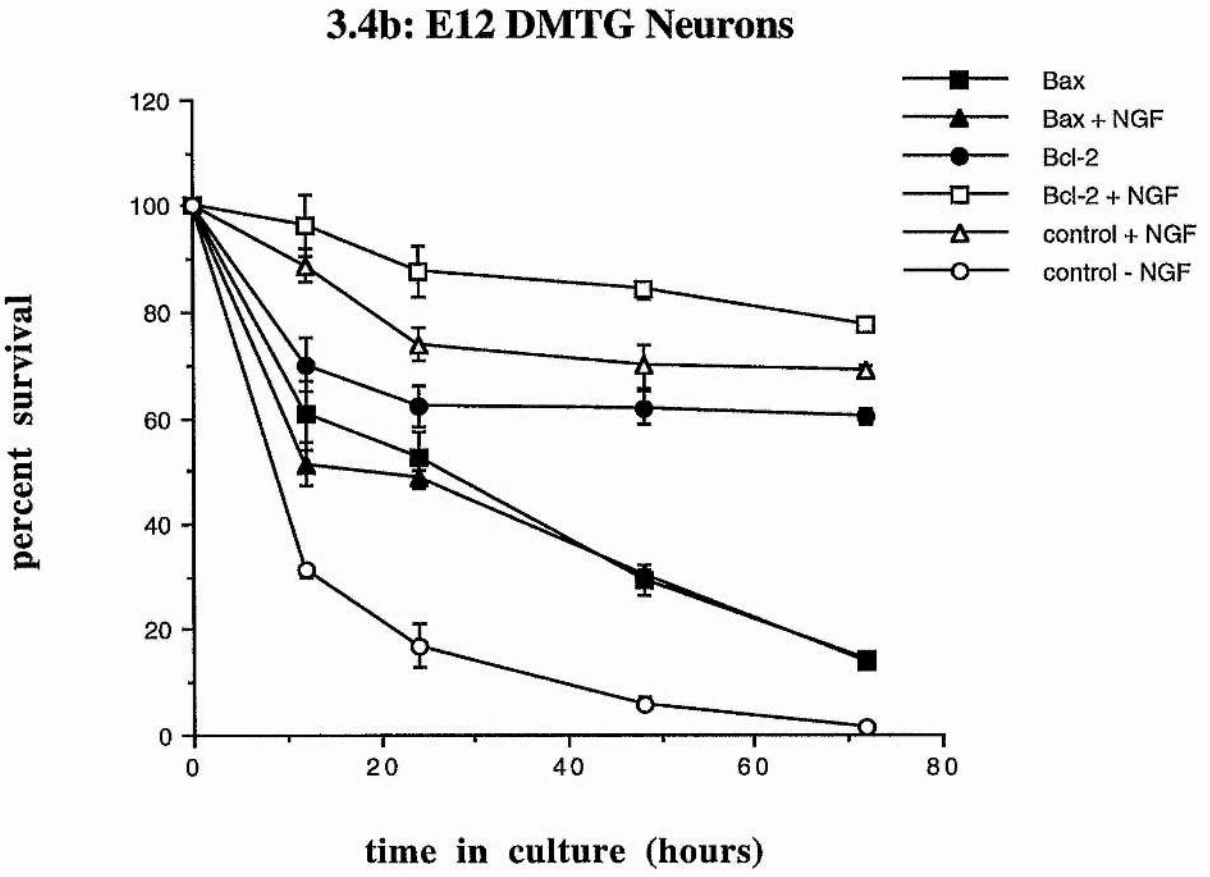


Figure 3.4b: The effects of overexpressing Bcl-2 and Bax on the survival responses of E12 DMTG neurons to NGF (5ng/ml). The number of neurons surviving at intervals after washing is expressed as the percentage of the initial number of neurons at the time of washing. Each graph shows the results of three separate experiments. In each experiment, three petri dishes were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

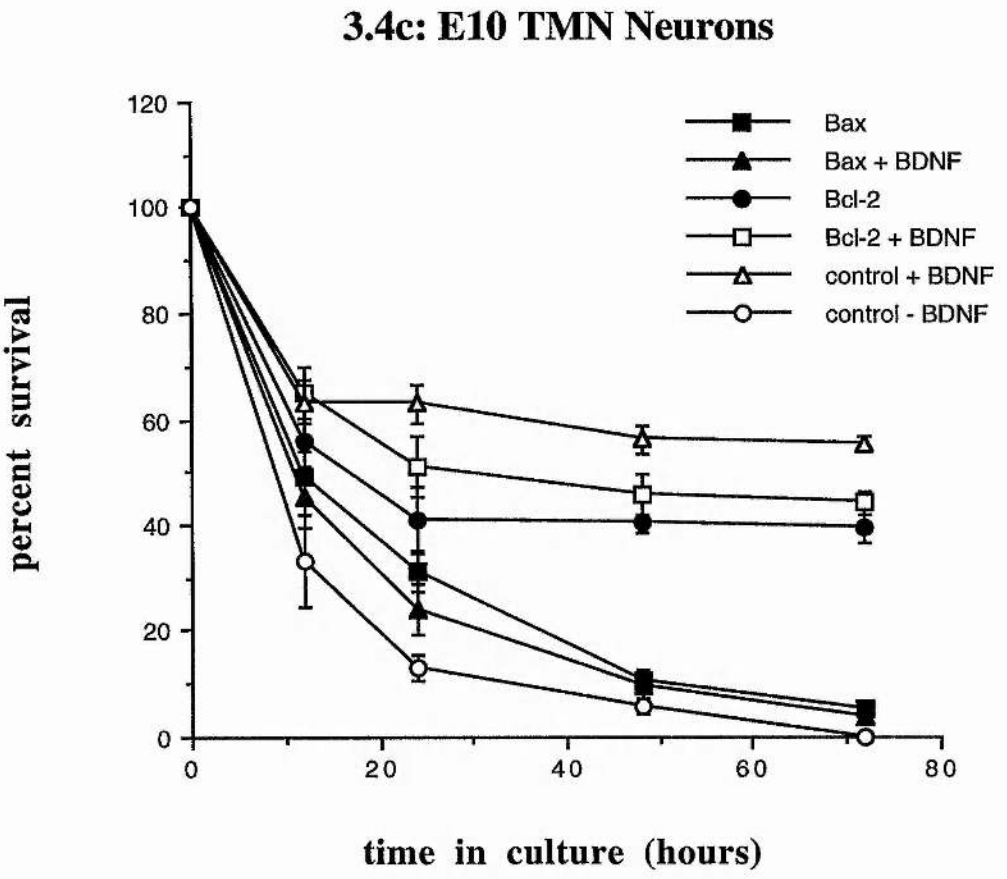


Figure 3.4c: The effects of overexpressing Bcl-2 and Bax on the survival responses of E10 TMN neurons to BDNF (5ng/ml). The number of neurons surviving at intervals after washing is expressed as the percentage of the initial number of neurons at the time of washing. Each graph shows the results of three separate experiments. In each experiment, three petri dishes were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

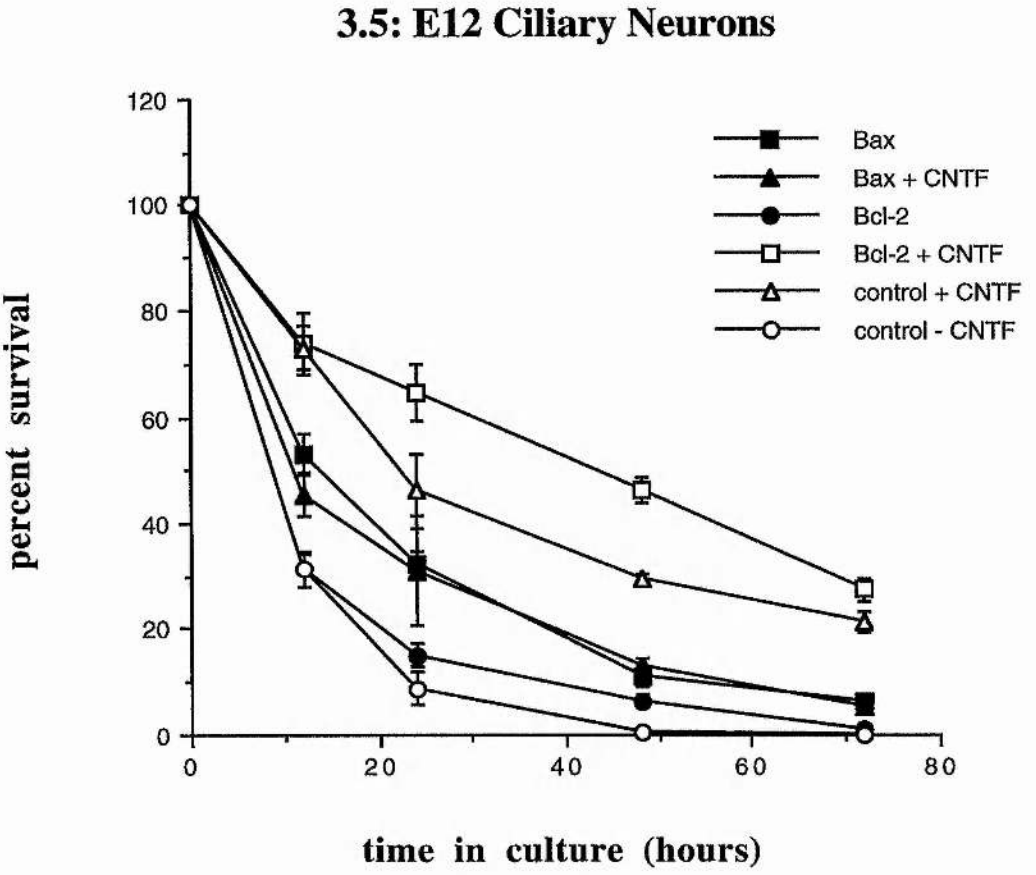


Figure 3.5 The effects of overexpressing Bax on the survival response of E12 ciliary neurons to CNTF (5ng/ml). The number of neurons surviving at intervals after washing is expressed as the percentage of the initial number of neurons at the time of washing. Each graph shows the results of three separate experiments. In each experiment, three petri dishes were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

The ratio of co-injected Bcl-2 and Bax expression vectors affects survival

Because Bax has been reported to inhibit the death repressor action of Bcl-2 in IL3-dependent cells (Oltvai et al., 1993), these experiments investigated whether Bax has a similar function in neurons by co-injecting neurotrophic factor-deprived DMTG neurons with Bcl-2 and Bax expression vectors. In these experiments, the ratio between the Bcl-2 and Bax expression vectors was altered whilst keeping the combined concentration of injected vectors the same (100 mg/ml). When either expression vector was injected in excess (2 : 1 or 1 : 2, Bcl-2 vector : Bax vector) many NGF-deprived neurons were rescued compared with control injected neurons, an excess of Bcl-2 over Bax being more effective than an excess of Bax over Bcl-2 (Figure 3.6). However, when the expression vectors were injected at a ratio of 1 : 1, very few neurons survived compared with control injected neurons.

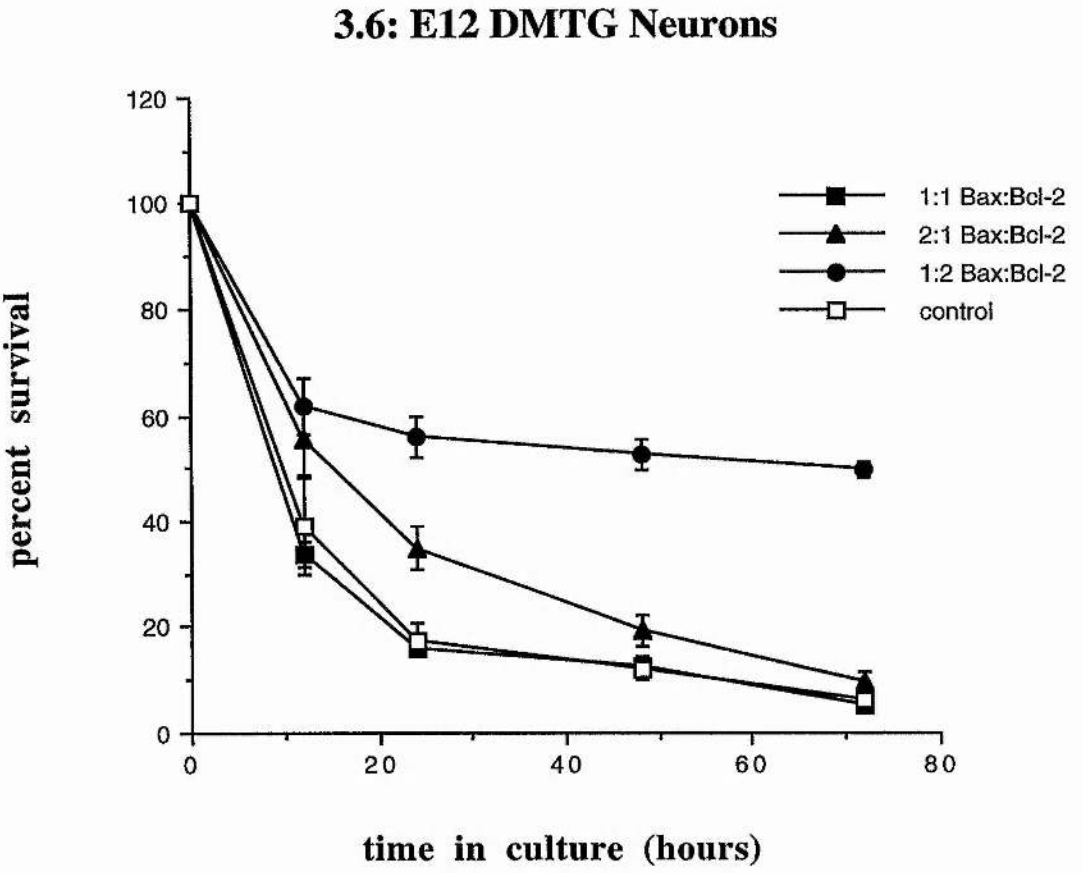


Figure 3.6: Effects of co-injecting NGF-deprived DMTG neurons with Bcl-2 and Bax expression vectors. The number of surviving neurons was monitored for 3 days following injection and is expressed as a percentage of the number of injected neurons. The graph shows the results of three separate experiments. In each experiment, three petri dishes were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

DISCUSSION

Microinjection was used to introduce expression vectors synthesising members of the bcl-2 family of intracellular proteins into cultured neurons in order to better understand how these proteins might influence neuronal survival or modulate the survival response of neurons to neurotrophic factors. Previous microinjection studies have shown that overexpression of bcl-2 is able to prevent the death of many sympathetic and sensory neurons deprived of members of the NGF family of neurotrophins, but is ineffective in rescuing neurons that were initially grown with CNTF, or GPA with bFGF (Garcia et al., 1992; Allsopp et al., 1993, 1995). These data presented here demonstrate that overexpression of bcl-xL or bcl-x_β is as effective as bcl-2 overexpression in rescuing NGF-deprived and BDNF-deprived neurons. Like bcl-2, neither bcl-xL nor bcl-x_β are able to rescue CNTF-deprived ciliary neurons. These results suggest that bcl-2, bcl-xL and bcl-x_β have similar potency and specificity in rescuing neurons deprived of different neurotrophic factors.

The similar efficiency of bcl-xL and bcl-x_β in preventing the death of neurotrophin-deprived neurons indicates that the C-terminal hydrophobic domain of bcl-x is not required for its death-suppressor function in neurons. Studies of bcl-2 suggest that its C-terminal hydrophobic domain is necessary for the attachment to the protein cytosolic side of cell membranes (Nguyen et al., 1993; Tanaka et al., 1993) but does not appear to be required for its death-suppressor function (Borner et al., 1994). The data presented in this study shows that bcl-x_s is as ineffective in rescuing growth factor deprived neurons as it is in rescuing growth factor deprived hematopoetic cells (Boise et al., 1993).

The most unexpected result of this study is that overexpression of bax is also able to prevent the death of some neurons deprived of neurotrophic factors. Not only does bax rescue some sensory neurons deprived of either NGF or BDNF, but also it was able to rescue some ciliary neurons deprived of CNTF. Although bax is not as effective as bcl-2, bcl-xL and bcl-x_β in rescuing neurotrophin-deprived sensory neurons and the effect on CNTF-deprived ciliary neurons is transient, this survival-

promoting effect of bax is statistically significant. Thus, contrary to the widely accepted view that bax promotes cell death based on studies of an IL-3-dependent cell line (Korsmeyer et al., 1993; Oltvai et al., 1993; Oltvai and Korsmeyer, 1994), these findings suggest that bax overexpression need not invariably cause apoptosis, but can promote the survival of neurons following neurotrophic factor deprivation. Moreover, bax seems to have a broader spectrum of survival-promoting activity among neurons than bcl-2, bcl-xL or bcl-x_β. However, because the effects of human bax are studied in chicken neurons one cannot rule out the caveat that evolutionary divergence in the bax genes might result in human bax acting as a dominant negative in chicken cells.

This study has shown that bax overexpression is also able to inhibit the survival response of neurons to neurotrophic factors; bax overexpression reduces the number of neurons surviving with neurotrophic factors to the number that survive when bax is overexpressed in the absence of neurotrophic factors. In contrast, bcl-2 overexpression does not antagonise the survival response of neurons to neurotrophic factors; the survival of bcl-2-overexpressing neurons is slightly better in the presence of neurotrophic factors. The mechanism by which bax promotes neuronal survival in the absence of neurotrophic factors whilst at the same time inhibiting the survival effect of these factors is unclear. Cultured neurons might express one or more members of the bcl-2 family of proteins that may be required for the survival response of neurons to neurotrophic factors. Sensory neurons and ciliary neurons, for example, are known to express bcl-2 mRNA (Allsopp et al., 1995). The demonstration that antisense bcl-2 RNA reduces the level of endogenous bcl-2 mRNA and inhibits the survival response of sensory neurons to BDNF but does not affect the survival response of ciliary neurons to CNTF, GPA or bFGF (Allsopp et al., 1995), suggests that endogenously expressed bcl-2 is required for the long-term survival response to BDNF but not to CNTF, GPA or bFGF. It is possible that overexpressed bax protein may compete with other members of the bcl-2 family of proteins and form heterodimers with these proteins which may interfere with their function and compromise the survival response to neurotrophic

factors. Although bax contains BH1 and BH2 domains, which are thought to be required for the survival effect of bcl-2 family members (Yin et al., 1994), and can promote neuronal survival in the absence of neurotrophic factors, bax is less effective in doing so than bcl-2 or bcl-x. Unfortunately, the small numbers of purified embryonic neurons available for these studies precludes direct measurements of the relative levels of bcl-2-related proteins in these neurons.

In contrast to the demonstration that bax inhibits the death repressor action of bcl-2 in IL3-dependent cells (Oltvai et al., 1993), this study has shown that co-injection of bax and bcl-2 expression vectors in sensory neurons does not accelerate cell death. When either expression vector was in excess (2 : 1 or 1 : 2, bcl-2 vector : bax vector) many NGF-deprived neurons were rescued (excess bcl-2 vector being more effective than bax vector). However, when the expression vectors were injected at a ratio of 1 : 1, very few additional neurons survived compared with uninjected controls. Although the relative levels of bcl-2 and bax proteins expressed in injected neurons are unknown and cannot be assessed because of the very small number of neurons available for analysis, these results raise the possibility that when either bcl-2 or bax proteins are expressed in excess in neurotrophic factor-deprived neurons, cell death is delayed.

Site-directed mutagenesis of Gly 145 in the BH1 domain or Trp 188 in the BH2 domain of bcl-2 prevents bcl-2 from forming heterodimers with bax but does not impede the formation of bcl-2 homodimers. Because these mutations inactivate the death repressor action of bcl-2, it has been proposed that bcl-2 must bind to bax in order to exert its death-repressor activity (Yin et al., 1994). However, there are alternative explanations for the effects of these mutations. First, it is possible that the bcl-2 homodimer rather than the bcl-2/bax heterodimer is the active suppressor of cell death and that these mutations inactivate the death-repressor function of bcl-2 without affecting homodimerisation. The demonstration that overexpression of bax prevents the death of neurotrophic factor-deprived neurons raises the possibility that bax homodimers may have a death-repressor function.

Second, mutations in the BH domains may not only prevent the formation of bcl-2/bax heterodimers but prevent bcl-2 from associating with other proteins that might be important for suppressing cell death. For example, bcl-2 has been shown to associate with the ras-related protein R-ras p23 (Fernadez-Sarabia & Bischoff, 1993) and with a novel protein, BAG-1, that enhances the survival-promoting effect of bcl-2 (Takayama et al., 1995).

The generation of mice with a null mutation in the bax gene has been reported (Knudson et al., 1995). These mice display both hyperplasia (increased numbers of B and T cells) and hypoplasia (testis atrophy associated with an increased numbers of apoptotic cells). These findings provide additional new evidence that, in addition to promoting cell death, bax may promote the survival of certain cell types. Analysis of the nervous system of these mice and studies of their neurons in culture will clarify the extent to which bax acts as a positive or negative regulator of cell survival in the nervous system.

bax is not the only family member that can cause death or survival depending on the assay system used. Anti-apoptotic effects of bak (Keifer et al., 1995) and pro-apoptotic effects of bcl-2 have also been observed. Thus it is possible that these proteins can have positive or negative effects on cell survival depending upon the cellular context.

IV. BCL-2 TRANSGENIC MICE: NEURONAL SURVIVAL STUDIES

CHAPTER FOUR

BCL-2 TRANSGENIC MICE:

NEURONAL SURVIVAL STUDIES

INTRODUCTION

The Bcl-2 protein is widely expressed in a number of different tissues and there is considerable evidence to suggest that it plays an important role in regulating cell survival in the immune system. Whereas mice with targeted null mutations in the Bcl-2 gene have markedly reduced number of B and T cells due to increased apoptosis (Nakayama et al., 1993, 1994; Veis et al., 1993), mice carrying a transgene causing high levels of Bcl-2 expression in the immune system show extended survival of B and T cells (McDonnell et al., 1989, 1990).

Experimental overexpression of Bcl-2 in developing neurons has shown that Bcl-2 is capable of influencing neuronal survival. Microinjection of a Bcl-2 expression vector into cultured sympathetic and sensory neurons prevents their death following neurotrophin deprivation (Allsopp et al., 1993; Garcia et al., 1992), and mice expressing a *bcl-2* transgene under the control of a neuron-specific enolase promoter have enlarged brains and increased numbers of neurons in several regions (Martinou et al., 1994).

Although Bcl-2 is capable of influencing neuronal survival *in vitro* and its overexpression can prevent naturally occurring cell death *in vivo*, it is not clear to what extent Bcl-2 plays a role in regulating neuronal survival in the embryo. In the developing nervous system, Bcl-2 is expressed in proliferating neuroepithelial cells of ventricular zones and in postmitotic cells of several other regions including the cortical plate, cerebellum, hippocampus and spinal cord (Merry et al., 1994). Bcl-2

expression decreases in CNS neurons postnatally, but is retained in sensory and sympathetic neurons throughout life. Although this pattern of expression is consistent with a role for Bcl-2 in regulating neuronal survival during development and sustaining the survival of some neurons in the adult, *bcl-2* $^{-/-}$ mice appear to have grossly normal nervous systems and do not exhibit any obvious neurological abnormalities (Nakayama et al., 1993, 1994; Veis et al., 1993). Statistically significant deficiencies in sensory, motor and sympathetic neurons have, however, recently been described in postnatal Bcl-2-deficient mice (Michaelidis et al., 1996). Neurotrophins have not been shown to influence either Bcl-2 mRNA levels or those of the other members of this gene family in hippocampal and cerebellar granular cells (Ohga et al., 1996). Focusing on the sympathetic nervous system, *in vitro* analysis of primary neuronal cultures from superior cervical ganglia of Bcl-2-deficient mice has shown that Bcl-2 is an important regulator of sympathetic neuron survival after NGF deprivation during the period of naturally occurring neuronal death (Greenlund et al., 1995).

To clarify the role of Bcl-2 in regulating the survival of primary sensory neurons during embryonic development, the *in vitro* and *in vivo* survival of sensory neurons from animals carrying a null mutation in the Bcl-2 gene has been compared with the survival of sensory neurons from wild type littermates, at several embryonic ages. In particular, this study has focused on two well-characterised population of cranial sensory neurons; those of the trigeminal and nodose ganglia. From E12 onwards, the majority of mouse trigeminal ganglion neurons are supported by NGF in culture (Buchman & Davies, 1993). The total number of neurons in the trigeminal ganglia peaks between E13 and E14 and falls by half by birth as a result of naturally occurring cell death (Davies & Lumsden, 1984). These neurons that survive this period of cell death do so because they acquire adequate supplies of NGF from their peripheral targets (Buchman & Davies, 1993, Davies et al., 1987b; Pinon et al., 1996). The majority of nodose ganglion neurons are supported by BDNF in culture

from E12 onwards and depend upon BDNF for survival *in vivo* during embryonic development (Davies et al., 1993; Ernfors et al., 1994; Jones et al., 1994).

The results presented in this chapter show that the survival response of Bcl-2-deficient trigeminal and nodose neurons to neurotrophins is less sustained than that of wild type neurons during the early fetal period, and that there is a significant reduction in the number of neurons in the trigeminal ganglia of *bcl-2* $^{-/-}$ mice during embryonic development. Together these data suggest a role for Bcl-2 in maintaining the survival of some embryonic sensory neurons. The *in vivo* investigation was carried out in collaboration with Luzia G.P. Pinon.

RESULTS

Response of Bcl-2-deficient sensory neurons to neurotrophins *in vitro*

Mice that were heterozygous for a Bcl-2 null mutation (Nakayama et al., 1993, 1994) were crossed to obtain *bcl-2*$^{-/-}$, *bcl-2*$^{+/-}$ and *bcl-2* $^{+/+}$ embryos. Separate, low-density, dissociated cultures were established from the trigeminal and nodose ganglia of each embryo and the survival of the neurons growing with and without neurotrophins monitored for three days in culture. The genotypes of the embryos were subsequently determined by a PCR-based approach using DNA extracted from embryonic tissues.

Trigeminal Neurons

Dissociated cultures of trigeminal ganglion neurons were established from embryos at E14, E16 and E18. The neurons were grown with or without NGF for 72 hours, and the number of surviving neurons counted at intervals during this time. At E14, which is at the peak of naturally occurring neuronal death in the trigeminal ganglion (Pinon et al., 1996), about half the initial number of neurons from wild type embryos were still surviving with NGF after 72 hours incubation (Fig. 4.1). In marked contrast, neurons from *bcl-2*$^{-/-}$ embryos died much more rapidly in the presence of

NGF, and all were dead by 48 hours. Neurons from *bcl-2*$^{+/-}$ embryos died at an intermediate rate in the presence of NGF, and all were dead by 72 hours. Although Bcl-2-deficient neurons died rapidly in the presence of NGF, they survived better than neurons grown without NGF in the culture medium. In these control cultures, all neurons were dead by 24 hours, and there was no difference in the rate of neuronal death between neurons from wild type and Bcl-2-deficient embryos (Fig. 4.1b). These results suggest that endogenous Bcl-2 expression is required for the sustained survival response of trigeminal neurons to NGF at E14.

In E16 cultures, the *bcl-2* mutation had a much smaller effect on the NGF survival response (Fig.4.2a). Although, there were significantly fewer Bcl-2-deficient neurones surviving with NGF after 24,48 and 72 hours incubation compared with wild type neurones ($p<0.05$. t-test), there was on average only a 20% reduction in the number of surviving neurons at these time points. Both wild type and Bcl-2-deficient neurons died rapidly in the absence of NGF at this stage, and the *bcl-2* null mutation did not effect the rate of death in control cultures (Fig. 4.2b).

By E18, which is near the end of the period of naturally occurring neuronal death in the trigeminal ganglion (Davies & Lumsden, 1984; Pinon et al., 1996), there were no significant differences between the numbers of wild type and Bcl-2-deficient neurones surviving in the presence of NGF (Fig. 4.3a). Again there were no differences between the three genotypes in the control cultures (Fig. 4.3b). These results suggest that by the end of the period of naturally occurring neuronal death, *bcl-2* is not required for a sustained survival response of trigeminal ganglion neurons to NGF.

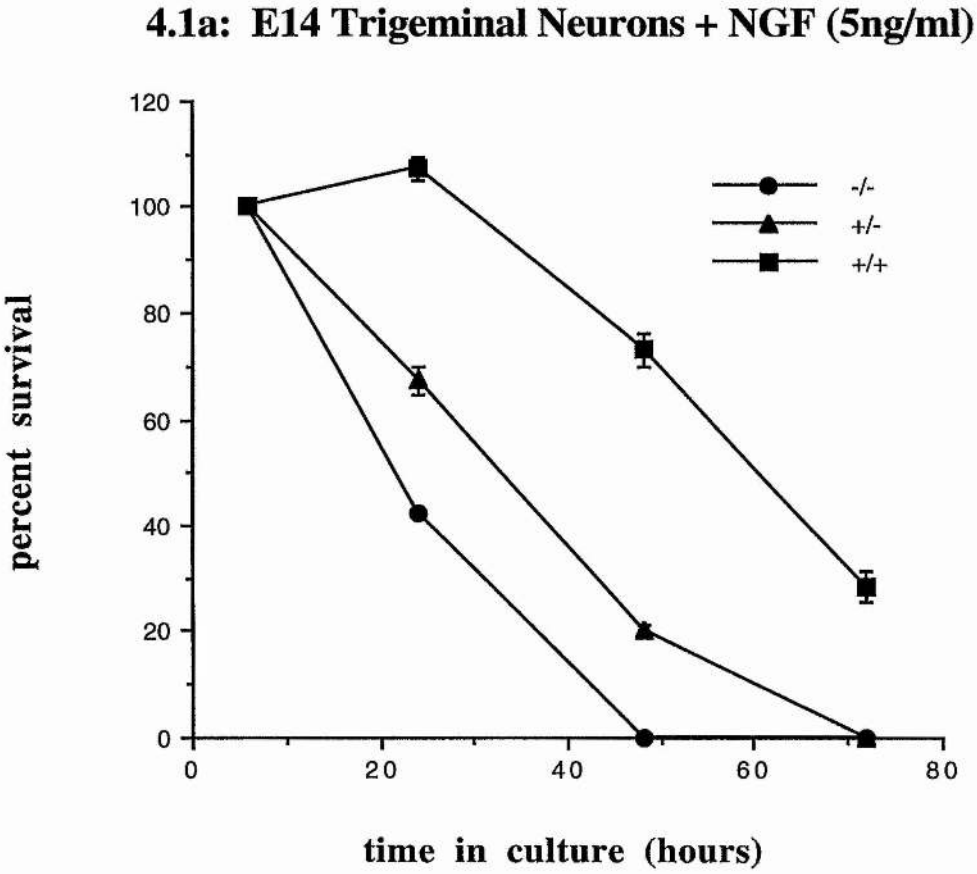


Figure 4.1a: Percent survival of E14 trigeminal neurons from *bcl-2-/-* (n= 11), *bcl-2+/-* (n= 25), and *bcl-2 +/+* (n= 12) embryos grown for 72 hours in culture with NGF (5ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

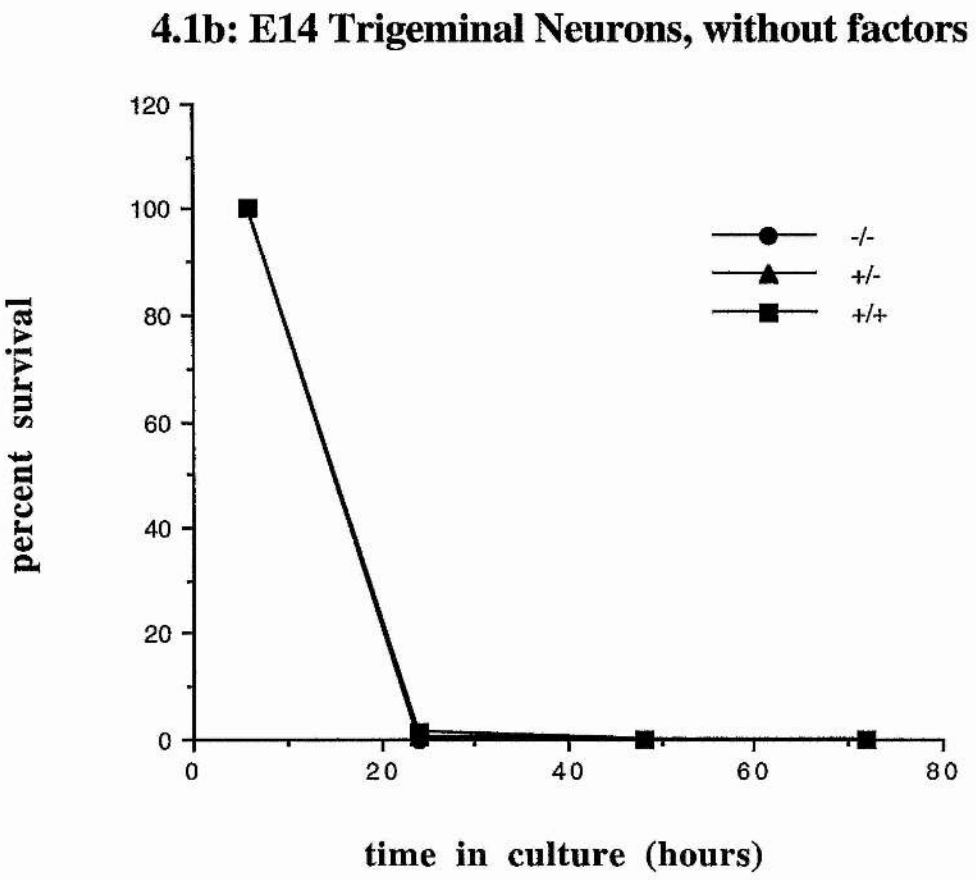


Figure 4.1b: Percent survival of E14 trigeminal neurons from *bcl-2-/-* (n= 11), *bcl-2+/-* (n= 25), and *bcl-2 +/+* (n= 12) embryos grown for 72 hours in culture without factors. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

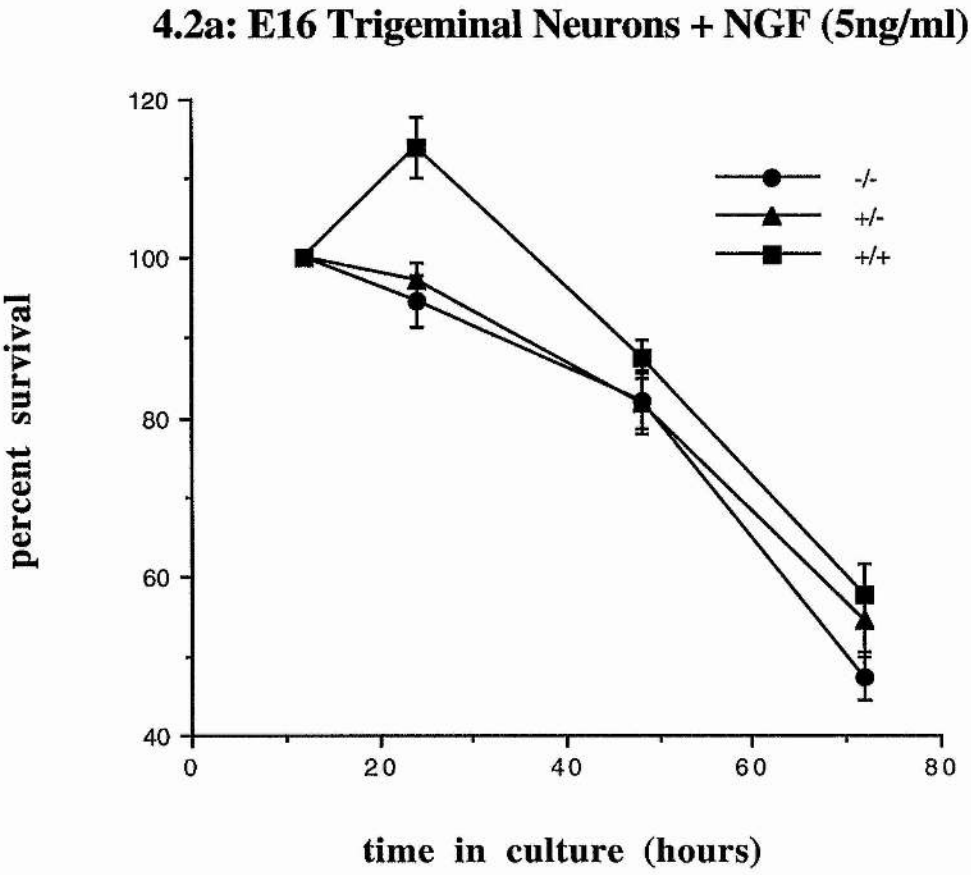


Figure 4.2a: Percent survival of E16 trigeminal neurons from *bcl-2-/-* (n= 6), *bcl-2+/-* (n= 13), and *bcl-2 +/+* (n= 7) embryos grown for 72 hours in culture with NGF (5ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

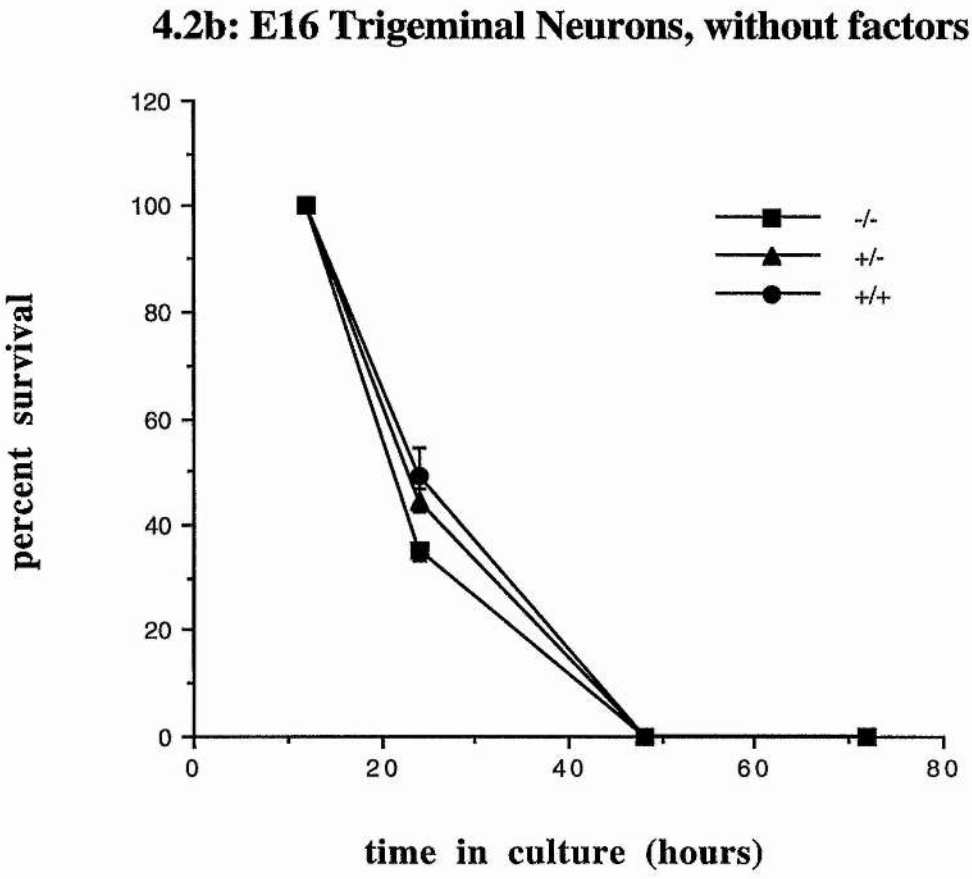


Figure 4.2b: Percent survival of E16 trigeminal neurons from *bcl-2-/-* (n= 6), *bcl-2+/-* (n= 13), and *bcl-2 +/+* (n= 7) embryos grown for 72 hours in culture without factors. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

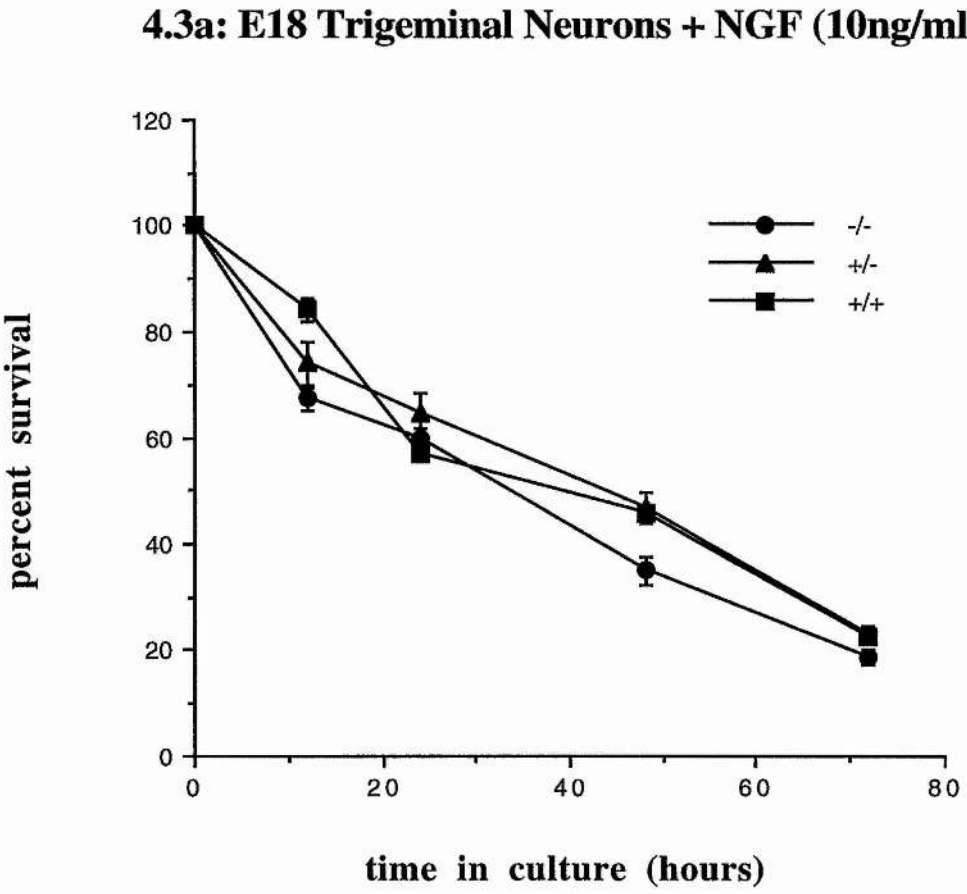


Figure 4.3a: Percent survival of E18 trigeminal neurons from *bcl-2-/-* (n= 6), *bcl-2+/-* (n= 13), and *bcl-2 +/+* (n= 7) embryos grown for 72 hours in culture with NGF (10ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

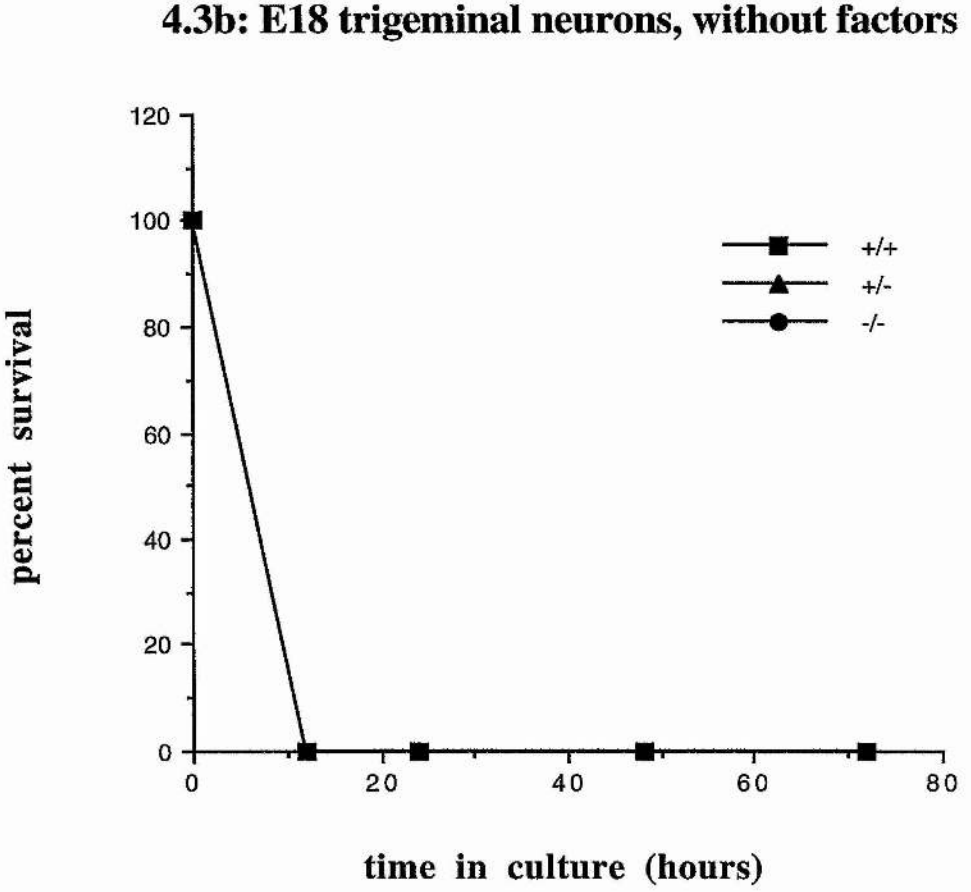


Figure 4.3b: Percent survival of E18 trigeminal neurons from *bcl-2-/-* (n= 8), *bcl-2+/-* (n= 14), and *bcl-2 +/+* (n= 5) embryos grown for 72 hours in culture without factors. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

Nodose Neurons

Very similar trends were observed in the BDNF survival responses of nodose neurons from *bcl-2*$^{-/-}$, *bcl-2* $^{+/-}$ and *bcl-2*$^{+/+}$ embryos at E14, E16 and E18 as were seen in the NGF survival responses of trigeminal neurons over this period in development.

In E14 cultures, the majority of nodose neurons from wild type embryos were still surviving in the presence of BDNF after 72 hours incubation, whereas neurons from *bcl-2*$^{-/-}$ embryos died much more rapidly in the presence of BDNF, and all were dead by 48 hours (Fig 4.4). Neurons from *bcl-2*$^{+/-}$ embryos died at an intermediate rate with less than 10% surviving by 72 hours. Although Bcl-2-deficient neurons died rapidly in the presence of BDNF, they survive better than those grown without BDNF. In these control cultures, all neurons were dead by 24 hours irrespective of the genotype of the embryo from which they were obtained. Like the survival response of E14 trigeminal neurons to NGF, these results suggest that endogenous Bcl-2 expression is required for the sustained survival response of early fetal nodose neurons to BDNF.

In E16 cultures, the *bcl-2* null mutation had a less dramatic effect on the response of nodose neurons to BDNF (Fig. 4.5). Although there were 10 to 15% fewer neurons from bcl-2 null mice surviving with BDNF compared neurons from wild type mice at 24 and 48 hours, by 72 hours there was a greater than 3 fold difference in the number of neurons from *bcl-2*$^{-/-}$ and *bcl-2* $^{+/+}$ embryos surviving with BDNF. At all time points, these differences were statistically significant ($p<0.05$).

By E18, there were no clear differences between the numbers of neurons from *bcl-2*$^{-/-}$and *bcl-2*$^{+/+}$ embryos surviving in the presence of BDNF, whereas in the absence of BDNF all neurons die rapidly (Fig. 4.6). As with the survival response of developing trigeminal neurons to NGF, these results suggest that by he late fetal period, Bcl-2 is not required for a sustained survival response of nodose neurons to BDNF.

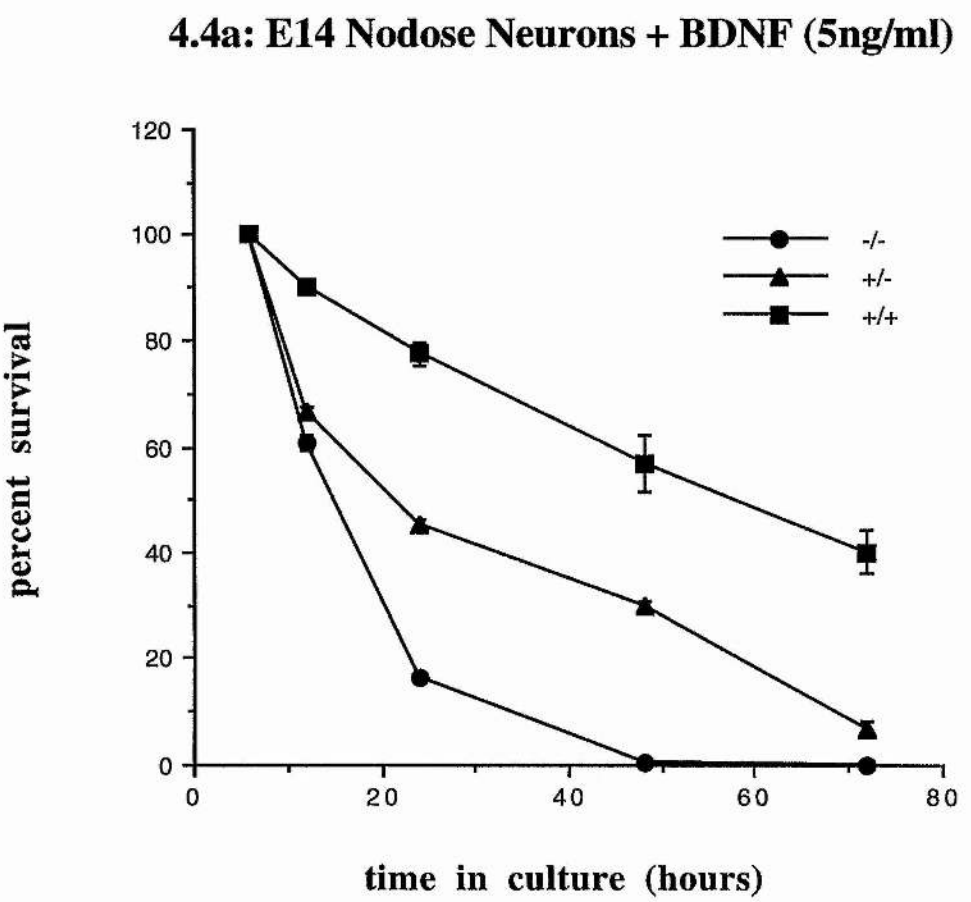


Figure 4.4a : Percent survival of E14 nodose neurons from *bcl-2-/-* (n= 11), *bcl-2+/-* (n= 25), and *bcl-2 +/+* (n= 12) embryos grown for 72 hours in culture with BDNF (5ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied

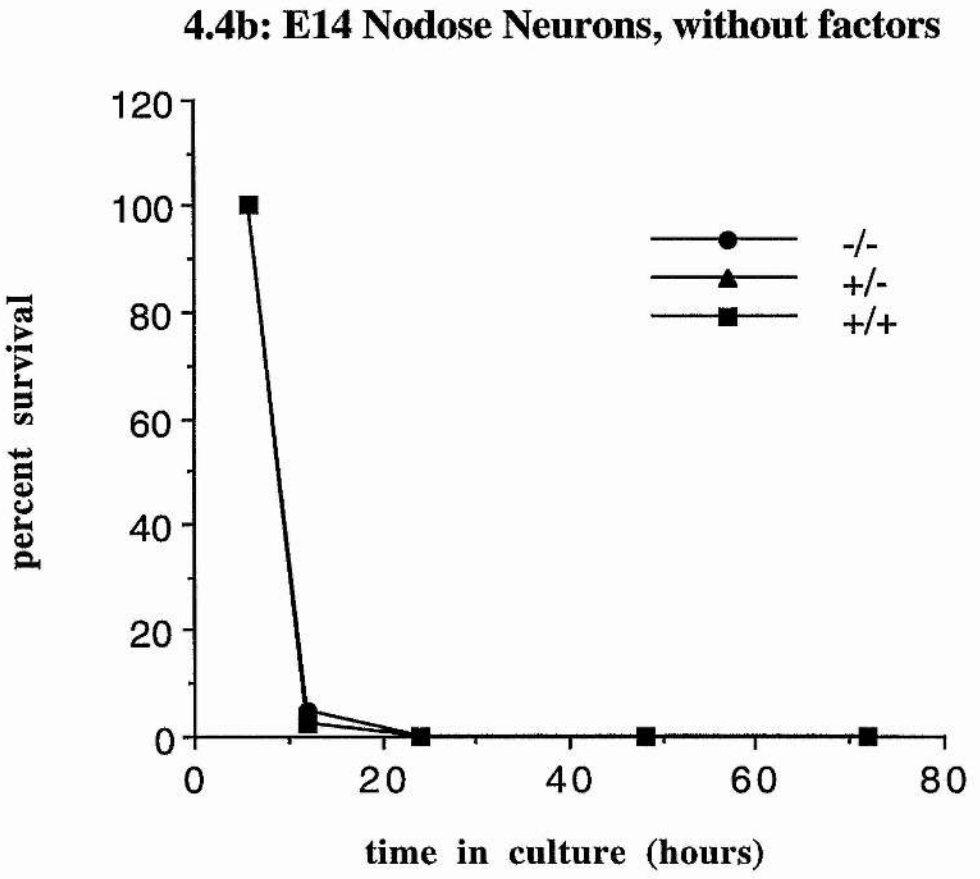


Figure 4.4b : Percent survival of E14 nodose neurons from *bcl-2-/-* (n= 11), *bcl-2+/-* (n= 25), and *bcl-2 +/+* (n= 12) embryos grown for 72 hours in culture without factors. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied

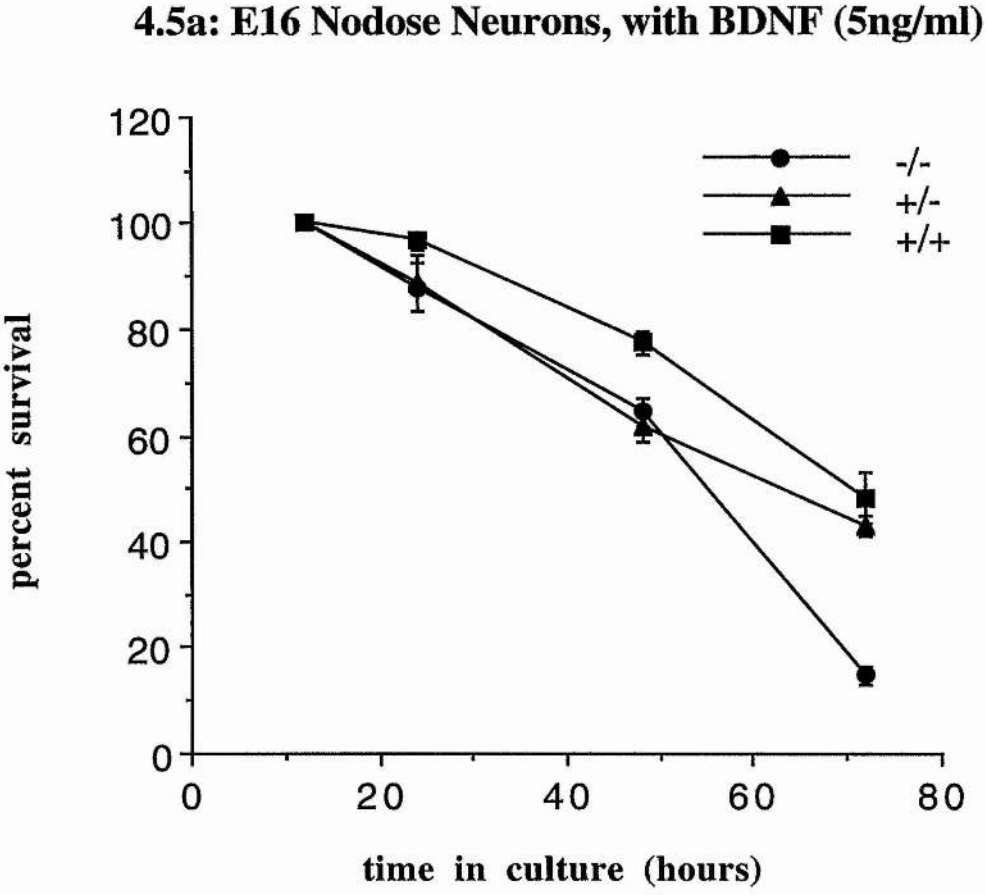


Figure 4.5a : Percent survival of E16 nodose neurons from *bcl-2-/-* (n= 6), *bcl-2+/-* (n= 13), and *bcl-2 +/+* (n= 7) embryos grown for 72 hours in culture with BDNF (5ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

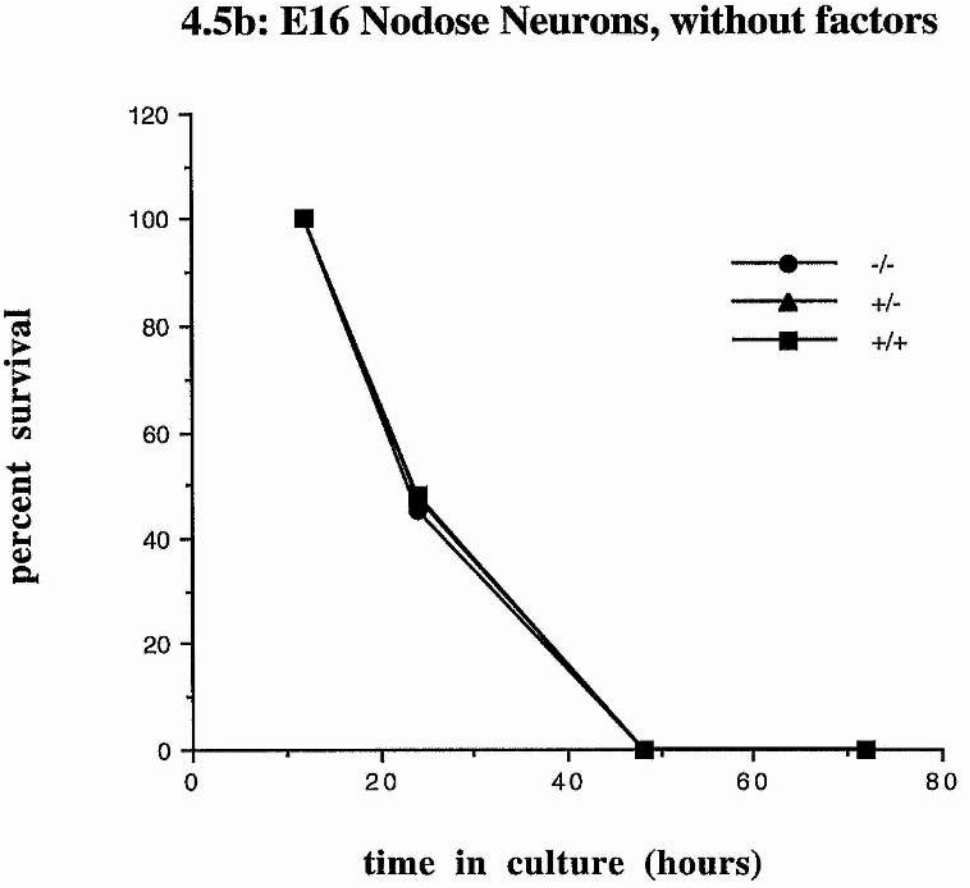


Figure 4.5b : Percent survival of E16 nodose neurons from *bcl-2-/-* (n= 6), *bcl-2+/-* (n= 13), and *bcl-2 +/+* (n= 7) embryos grown for 72 hours in culture without factors. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied

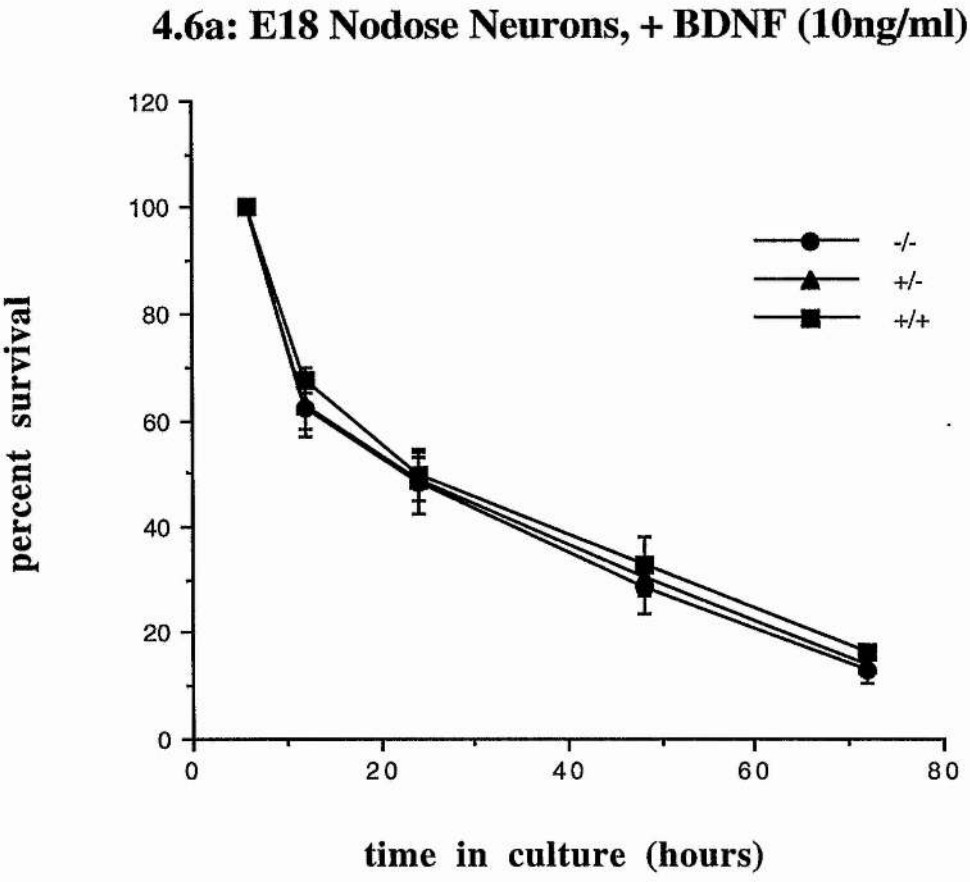


Figure 4.6a : Percent survival of E18 nodose neurons from *bcl-2-/-* (n= 5), *bcl-2+/-* (n= 14), and *bcl-2 +/+* (n= 8) embryos grown for 72 hours in culture with BDNF (10ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

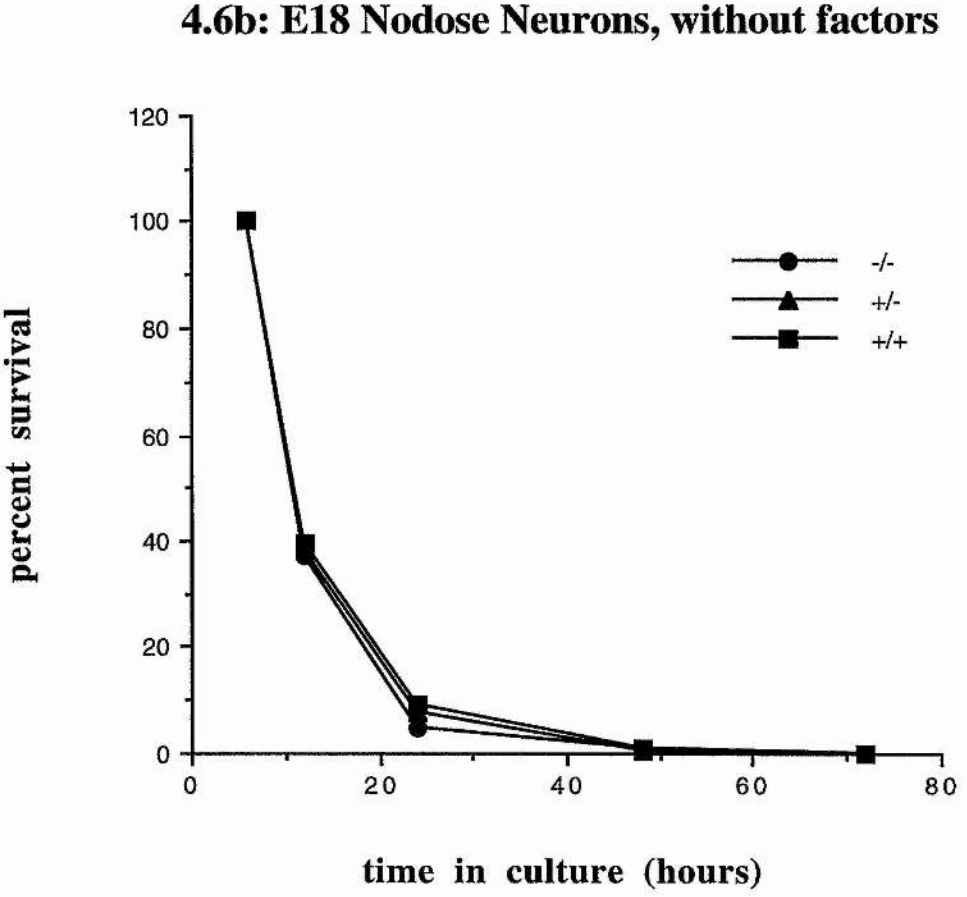


Figure 4.6b : Percent survival of E18 nodose neurons from *bcl-2-/-* (n= 5), *bcl-2+/-* (n= 14), and *bcl-2 +/+* (n= 8) embryos grown for 72 hours in culture without factors. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied

Bcl-2-deficient sensory neurons have normal neurotrophin dose response

Although Bcl-2 expression was required for the sustained survival response of early fetal trigeminal neurons to NGF it did not appear to affect the sensitivity to these neurons when this was counted at 12 hours after plating. The graph in Figure 4.7 show that there is no obvious difference in the dose responses of these neurons to NGF; the EC50 for all three genotypes was approximately 10pg/ml.

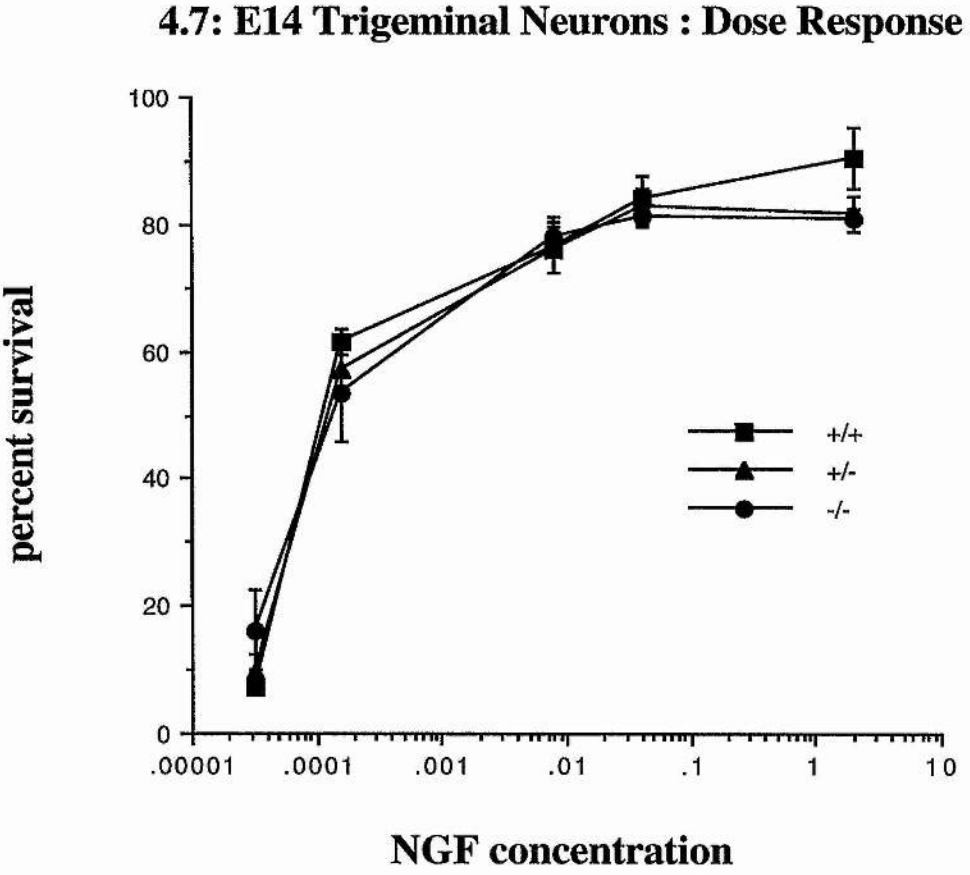


Figure 4.7: Dose responses of E14 trigeminal ganglion neurons from *bcl-2-/-* (n= 11), *bcl-2+/-* (n= 25), and *bcl-2+/+* (n= 12) embryos to a range of concentrations of NGF. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

Survival with cytokines

At E18, nodose and trigeminal neurons from wild type mice show a very limited response to cytokines such as CNTF, IL-6 and LIF. To ascertain whether cytokines had any survival effects in mice with a targeted null mutation in the Bcl-2 gene, triplicate control dishes and dishes supplemented with CNTF were set up for E18 trigeminal and nodose neurons. Under control conditions, there was no difference between the genotypes reflecting the results shown in figures 4.3 and 4.6. In the presence of CNTF, at 10ng/ml, there was no statistically significant difference between the survival of neurons from *bcl-2*$^{-/-}$, *bcl-2* $^{+/-}$ or *bcl-2* $^{+/+}$ embryos (Fig. 4.8). These results indicate that, at E18, there would appear to be no *in vitro* evidence for *bcl-2* having a role in the survival response of sensory neurons to cytokines.

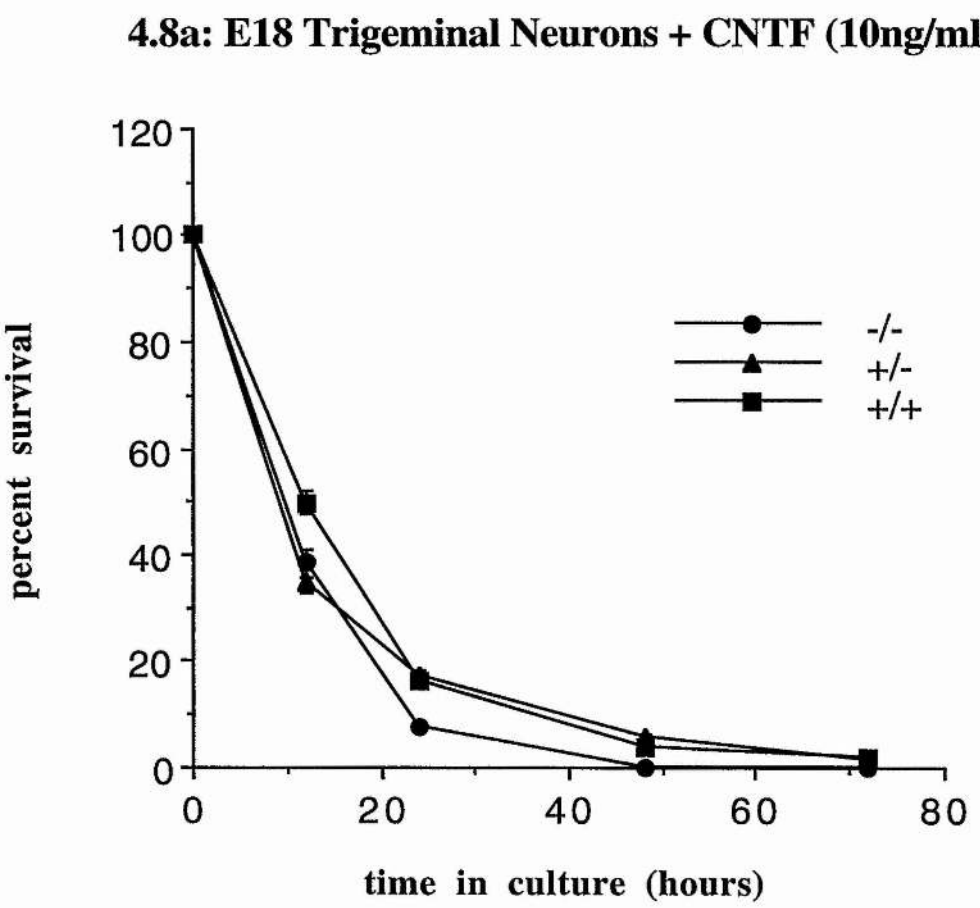


Figure 4.8a: Graph of the percent survival of E18 trigeminal neurons from *bcl-2-/-* (n= 8), *bcl-2+/-* (n= 14), and *bcl-2 +/+* (n= 5) embryos grown for 72 hours in culture with CNTF (10ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied

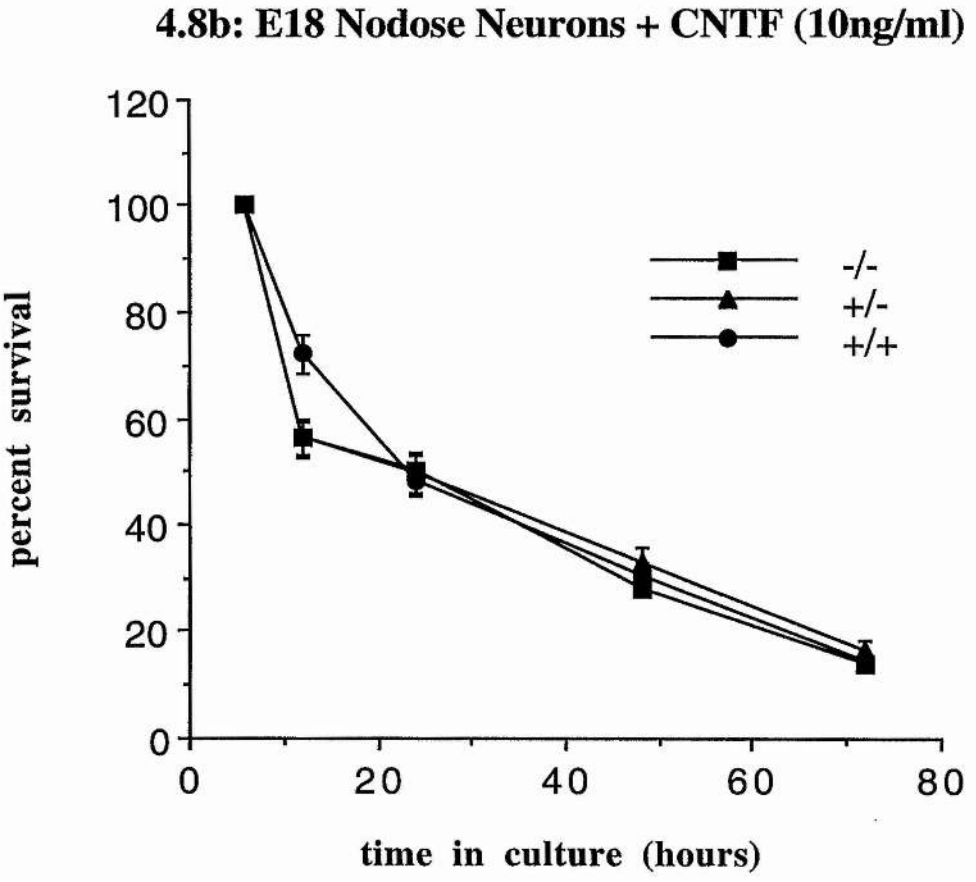


Figure 4.8b: Graph of the percent survival of E18 nodose neurons from *bcl-2-/-* (n= 8), *bcl-2+/-* (n= 14), and *bcl-2 +/+* (n= 5) embryos grown for 72 hours in culture with CNTF (10ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied

Sympathetic Neurons from bcl-2 null embryos show reduced survival in vitro

A very small study was carried out into the effects of a null mutation in the bcl-2 gene on the neurons derived from the sympathetic chain of embryos which were bcl-2 $^{-/-}$, bcl-2 $^{+/-}$ or bcl-2$^{+/+}$. At E14, there is decreased survival of neurons derived from bcl-2$^{-/-}$ embryos as compared neurons from wild type embryos, with neurons from bcl-2$^{+/-}$ embryos showing intermediary survival. This effect can be seen both in the presence of NGF at a concentration of 5ng/ml (Fig. 4.9a) and in the absence of growth factors (Fig. 4.9b). These results indicate a role for bcl-2 in the survival of sympathetic neurons at this time in development both in the presence and in the absence of NGF.

4.9a: E14 Symapthetic Chain Neurons, with NGF (5ng/ml)

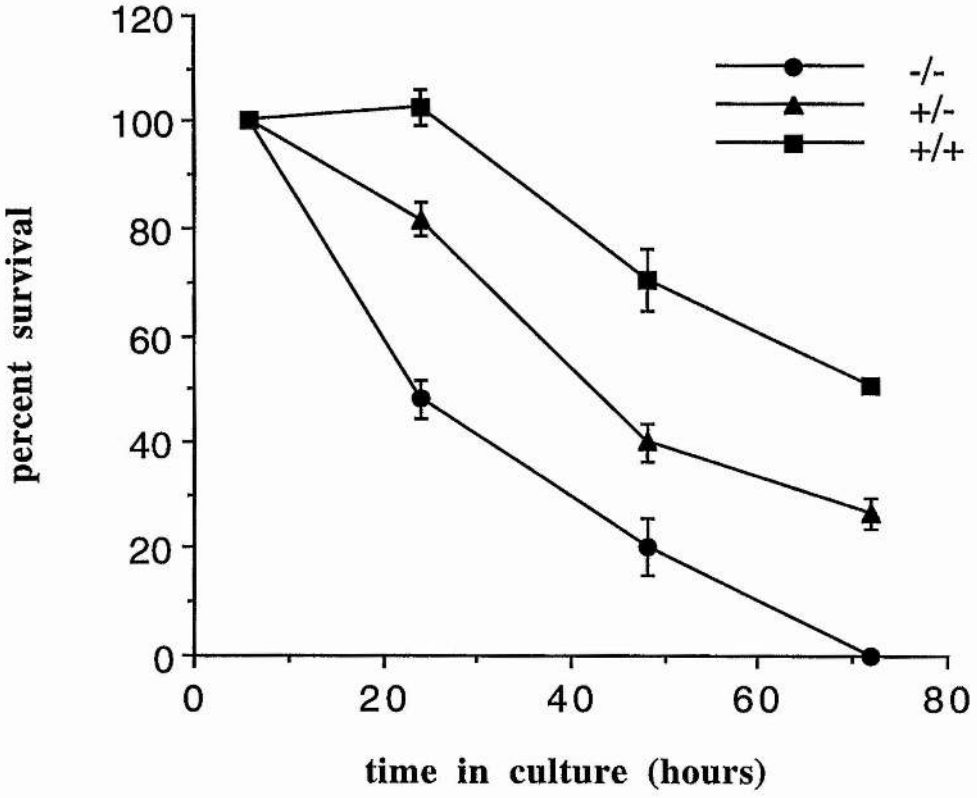


Figure 4.9a: Percent survival of E14 sympathetic chain neurons from *bcl-2-/-* (n= 3), *bcl-2+/-* (n= 8), and *bcl-2 +/+* (n= 4) embryos grown for 72 hours in culture with NGF (5ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

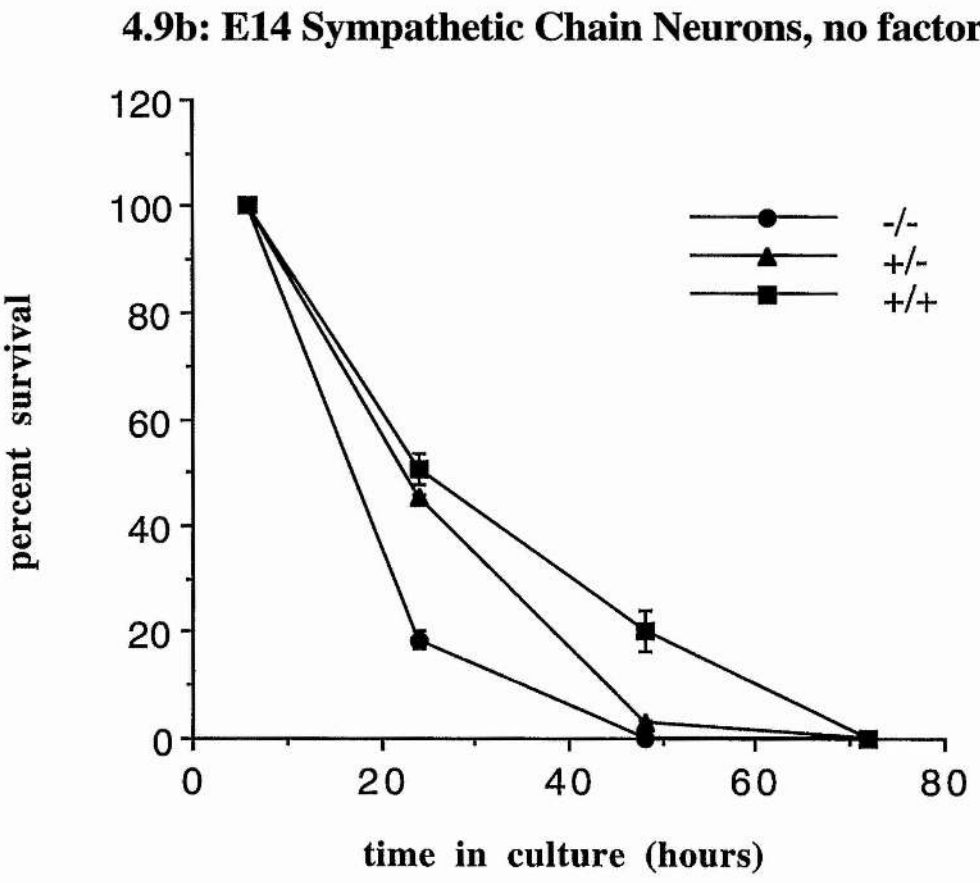


Figure 4.9b: Percent survival of E14 sympathetic chain neurons from *bcl-2-/-* (n= 3), *bcl-2+/-* (n= 8), and *bcl-2 +/+* (n= 4) embryos grown for 72 hours in culture without factors. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

Bcl-2 null embryos have reduced numbers of sensory neurons

To determine if the short-lived survival responses of Bcl-2-deficient neurons to neurotrophins *in vitro* are physiologically relevant, a comparative study was made both of the number of neurons undergoing apoptosis and the total number of neurons in the trigeminal ganglia of wild type and Bcl-2 mutant embryos at E14 and at later stages during the period of naturally occurring neuronal death (Davies & Lumsden, 1984; Pinon et al., 1996). Embryos were prepared for routine histology and the number of neurons with pyknotic nuclei and the total number of neurons counted in serial sections of the trigeminal ganglia. All histology slides were coded so that these estimates were made without prior knowledge of the genotype.

The estimates of total numbers of neurons in the trigeminal ganglia of wild type embryos over the age range were very similar to previously reported estimates (Davies & Lumsden, 1984; Pinon et al., 1996), showing an approximate 50% decrease between E14 and E18 (Fig. 4.10). Although there were fewer neurons in the trigeminal ganglia of *bcl-2*$^{-/-}$ embryos compared with wild type embryos at E14, this difference was not statistically significant ($p<0.1$, t-test $n=22$). There were, however, statistically significant reductions of approximately 25% in the number of neurons in the trigeminal ganglia of *bcl-2*$^{-/-}$ embryos compared with wild type embryos at E16 ($p<0.05$, t-test, $n=28$) and E18 ($p<0.001$, t-test, $n=18$). There was also a significant increase in the number of neurons with pyknotic nuclei in the trigeminal ganglia of *bcl-2*$^{-/-}$ embryos compared with wild type embryos at E14 (28% increase, $p<0.01$, t-test, $n=18$). Although there were slightly more pyknotic neurons in the trigeminal ganglia of *bcl-2*$^{-/-}$ embryos as compared to wild type at E16 and E18, these differences were not statistically significant. These results suggest that in the absence of Bcl-2, a proportion of trigeminal neurons undergo apoptosis at E14 and this is reflected in a reduction in the total number of neurons in the ganglia at later ages.

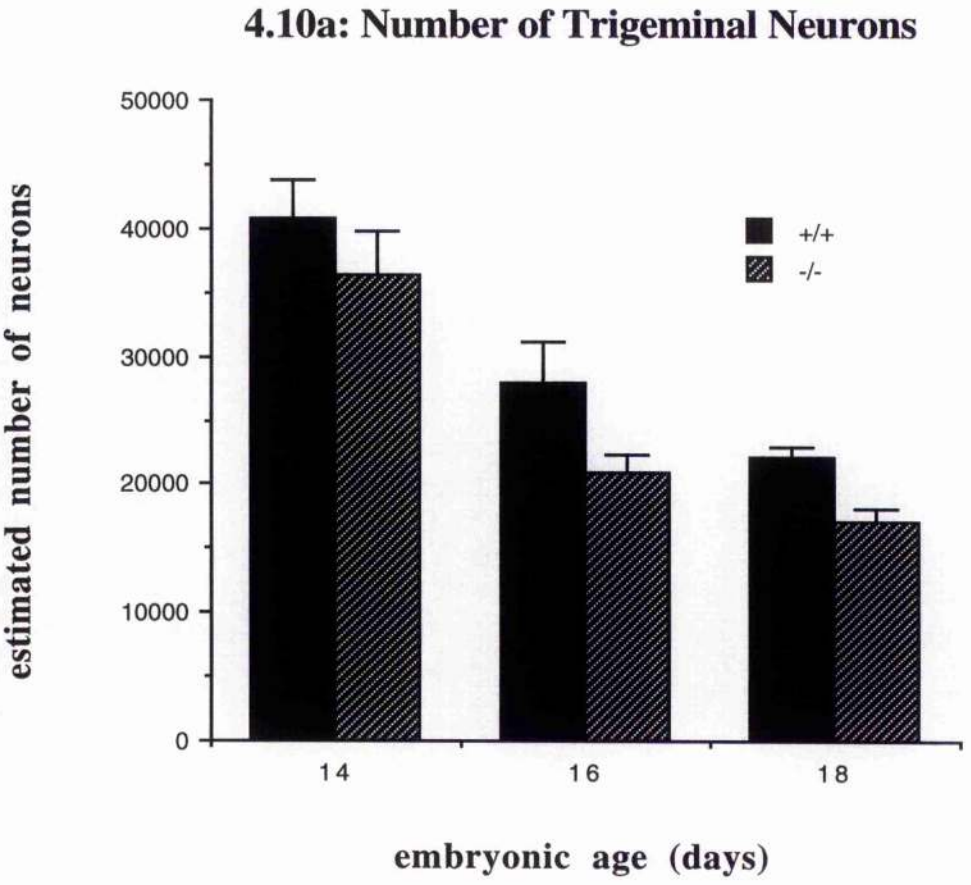


Figure 4.10a: Estimated number of neurons in the trigeminal ganglia of *bcl-2 -/-* and *bcl-2+/+* embryos at E14, E16 and E18. The means and SEMs of counts are shown. At E14, 16 wild type and 14 Bcl-2-deficient ganglia were studied; at E16, 10 wild type and 13 knock out ganglia were studied and at e18, 13 wild type and 16 knock out ganglia were studied.

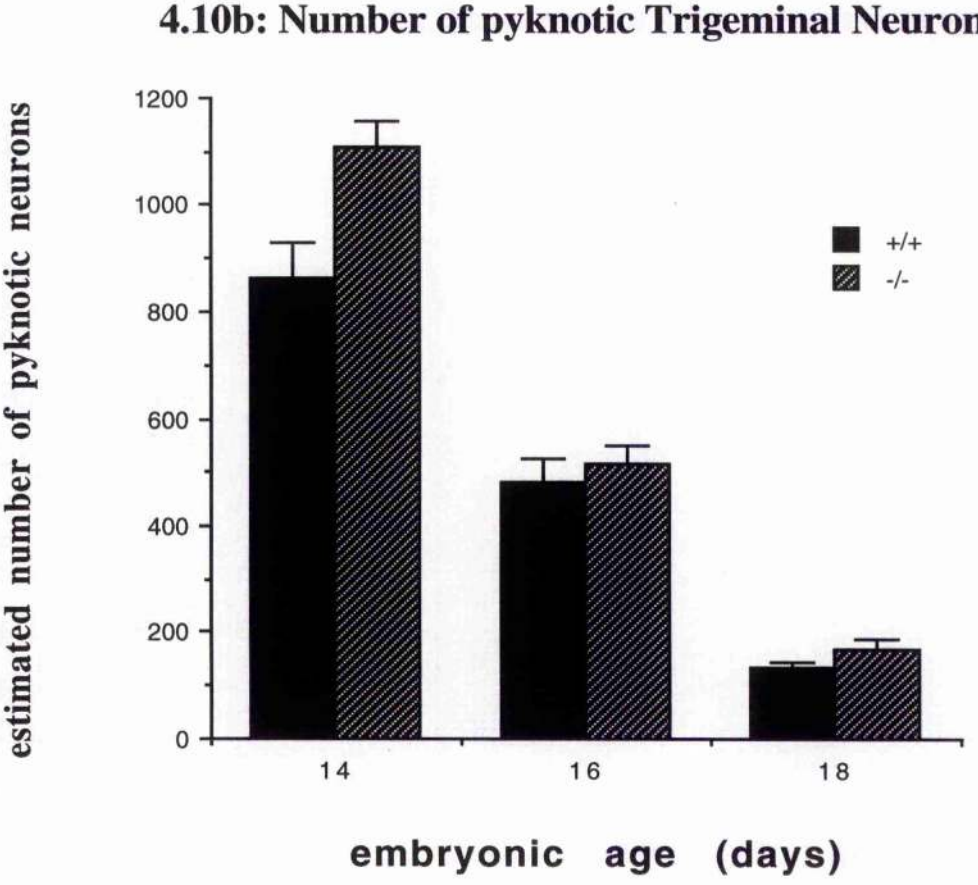


Figure 4.10b: Estimated number of pyknotic neurons in the trigeminal ganglia of *bcl-2 -/-* and *bcl-2+/+* embryos at E14, E16 and E18. The means and SEMs of the counts are shown. At E14, 16 wild type and 14 Bcl-2-deficient ganglia were studied; at E16, 10 wild type and 13 knock out ganglia were studied and at e18, 13 wild type and 16 knock out ganglia were studied.

DISCUSSION

The data presented in this chapter reveals that bcl-2 is required for the sustained survival of E14 trigeminal, nodose and sympathetic neurons in the presence of neurotrophins. bcl-2 deficient trigeminal neurons initially display a survival response to neurotrophins that has similar dose response characteristics to that of wild type neurons, but these neurons die more rapidly in the presence of neurotrophins compared to wild type neurons. Likewise, the neurons derived from the lumbar sympathetic chain and the nodose ganglion which have a null mutation in their bcl-2 gene do not show sustained survival in vitro in the presence of NGF whereas those neurons derived from wild type embryos do. However, at later developmental stages, the difference in the survival response between wild type and bcl-2 deficient neurons is less pronounced, and by E18, bcl-2 does not appear to be required for neurotrophin promoted survival since wild type and bcl-2-deficient trigeminal and nodose neurons survive equally well with NGF and BDNF, respectively. It has previously been shown that the number of neurons in the trigeminal ganglion peaks between E13 and E14 and falls by half to reach a stable number by birth as a result of naturally occurring neuronal death (Davies & Lumsden, 1984). Accordingly, the number of apoptotic neurons in the trigeminal ganglion peaks at E14 and declines to negligible levels by birth (Pinon et al., 1996). The in vitro findings therefore suggest that bcl-2 expression is required for the sustained survival response of a subset of trigeminal, nodose and sympathetic neurons to neurotrophins during the early phase of naturally occurring neuronal death.

The finding that the survival of bcl-2-deficient cranial sensory neurons to neurotrophins is short lived during the early stages of naturally occurring neuronal death accords, with the previous demonstration that microinjection of antisense bcl 2 expression constructs into E10 chicken trigeminal mesencephalic nucleus (TMN) neurons cause a substantial and specific reduction in the number of these neurons surviving with BDNF (Allsopp et al., 1995). Naturally occurring neuronal death takes

place in the chicken TMN between E9 and E14 (Rogers & Cowen, 1974), thus reduced Bcl-2 expression appears to compromise the neurotrophin survival response during the early stages of naturally occurring neuronal death. Interestingly, earlier in development, shortly after differentiating from progenitor cells, sensory neurons from *bcl-2*$^{-/-}$ embryos and wild type embryos survive equally well in culture.

Although not required for the sustained neurotrophin survival response of mouse cranial sensory neurons at the end of the period of naturally occurring neuronal death, injection of a Bcl-2 expression vector into the trigeminal neurons of E18 and newborn wild type mice is still able to prevent their death following NGF deprivation (J. Adu and A. M. Davies, unpublished observations). Likewise, DRG neurons isolated from transgenic mice expressing Bcl-2 under the control of the neuron-specific enolase promoter showed enhanced survival in the absence of NGF (Farlie et al., 1995).

To ascertain the physiological significance of the *in vitro* observations, a comparison was made of the numbers of surviving and dying neurons in the trigeminal ganglia of wild type and *bcl-2* mutant embryos during the period of naturally occurring neuronal death. In contrast to initial reports that the nervous system of *bcl-2*$^{-/-}$ animals is grossly normal (Nakayama et al., 1994,1994; Veis et al., 1993), there is a significant increase in the number of dying neurons in the trigeminal ganglia of *bcl-2*$^{-/-}$ embryos at E14 and this is reflected in a 25% reduction in the total number of neurons in the trigeminal ganglia of Bcl-2-deficient embryos at E16 and E18. These findings suggest that Bcl-2 expression is necessary for sustaining the survival of a proportion of trigeminal ganglion neurons during the early stages of naturally occurring neuronal death in the trigeminal ganglion. Thus, the existence of specific neuronal deficiencies in *bcl-2*$^{-/-}$ mice are consistent with the impaired survival response of Bcl-2-deficient neurons to neurotrophins *in vitro* providing evidence for a physiological role for Bcl-2 in sustaining the survival of neurons during embryonic development.

Although bcl-2 expression is only required for the survival of a subset of trigeminal neurons during development, the related protein bcl-x (Boise et al., 1993), has been shown to play a prominent role in regulating the survival of embryonic sensory neurons. bcl-x$^{-/-}$ mice die in utero at E13 with extensive neuronal apoptosis in the central nervous system and dorsal root ganglia (Motoyama et al., 1995), suggesting that the survival of many neurons is dependent on bcl-x expression during the earliest stages of their development. Because bcl-x expression is retained in dorsal root and cranial sensory ganglia in postnatal and adult mice (Krajewski et al., 1994; Gillardon et al., 1996), it is possible that bcl-x is also required for the survival of many sensory neurons at later stages of embryonic development and in the adult.

bcl-2 expression is also retained in sensory, sympathetic and motor neurons throughout life (Merry et al., 1994), and a recent study of bcl-2 null mice has revealed the appearance of statistically significant deficiencies in these populations of neurons during the postnatal period (Michaelidis et al., 1996). Between P3 and P9 the number of motorneurons in the facial nucleus of bcl-2$^{-/-}$ falls to 71% of that in wild type mice and between P3 and P10 the number of sympathetic neurons in the superior cervical sympathetic ganglion falls to 60% of normal. There is also a significant reduction in the number of DRG neurons, but this does not become apparent until P44. Although further decreases in the neuronal complement of cranial sensory ganglia that may take place postnatally in bcl-2$^{-/-}$ mice have not been investigated here, it has been shown that bcl-2 expression is required for maintaining the correct number of neurons in cranial sensory ganglia during the period of naturally occurring neuronal death.

V. BCL-2 TRANSGENIC MICE:

NEURONAL MATURATION AND NEURITE OUTGROWTH

CHAPTER FIVE

BCL-2 TRANSGENIC MICE:

NEURONAL MATURATION AND NEURITE OUTGROWTH

INTRODUCTION

Extensive evidence has established that bcl-2 plays a key role in the prevention of apoptosis, both during development and normal tissue homeostasis, as well as in certain diseases (Thompson, 1996). However, evidence for bcl-2 having further functions has recently been reported.

For example, bcl-2 overexpression in a neural crest-derived cell line enhances the outgrowth of neurite-like processes and increases the expression of neuron-specific enolase (Zhang et al., 1996). bcl-2 also enhances the differentiation of the PC12 neural cell line into sympathetic-like neurons in serum-free, but not serum-containing, media (Batistatou et al., 1993). In addition, midbrain-derived dopaminergic cell lines stably expressing bcl-2 extend longer neurites than control transfected cells but do not show increased expression of many neuron-specific proteins (Oh et al., 1996). The above data raises the possibility that bcl-2 may play a role in regulating differentiation and/or axonal growth in the developing nervous system. However, the physiological and developmental significance of overexpressing bcl-2 in tumour cell lines is difficult to interpret. For these reasons, a comparative in vitro study of newly differentiated sensory neurons of bcl-$2^{-/-}$ and wild type mouse embryos was undertaken to determine if the absence of bcl-2 affects the early development of these neurons. This study focused on the morphological changes that occur in the mouse trigeminal ganglion, as this population has been extensively studied and is well understood (Davies & Lumsden, 1984, 1986; Davies, 1987b; Pinon et al., 1996; Wilkinson et al., 1996). The majority of neurons in the trigeminal ganglion differentiate from progenitor cells

between E9.5 and E13.5 with the first axons emerging from the trigeminal ganglion at E9.5. The total number of neurons in the ganglion peaks between E12 and E14 and decreases by 50% to reach a stable number by birth as a result of naturally occurring cell death which peaks between E12 and E14. These studies focus on neurons derived from embryos aged between E11 and E14, a period when most neurons are differentiating from their progenitor cells and extending axons to their target fields.

Previous studies have established that shortly after differentiating from progenitor cells, sensory neurons undergo a distinct morphological change. Initially, they have small, spindle-shaped, phase-dark cell bodies which become large spherical and phase-bright as they mature (Wright et al., 1992). It is shown here that early sensory neurons cultured from the trigeminal ganglia of *bcl-2*$^{-/-}$ embryos at E11 and E12 undergo this change more slowly than trigeminal neurons from wild type embryos of the same ages. The delay is not due to any survival effects of Bcl-2. Accordingly, there was a significantly smaller number of mature-type neurons in the trigeminal ganglia of E12 and E13 *bcl-2*$^{-/-}$ embryos, yet the total cellular content of these ganglia was similar to that of ganglia derived from wild type embryos. Furthermore, the trigeminal ganglia of both *bcl-2*$^{-/-}$ and wild type embryos contain similar numbers of neurofilament positive cells. The absence of Bcl-2 did not cause a uniform delay in the developmental program of sensory neurons because the time course of NGF receptor expression (both trkA and p75) was unaffected in the trigeminal neurons of *bcl-2* null embryos. These findings indicate that Bcl-2 expression is required for the normal progression of a particular early maturational change in embryonic sensory neurons. Furthermore, Bcl-2 is shown to have a role in regulating axonal growth rate in embryonic neurons. Using neurons derived from the trigeminal ganglia of E11 and E12 mice, it is clear that in the absence of Bcl-2 there is a marked decrease in neurite growth rate, irrespective of whether the neurons are grown with NGF, BDNF, or NT-3. These results, taken in conjunction with the

maturation studies indicate a role, or several roles, for bcl-2 apart from its well documented role in preventing apoptosis.

RESULTS

Neuronal Maturation in vitro

Newly-differentiated sensory neurons undergo a distinct maturational change in vitro . Those which are defined here as immature have small, spindle-shaped phase dark cell bodies whereas those which are defined as mature have large spherical phase-bright cell bodies (Fig. 5.1). The proportion of the morphologically distinct immature neurons in dissociated cultures from bcl-2-deficient embryos was consistently higher than that observed in cultures derived from wild type embryos of the same age (Fig. 5.2). Furthermore, the proportion of immature neurons in cultures derived from embryos heterozygous for a null mutation in the bcl-2 gene was intermediate between that observed for wild type embryos and that observed for bcl-2-deficient embryos, suggesting a gene dosage effect.

An elevated number of immature neurons in dissociated cultures of early sensory ganglia from bcl-$2^{-/-}$ embryos could be due to differences in progenitor cell differentiation or neuronal survival in these cultures. To address directly the question of whether bcl-2-deficient sensory neurons mature more slowly than wild type neurons, defined cohorts of immature neurons in cultures of E11 and E12 trigeminal neurons were identified 18 hours after plating and the fate of each individual cell followed for up to 3 days (Fig. 5.3 and 5.4). These data show that bcl-2-deficient neurons retain an immature morphology much longer than wild type neurons. The marked difference in the rate of neuronal maturation cannot be attributed to differences in the survival of bcl-2-deficient and wild type neurons as very few neurons died in the cohorts over the period of the study (Fig. 5.3 and 5.4). Because all data were obtained prior to genotyping, these observations were not influenced by observer bias.

A B C

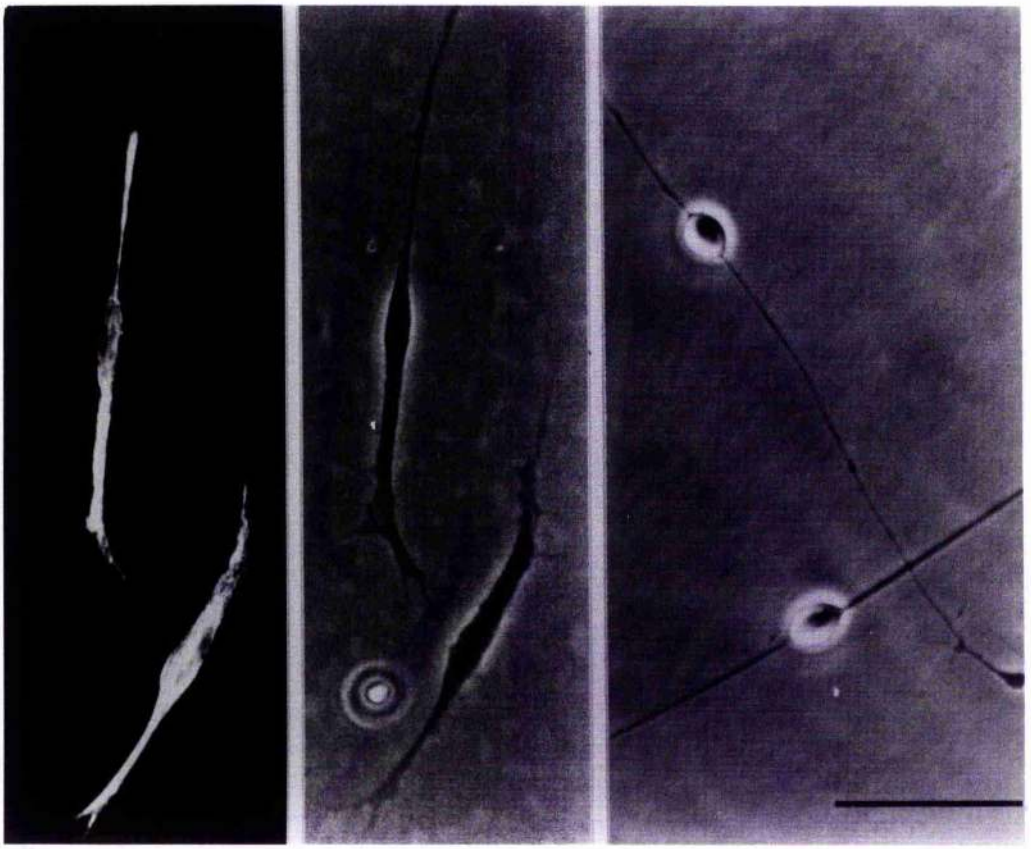


Figure 5.1: Photomicrographs of E11 trigeminal ganglion cultures showing the typical morphology of immature (A,B) and mature (C) early sensory neurons. The paired immunofluorescence (A) and phase contrast (B) photomicrographs demonstrate that cells identified as immature neurons are stained with anti-neurofilament antiserum (Wright et al., 1992). Scale bar represents 50μm.

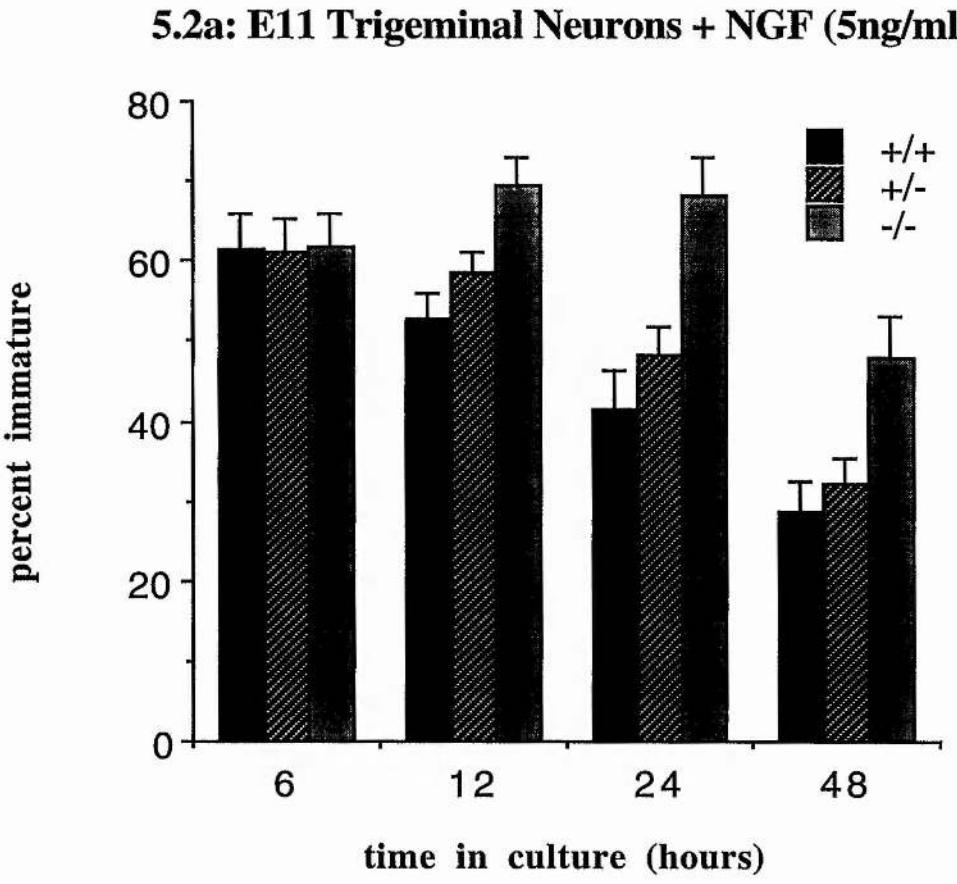


Figure 5.2a: Percentage of neurons that are immature in dissociated cultures of E11 trigeminal ganglia derived from embryos of each of the three genotypes. Neurons were cultured in the presence of NGF (5ng/ml). The bar charts represent the results of cultures established from 13 bcl-$2^{-/-}$, 26 bcl-$2^{+/-}$, and 14 wild type embryos. The means and standard errors of the means are shown. Each point on the graph represents the mean of a number of embryos. For each embryo, triplicate cultures were established for all conditions studied.

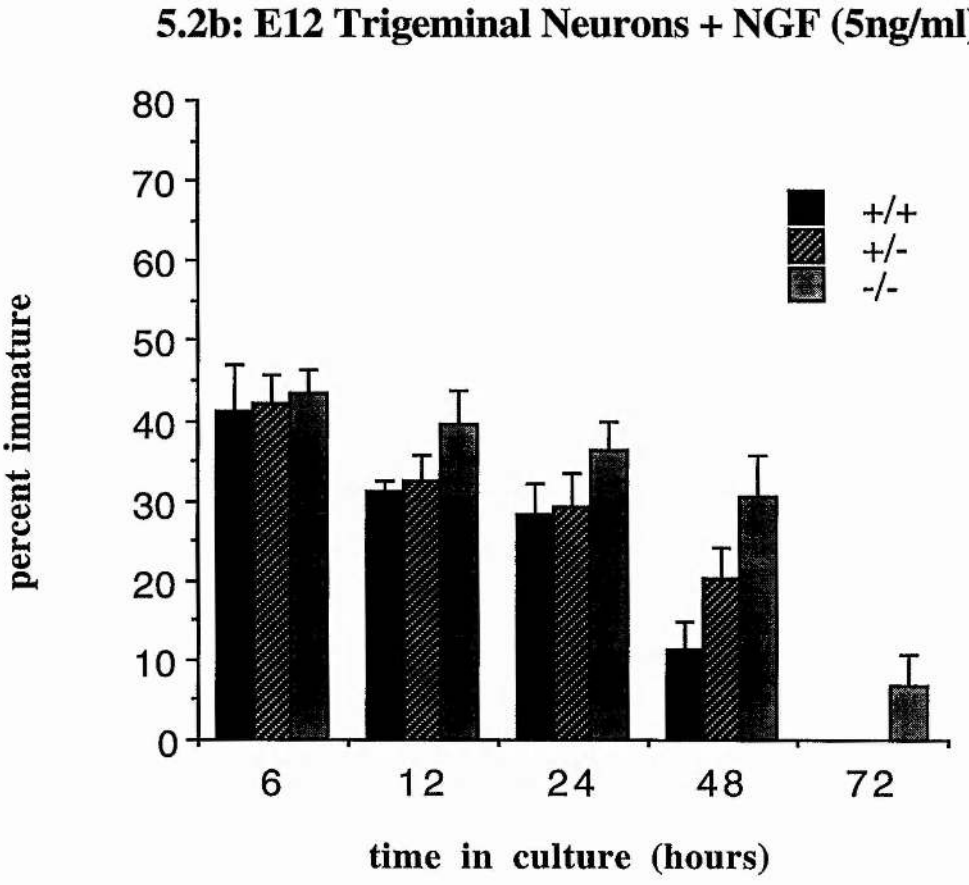


Figure 5.2b: Percentage of neurons that are immature in dissociated cultures of E12 trigeminal ganglia derived from embryos of each of the three genotypes. Neurons were cultured in the presence of NGF (5ng/ml). The bar charts represent the results of cultures established from 17 bcl-$2^{-/-}$, 29 bcl-$2^{+/-}$, and 18 wild type embryos. The means and standard errors of the means are shown. Each point on the graph represents the mean of a number of embryos. For each embryo, triplicate cultures were established for all conditions studied.

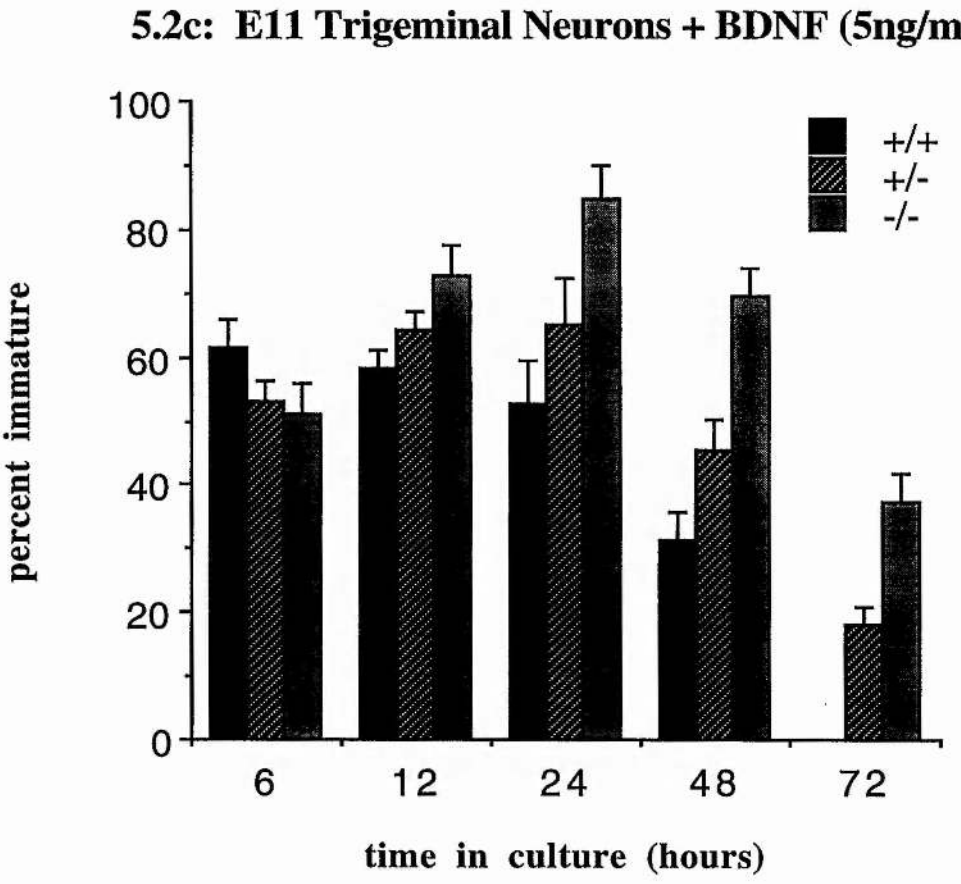


Figure 5.2c: Percentage of neurons that are immature in dissociated cultures of E11 trigeminal ganglia derived from embryos of each of the three genotypes. Neurons were cultured in the presence of BDNF (5ng/ml). The bar charts represent the results of cultures established from 13 bcl-$2^{-/-}$, 26 bcl-$2^{+/-}$, and 14 wild type embryos. The means and standard errors of the means are shown. Each point on the graph represents the mean of a number of embryos. For each embryo, triplicate cultures were established for all conditions studied.

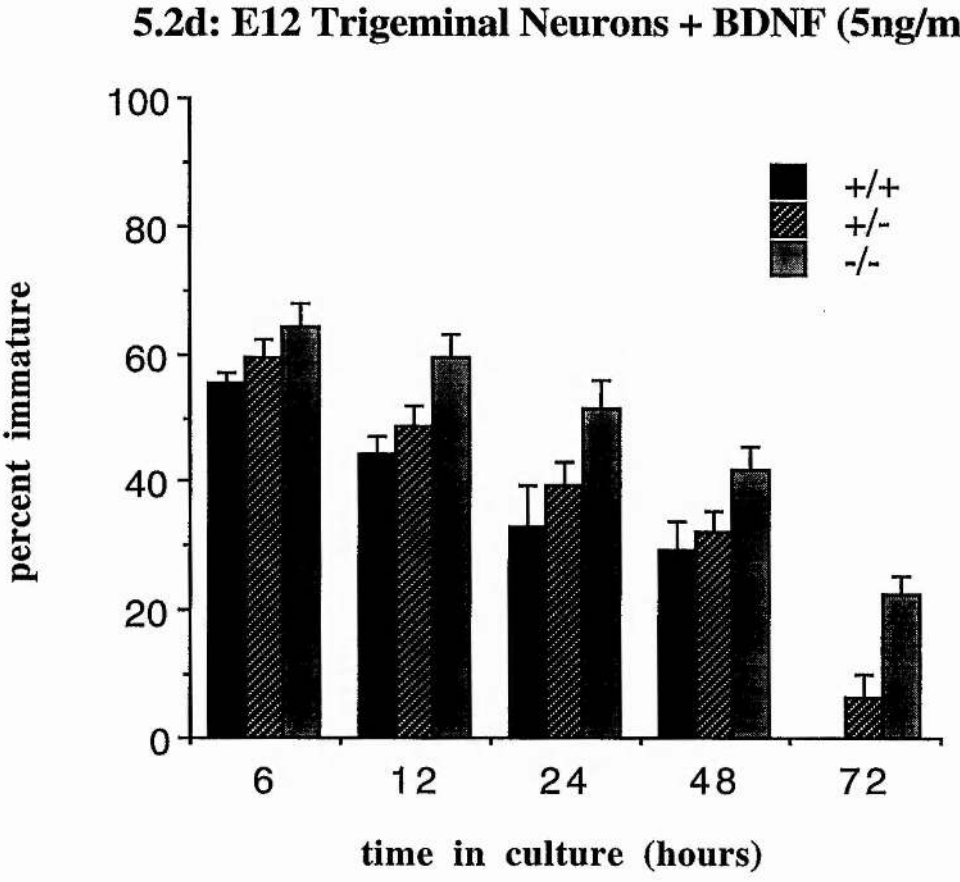


Figure 5.2d: Percentage of neurons that are immature in dissociated cultures of E11 trigeminal ganglia derived from embryos of each of the three genotypes. Neurons were cultured in the presence of BDNF (5ng/ml). The bar charts represent the results of cultures established from 17 bcl-$2^{-/-}$, 29 bcl-$2^{+/-}$, and 18 wild type embryos. The means and standard errors of the means are shown. Each point on the graph represents the mean of a number of embryos. For each embryo, triplicate cultures were established for all conditions studied.

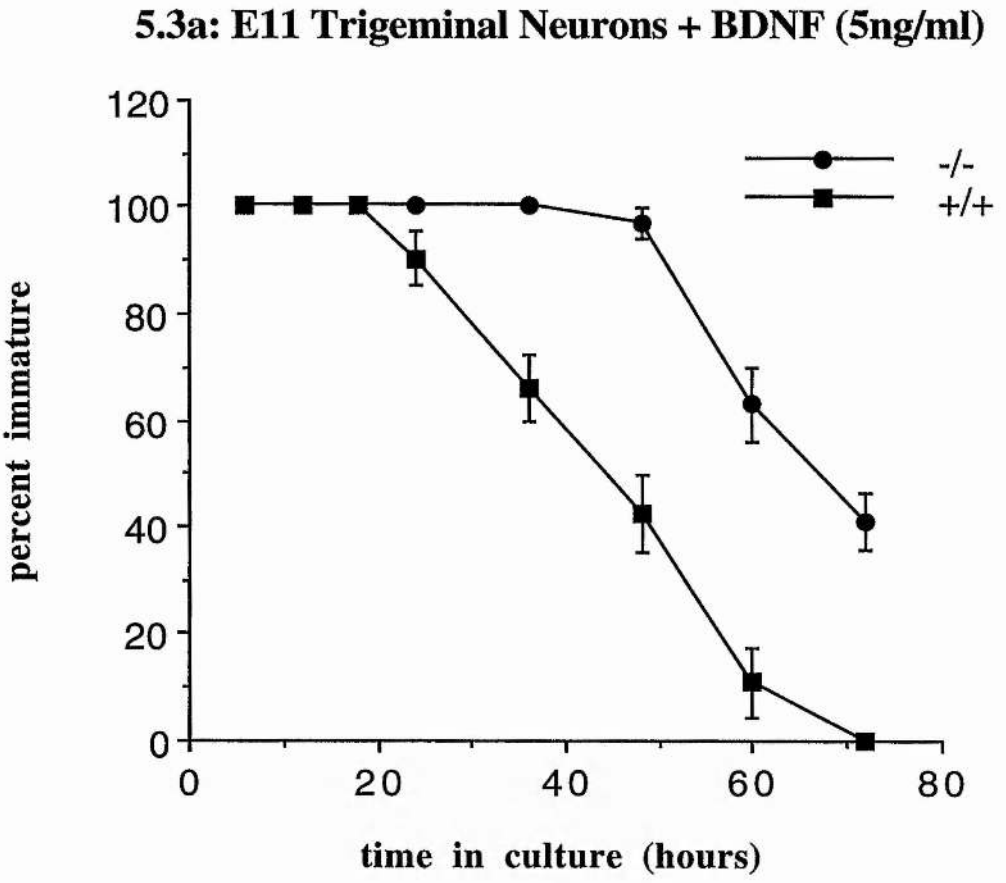
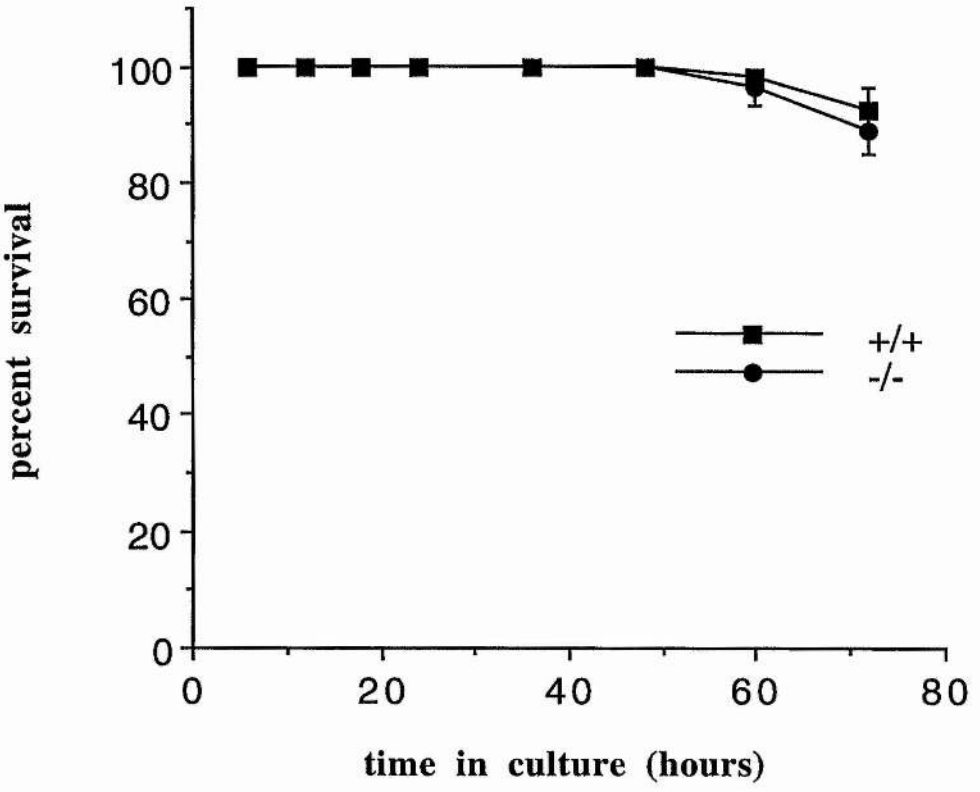


Figure 5.3: Results of neuron cohort experiments to compare the maturation and survival of trigeminal neurons from wild type and *bcl-2*$^{-/-}$ embryos at E11. The number of immature neurons remaining in the cohorts (a) is expressed as a percentage of the initial cohort size up to 72 hours after plating. Likewise, the total number of neurons in the cohort (b) is again expressed as a percentage. The means and standard errors of the means are shown. Each point on the graph represents the mean of a number of embryos. For each embryo, triplicate cultures were established for all conditions studied. In total, at E11, 24 Bcl-2-deficient embryos and 23 wild type embryos were studied. The initial cohort size in each culture ranged from 20 to 60 neurons.

5.3b: E11 Trigeminal Neurons + BDNF (5ng/ml)



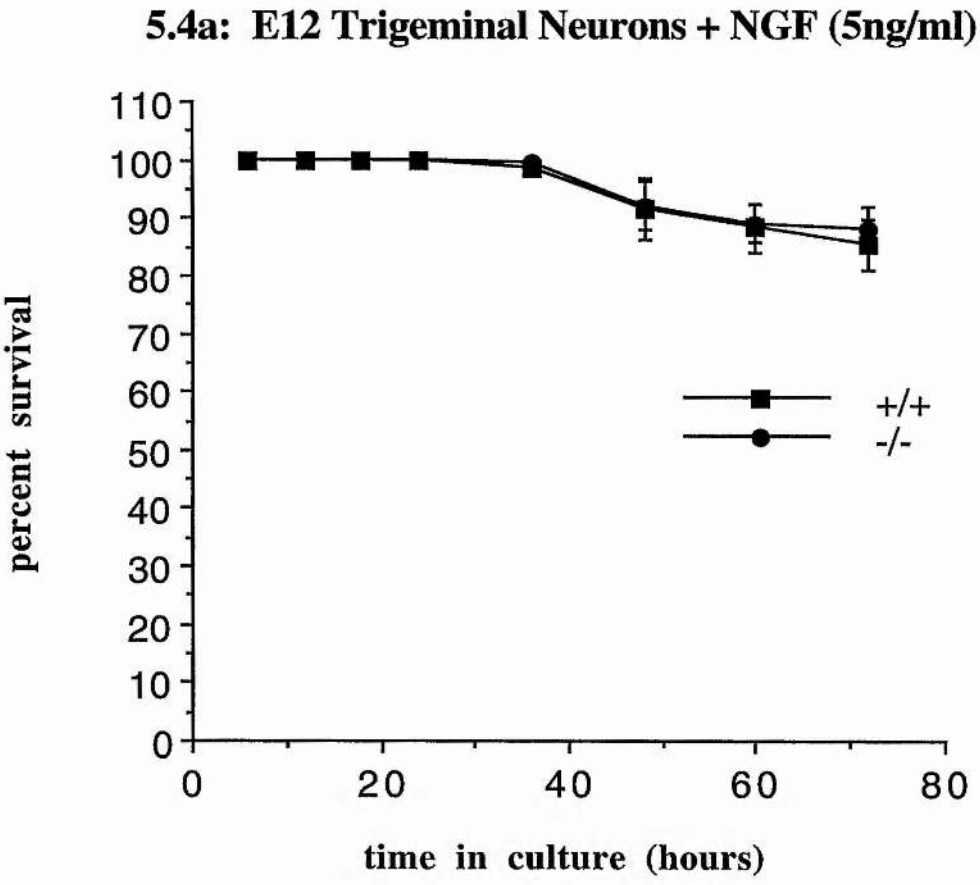
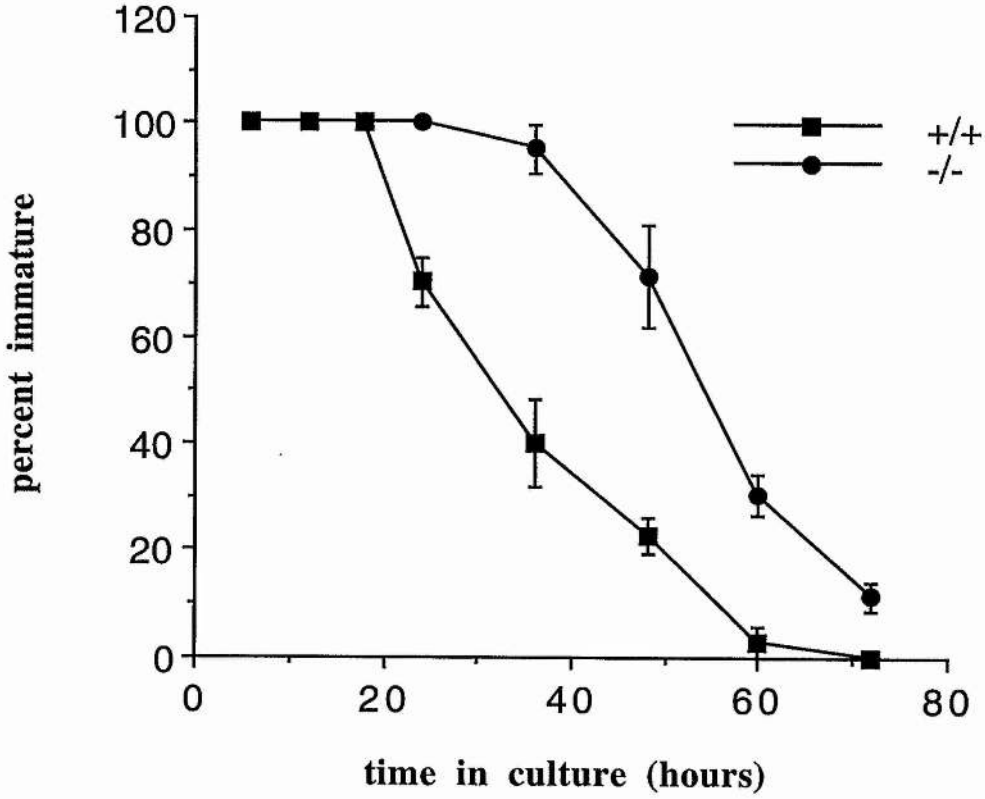


Figure 5.4: Results of neuron cohort experiments to compare the maturation and survival of trigeminal neurons from wild type and *bcl-2*$^{-/-}$ embryos at E12. The number of immature neurons remaining in the cohorts (a) is expressed as a percentage of the initial cohort size up to 72 hours after plating. Likewise, the total number of neurons in the cohort (b) is again expressed as a percentage. The means and standard errors of the means are shown. Each point on the graph represents the mean of a number of embryos. For each embryo, triplicate cultures were established for all conditions studied. A total of 22 Bcl-2-deficient embryos and 17 wild type embryos were studied. The initial cohort size in each culture ranged from 20 to 60 neurons.

5.4b: E12 Trigeminal Neurons + NGF (5ng/ml)



Development of Early Trigeminal neurons *in vivo*

To determine if neuronal maturation is delayed in the trigeminal ganglia of Bcl-2-deficient embryos *in vivo*, the number of morphologically recognisable cresyl fast violet stained mature neurons were counted in the trigeminal ganglia of Bcl-2-deficient and wild type embryos at several embryonic ages. The criteria used for determining what was a neuron were the presence of cresyl-stained Nissl substance in the cytoplasm and the presence of a densely-stained nucleolus in the nucleus whilst the nucleoplasm remains unstained. Neurogenesis in the mouse trigeminal ganglion and the recruitment of axons to the trigeminal nerve occurs between E9.5 and E13.5 (Davies & Lumsden, 1984; Wilkinson et al., 1996). The earliest age at which large numbers of neurons become distinguishable by morphological criteria from other cell types is E12 (Davies & Lumsden, 1984) and for this reason this is the youngest age studied here. Because of their small size, immature neurons cannot be distinguished from their progenitor cells and glial cells and are therefore not included in these counts. There are significantly greater numbers of morphologically distinct neurons in the trigeminal ganglia of wild type embryos at both E12 ($p=0.0008$, $n=17$, t-test) and E13 ($p,0.006$, $n=18$, t-test)(Fig. 5.5). By E14, however, the number of neurons of the trigeminal ganglia of Bcl-2-deficient embryos and wild type embryos was not significantly different ($p>0.1$, $n=22$, t-test). Because all data was obtained before the genotype were determined, the data is not influenced by observer bias.

To ascertain if the reduced number of morphologically identifiable neurons in the early trigeminal ganglia of Bcl-2-deficient embryos was due to a reduction in the total number of neurons in the ganglion, all neurons (both immature and mature) were positively identified using neurofilament staining at E12 (Fig. 5.6). Estimates of the total number of neurofilament-positive cells in the ganglia of wild type and Bcl-2-deficient embryos showed no significant difference at this stage (Fig. 5.5), these observations suggest that the reduction in the numbers of morphologically

recognisable neurons in the trigeminal ganglia of Bcl-2-deficient embryos during the early stages of ganglion formation is not due to a reduction in the total number of neurons in the ganglion. These *in vivo* observations are consistent with the results of the *in vitro* studies which suggest that Bcl-2-deficient neurons retain an immature morphology longer *in vivo* than wild type neurons.

5.5a: Number of Morphologically Mature Trigeminal Neurons

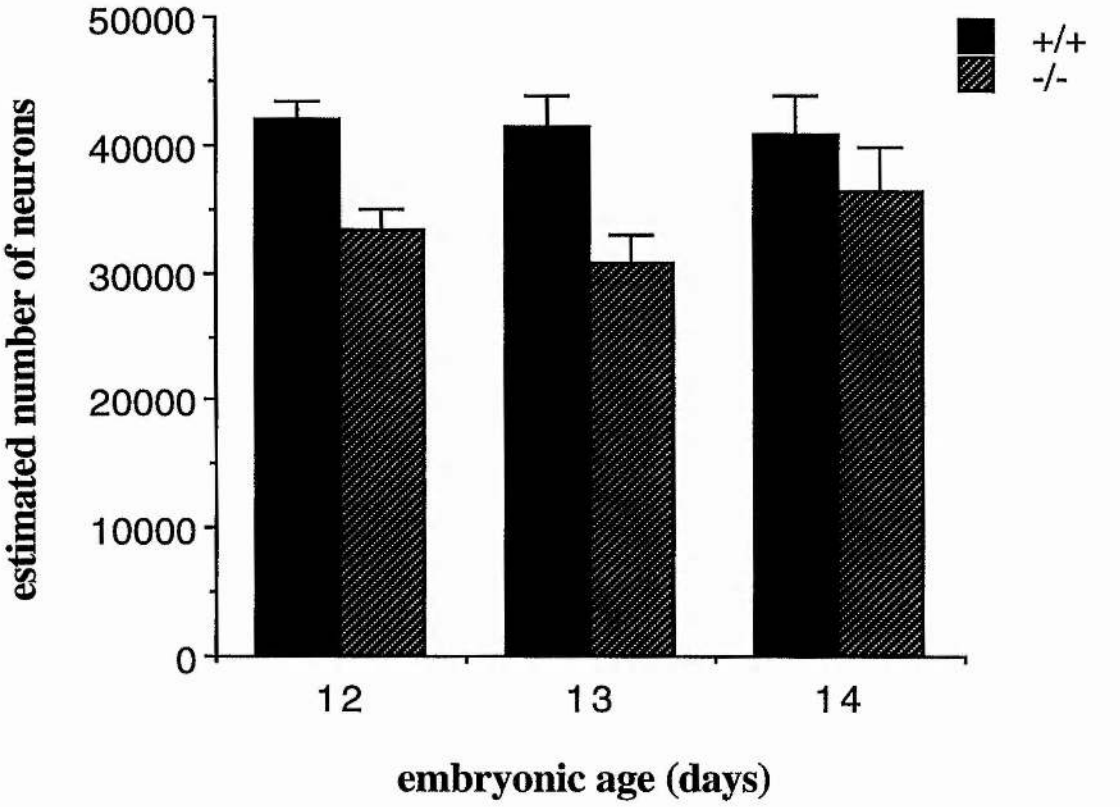


Figure 5.5a: Number of morphologically recognisable neurons in cresyl fast violet stained sections of the trigeminal ganglia of wild type and Bcl-2-deficient embryos at E12, E13 and E14. The number of Bcl-2-deficient embryos studied at each age were as follows: E12 n=4, at E13 n=6, at E14 n=5. For the wild type embryos, at E12 n=6, at E13 n=6, and at E14 n=4. The means and standard errors of the means are shown.

5.5b: Number of Neurofilament Positive Cells

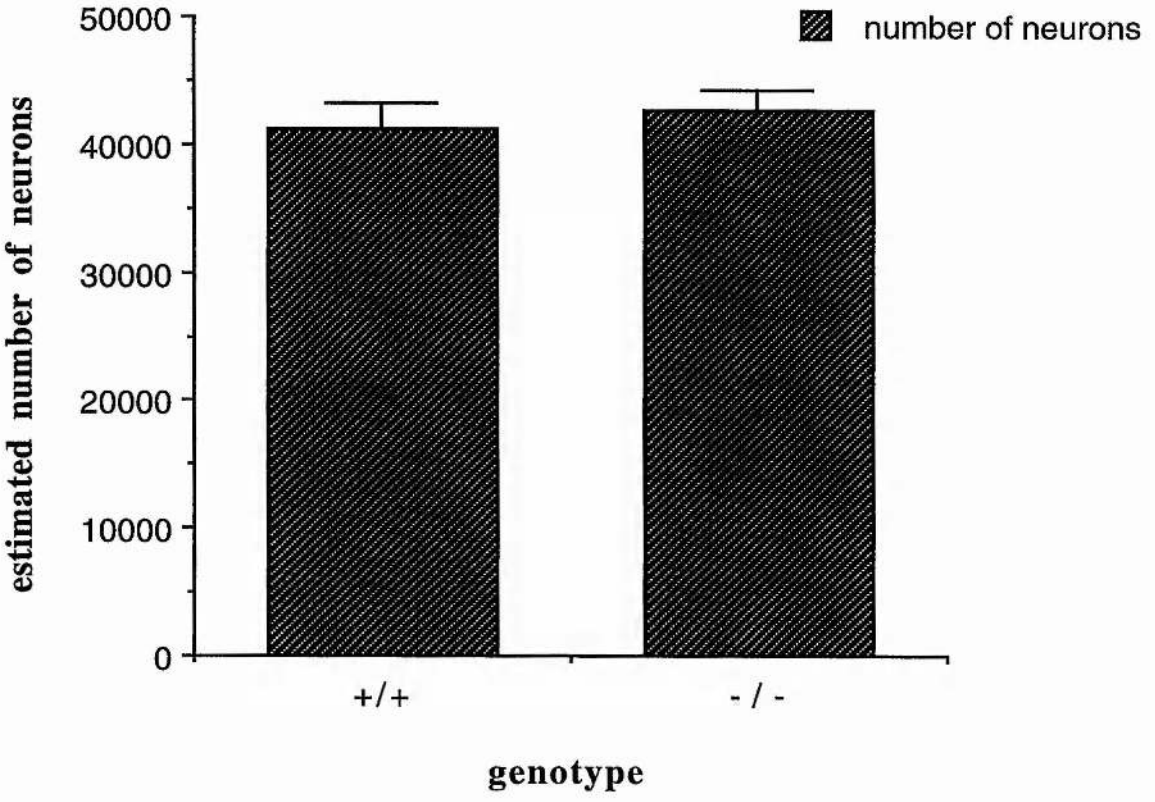
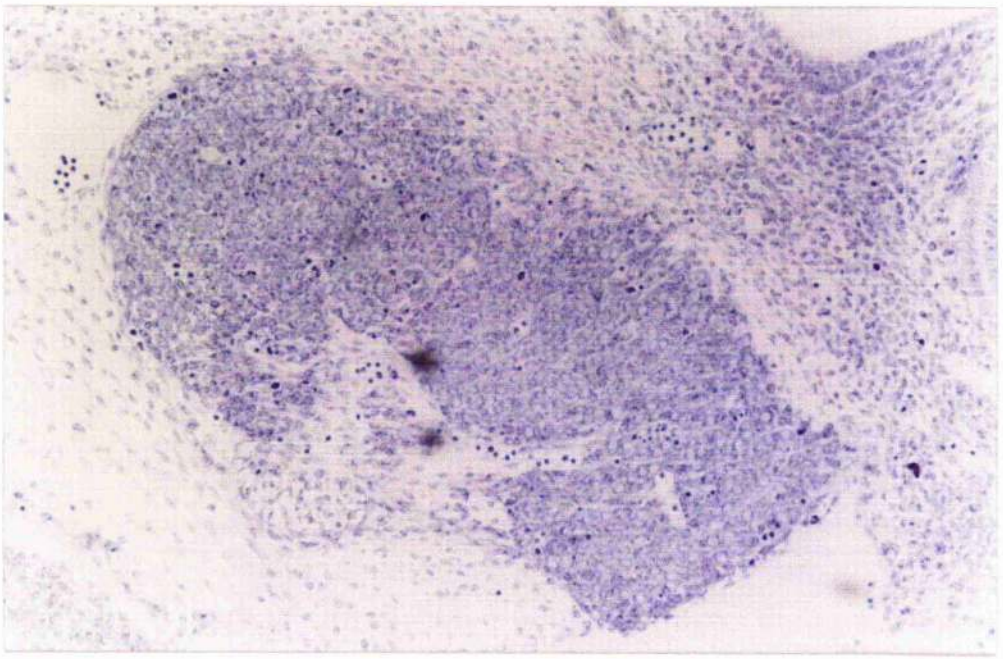


Figure 5.5b: Number of neurofilament positive neurons in anti-neurofilament stained sections of the trigeminal ganglia of wild type and Bcl-2-deficient embryos at E12.

The number of Bcl-2-deficient embryos studied was 6 and for the wild type embryos n=5. The means and standard errors of the means are shown.

A



B

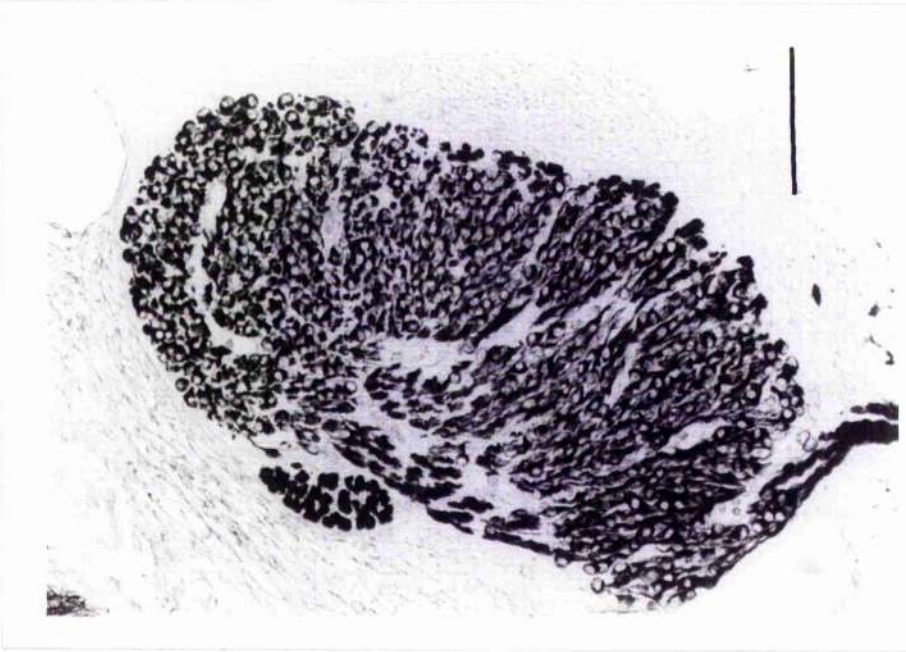


Figure 5.6: Photomicrograph of a section of the trigeminal ganglia of an E12 embryo stained for cresyl fast violet (A) and neurofilament (B). Scale bar represents 100μm.

Quantification of trkA and p75 mRNAs

This work was done by Dr. S. Wyatt using competitive RT-PCR to quantify the level of the mRNAs encoding the NGF receptors, p75 and trkA. The aim of this study was to determine if the delayed morphological maturation of early sensory neurons in Bcl-2-deficient embryos was a feature of a more generalised delay in the development of these neurons. The levels of the mRNAs for both wild type and Bcl-2-deficient embryos increased to the same extent between E12 and E14 (Fig. 5.7) as neurons within the trigeminal ganglia acquire NGF dependence.

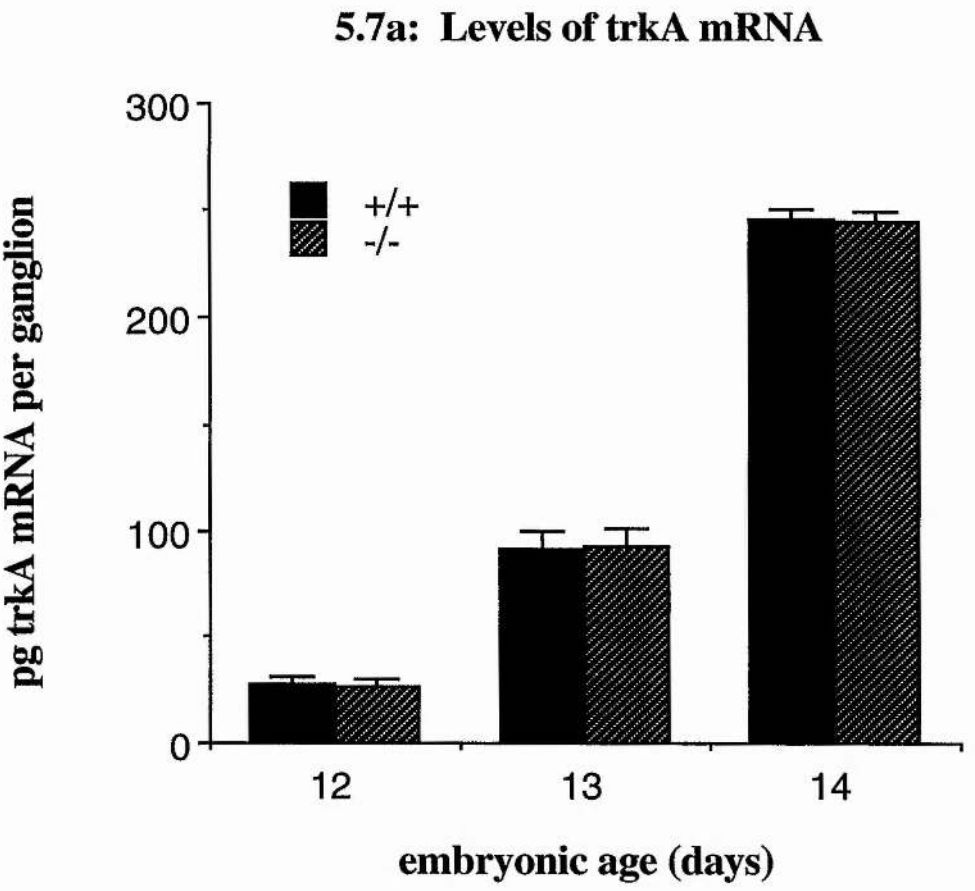


Figure 5.7a: Level of trkA mRNA in the trigeminal ganglia of wild type and Bcl-2-deficient embryos at E12, E13, and E14. For Bcl-2-deficient embryos at E12 n=4, at E13 n=6, and at E14 n=6. For wild type embryos at E12 n=5, at E13 n=5 and at E14 n=7. Each data point represents the mean of a number of embryos. The means and standard errors of the means are shown.

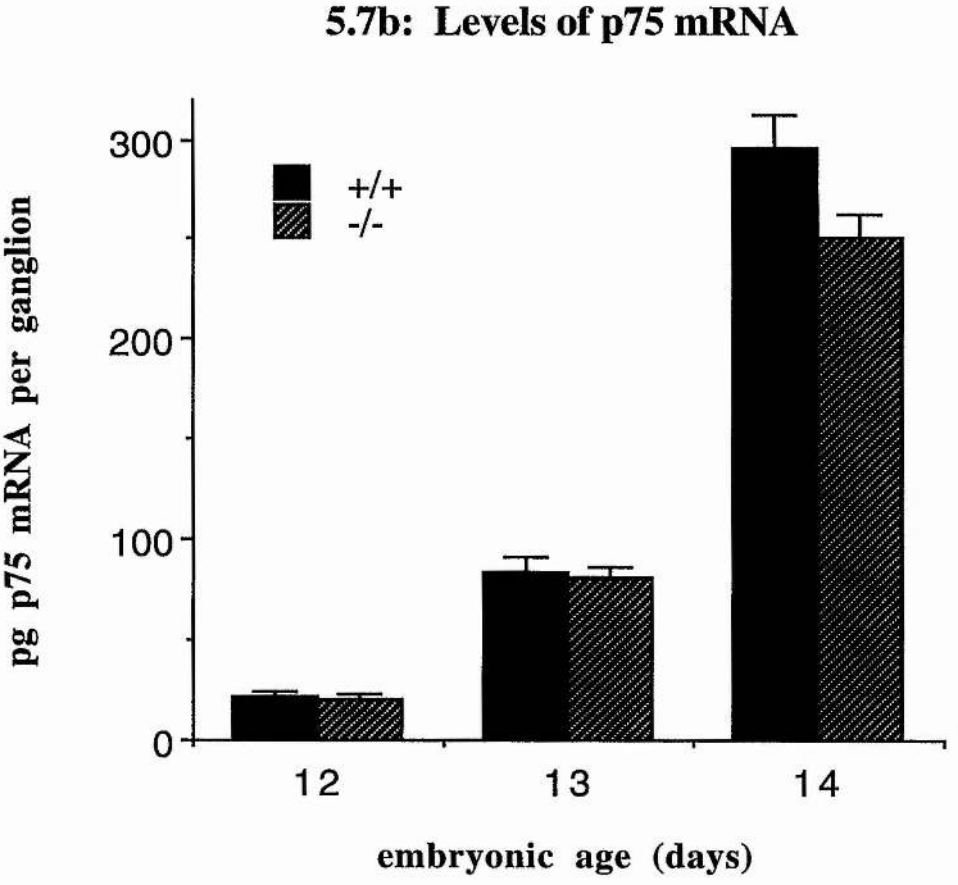


Figure 5.7b: Level of p75 mRNA in the trigeminal ganglia of wild type and Bcl-2-deficient embryos at E12, E13, and E14. For Bcl-2-deficient embryos at E12 n=4, at E13 n=6, and at E14 n=6. For wild type embryos at E12 n=5, at E13 n=5 and at E14 n=7. Each data point represents the mean of a number of embryos. The means and standard errors of the means are shown.

Analysis of Neurite Growth Rate

A detailed comparative study of neurite growth rate in low-density dissociated cultures of trigeminal ganglion neurons, established form Bcl-2-deficient and wild type mouse embryos, was performed in collaboration with Mark Hilton. The ages studied, E11 and E12, reflect a time in development when many trigeminal axons are growing towards their peripheral and central targets *in vivo.* Figure 5.8 shows that at both ages and in the presence of NGF (a), BDNF (b) or NT-3 (c), Bcl-2-deficient neurons extended neurites at a slower rate than either heterozygous or wild type neurons. After 48 hours of incubation, neurites from Bcl-2-deficient neurons were between 44 and 60% shorter than those of wild type neurons in E11 cultures and 35% shorter in E12 cultures. The differences between the wild type and Bcl-2-deficient neurons were statistically significant at 12, 24, and 48 hours incubation in all cases ($p<0.0001$ in all cases, t-tests). Because only neurons that survived the 48 hour duration of the study were considered, the differences in neurite outgrowth do not reflect differences in survival. Furthermore, these results were not influenced by observer bias because the genotypes were ascertained after all the measurements were made. These results appear to indicate a role for Bcl-2 in promoting neurite outgrowth in early trigeminal neurons. However, the possibility that this is another maturation effect cannot be discounted as no distinction was made between mature and immature neurons although this currently being studied. One point which may argue that the differences in neurite outgrowth are not simply a reflection of the level of maturation is the observation that the differences in neurite length are not Bcl-2 dose dependent, unlike maturation. This suggests that neurite growth rate differences are a distinguishable effect from maturation.

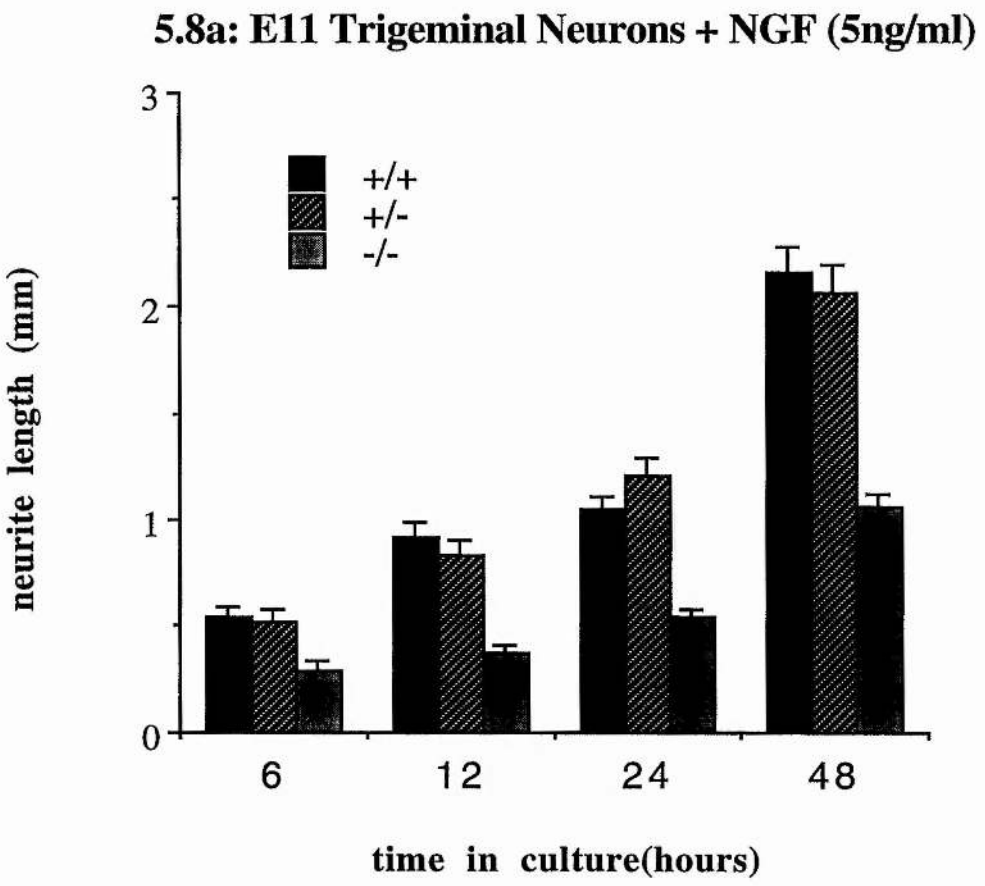


Figure 5.8a: Results of neuron cohort experiments to compare axonal growth rates of trigeminal neurons from Bcl-2-deficient, heterozygous and wild type mice, at E11 in the presence of NGF (5ng/ml). The means and standard errors of the means are shown. Each point on the graph represents the mean of a number of embryos. For each embryo, triplicate cultures were established for all conditions studied. For Bcl-2-deficient embryos n=5 and for wild type embryos n=5.

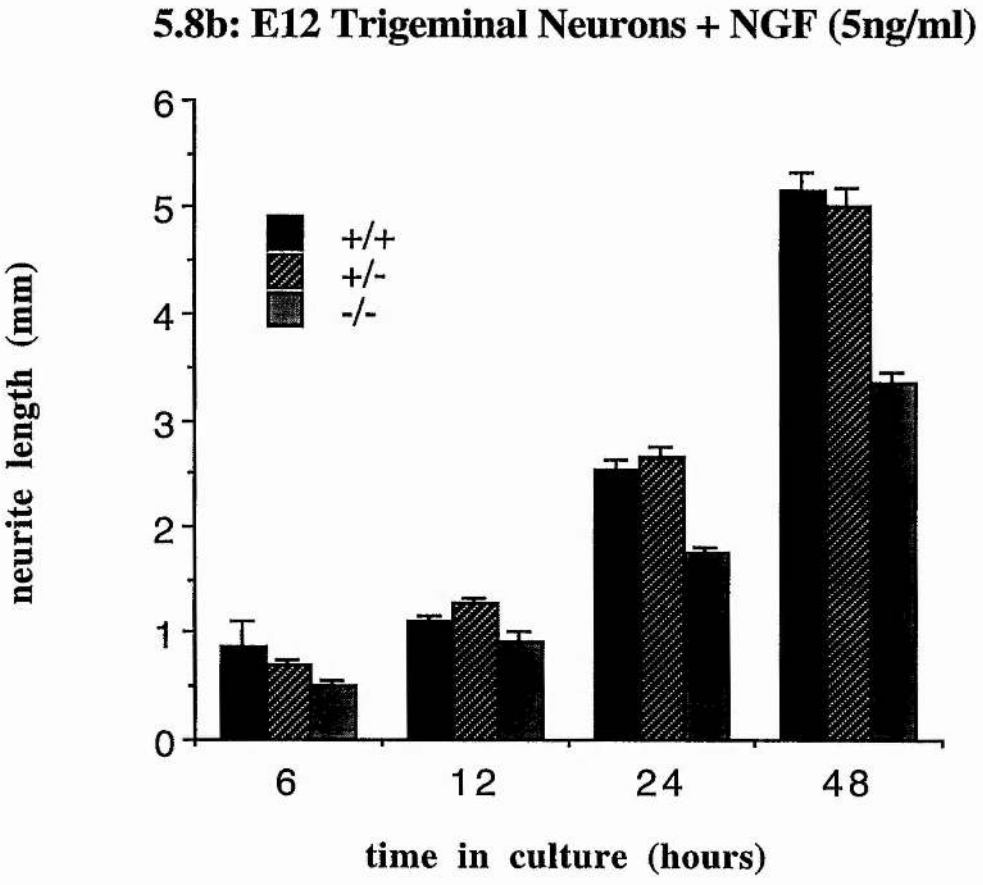


Figure 5.8b: Results of neuron cohort experiments to compare axonal growth rates of trigeminal neurons from Bcl-2-deficient, heterozygous and wild type mice, at E12 in the presence of NGF (5ng/ml). The means and standard errors of the means are shown. Each point on the graph represents the mean of a number of embryos. For each embryo, triplicate cultures were established for all conditions studied. For Bcl-2-deficient embryos n=7 and for wild type embryos n=6.

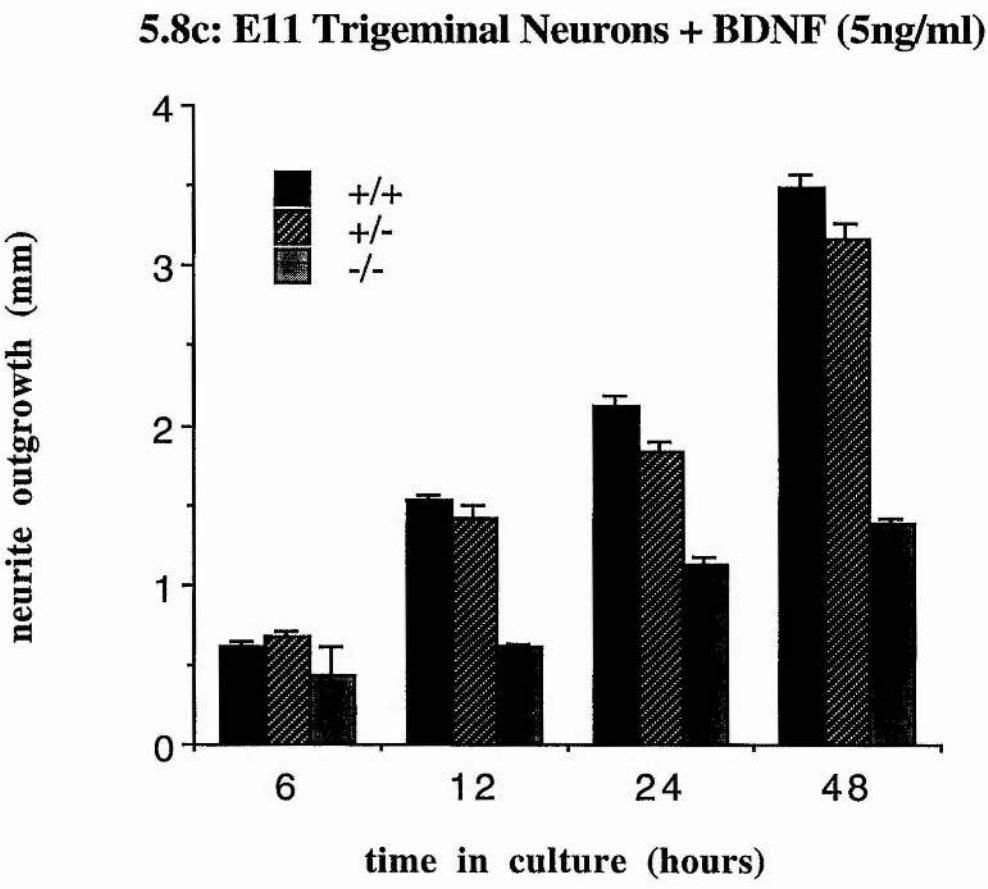


Figure 5.8c: Results of neuron cohort experiments to compare axonal growth rates of trigeminal neurons from Bcl-2-deficient, heterozygous and wild type mice, at E11 in the presence of BDNF (5ng/ml). The means and standard errors of the means are shown. Each point on the graph represents the mean of a number of embryos. For each embryo, triplicate cultures were established for all conditions studied. For Bcl-2-deficient embryos n=5 and for wild type embryos n=5.

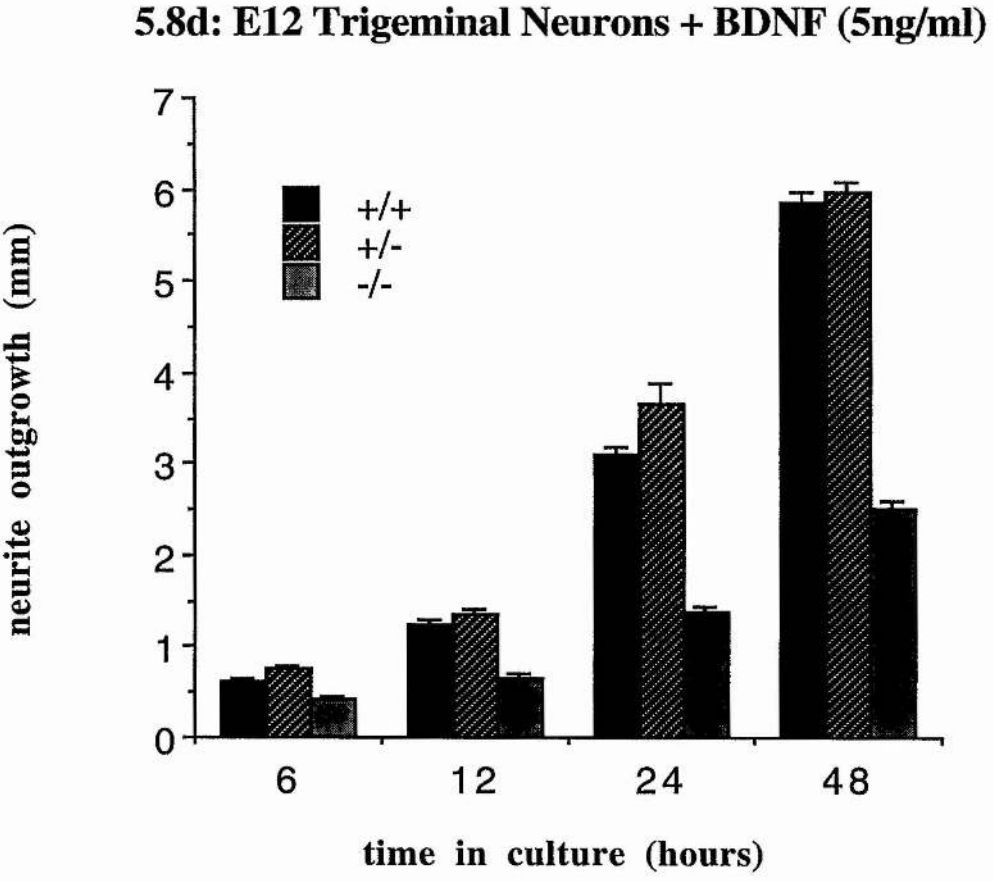


Figure 5.8d: Results of neuron cohort experiments to compare axonal growth rates of trigeminal neurons from Bcl-2-deficient, heterozygous and wild type mice, at E12 in the presence of BDNF (5ng/ml). The means and standard errors of the means are shown. Each point on the graph represents the mean of a number of embryos. For each embryo, triplicate cultures were established for all conditions studied. For Bcl-2-deficient embryos n=7 and for wild type embryos n=6.

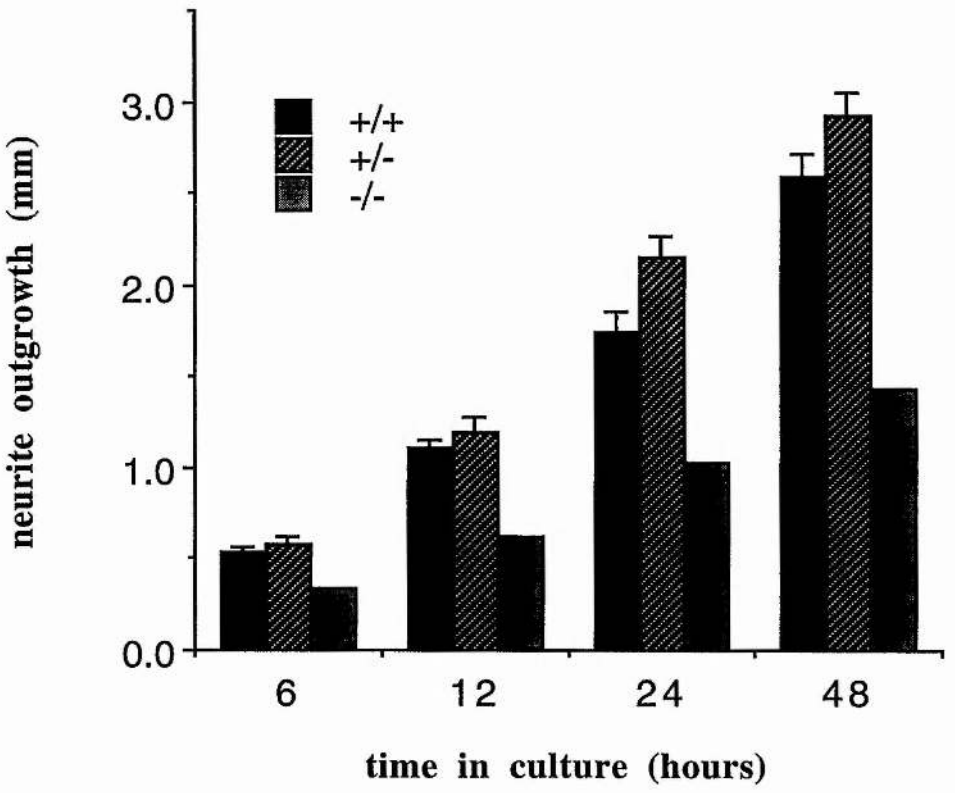


Figure 5.8e: Results of neuron cohort experiments to compare axonal growth rates of trigeminal neurons from Bcl-2-deficient, heterozygous and wild type mice, at E11 in the presence of NT-3 (5ng/ml). The means and standard errors of the means are shown. Each point on the graph represents the mean of a number of embryos. For each embryo, triplicate cultures were established for all conditions studied. For Bcl-2-deficient embryos n=5 and for wild type embryos n=5.

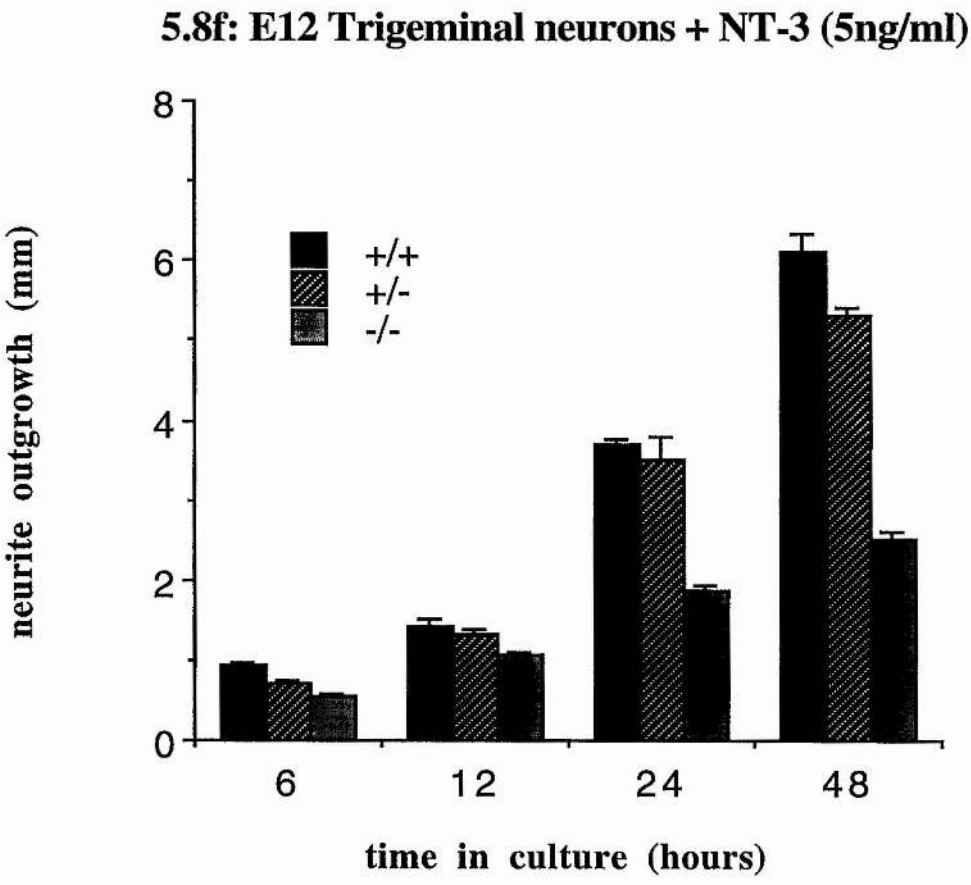


Figure 5.8f: Results of neuron cohort experiments to compare axonal growth rates of trigeminal neurons from Bcl-2-deficient, heterozygous and wild type mice, at E11 in the presence of NGF (5ng/ml). The means and standard errors of the means are shown. Each point on the graph represents the mean of a number of embryos. For each embryo, triplicate cultures were established for all conditions studied. For Bcl-2-deficient embryos n=7 and for wild type embryos n=6.

DISCUSSION

The data presented in this chapter provide compelling evidence for at least one novel role for bcl-2 during the early stages of neuronal development that is distinct from its well-recognised anti-apoptotic function. Newly differentiated sensory neurons initially have small, spindle-shaped phase dark cell bodies which subsequently enlarge becoming spherical and phase bright. By tracking the morphology of individual neurons at intervals in cultures established from the early trigeminal ganglia of bcl-2-deficient and wild type embryos, it has been demonstrated that this clearly recognisable morphological change is markedly delayed in bcl-2-deficient neurons. This delay is not due to a decrease in the viability of bcl-2-deficient neurons because the early trigeminal neurons of bcl-2-deficient and wild type embryos survive equally well in vitro. Thus, the absence of bcl-2 has a marked effect on the in vitro development of early sensory neurons that is unrelated to its role in regulating cell survival. Interestingly, later in development, during the peak period of naturally occurring neuronal death, bcl-2-deficient trigeminal neurons do die more rapidly than wild type neurons in NGF-supplemented medium. This decrease in the in vitro viability of older embryonic bcl-2-deficient trigeminal neurons is correlated with an increase in the number of dying neurons in the trigeminal ganglion at this stage in vivo, and a reduction in the total number of neurons in the ganglion before birth (Pinon et al., 1997). Reductions in the number of neurons in dorsal root and sympathetic ganglia and in motoneuron populations have also been reported in bcl-2-deficient mice postnatally (Michaelidis et al., 1996).

The in vitro observations of the delayed morphological maturation of early bcl-2-deficient sensory neurons were supported by an in vivo analysis of the trigeminal ganglia of bcl-2-deficient and wild type embryos. There are fewer morphologically recognisable neurons in the trigeminal ganglia of bcl-2-deficient embryos as compared with wild type embryos at both E12 and E13 as analysed in cresyl fast

violet stained sections. This is the stage when many neurons are differentiating from progenitor cells and starting to extend axons to their targets (Davies, 1987b; Davies & Lumsden, 1984). Because of their small size, immature neurons are not distinguishable from progenitor cells and other non-neuronal cells in this histological analysis. The total neuronal complement of the trigeminal ganglion of Bcl-2-deficient embryos was, however, normal during this period of development as revealed by counting the number of neurofilament-positive cells in the trigeminal ganglia of Bcl-2-deficient and wild type embryos. The most likely explanation for these findings is that, as *in vitro*, the morphological maturation of early sensory neurons is delayed *in vivo* in the absence of Bcl-2.

Of particular interest is the demonstration that the delay in the morphological maturation of early Bcl-2-deficient neurons is not a consequence of a general retardation of the development of Bcl-2-deficient embryos or of the sensory neurons within these embryos. There was no apparent difference in the stage-specific external features (Theiler, 1972) of Bcl-2-deficient and wild type embryos in each litter and the time course and level of expression of both p75 and trkA mRNAs were virtually identical in embryos of both genotypes. Because these mRNAs are expressed predominantly, if not exclusively, in the neurons of the embryonic trigeminal ganglion and their levels increase markedly with the acquisition of NGF survival response in these neurons (Wyatt & Davies, 1993; Wyatt et al., 1990), these findings also reinforce the evidence based on the GAPDH measurements that the neuronal complement of the early trigeminal ganglion is similar in both Bcl-2-deficient and wild type embryos.

In addition to, or perhaps as a consequence of, the effects on neuronal maturation, Bcl-2 has been shown to influence the rate or neurite outgrowth *in vitro*. Neurons from Bcl-2-deficient embryos extend shorter neurites than neurons from wild type or heterozygous embryos at E11 and E12, in the presence of NGF (5ng/ml), BDNF (5ng/ml) or NT-3 (5ng/ml). Although this finding may be a consequence of the

delay in the maturation of Bcl-2-deficient neurons, the fact that this is not a gene dose dependent effect, unlike maturation, argues for a role for Bcl-2 in neurite outgrowth that is distinct from its role in neuronal maturation.

Several studies of the consequences of manipulating the expression of Bcl-2 in various cell lines and neurons have raised the possibility that Bcl-2 may have functions in addition to its anti-apoptotic role. The differentiation of the PC12 pheochromacytoma cell line into neurons is enhanced by overexpression of Bcl-2. However, this effect is only observed when the cells are grown in serum-free medium (Batistatou et al., 1993). Midbrain-derived dopaminergic cell lines stably expressing Bcl-2 extend longer neurites than control transfected cells but do not have increased expression of many neuron-specific proteins (Oh et al., 1996). Bcl-2 overexpression in a neural crest-derived cell line enhances the outgrowth of neurite-like processes and also increases the expression of neuron-specific enolase (Zhang et al., 1996). Several observations indicate that the level of Bcl-2 expression in mouse retinal ganglion cells (RGCs) influences the ability of their axons to regenerate into co-cultured tectal explants (Chen et al., 1997). In the early fetal stages, RGCs normally express high levels of Bcl-2 and are able to extend axons into co-cultured tectal tissue of the same age, whereas late fetal RGCs express low levels of Bcl-2 and are not able to grow axons into tectal tissue. The ability of early fetal RGCs to regenerate into tectal tissue is substantially reduced in retinal explants obtained from Bcl-2-deficient embryos, whereas late fetal and adult RGCs from transgenic mice overexpressing Bcl-2 in neurons are able to regenerate into tectal tissue.

In summary, the data presented in this chapter demonstrates at least one novel role for Bcl-2 in promoting the maturation of early sensory neurons of the trigeminal ganglion. A second role in neurite outgrowth is also suggested by the data although further work will be necessary to fully elucidate this. In particular it will be important to determine whether the reduced rate of neurite outgrowth in E11 and E12 Bcl-2-

deficient neurons is a consequence of slowed maturation or whether Bcl-2 plays a role in neurite outgrowth in mature sensory neurons. It will also be necessary to determine whether axonal growth rates are reduced *in vivo* in Bcl-2-deficient embryos.

VI. BAX TRANSGENIC MICE

SURVIVAL STUDIES

CHAPTER SIX

BAX TRANSGENIC MICE

NEURONAL SURVIVAL STUDIES

INTRODUCTION

To date, the role of Bax in regulating neuronal survival has been poorly elucidated. Data presented in Chapter Three suggests that supraphysiological expression of human Bax can promote survival of embryonic chicken neurons in the absence of trophic support. This finding was unexpected, since Bax has previously been shown to promote cell death in IL-3-dependent cell lines and inhibit the death repressor action of Bcl-2 (Oltvai et al., 1993). However, the interpretation of the data in Chapter Three is complicated by the species difference between the Bax cDNA that was available for injection and the host neurons, and also by the high, non-physiological levels of Bax ectopically expressed in the host neurons. It is possible that the survival-promoting effect of Bax is an artefact of the experimental system used in Chapter Three. Indeed, Bax overexpression has recently been shown to promote cell death in rat sympathetic neurons both in the presence and absence of NGF (Vekrellis et al., 1997).

In this chapter, Bax null mutant mice have been used to try and clarify the role Bax plays in regulating the survival of developing sensory neurons. These mice survive postnatally and show cellular hyperplasia in various cells of the immune system and cellular hypoplasia in the cells of the testis, rendering the males infertile (Knudson et al., 1995). Here, *bax* null mutant mice were used to make a detailed study of the cells of the nodose and trigeminal ganglia and to examine the role of Bax in these cells.

Cell culture experiments were carried out over a range of embryonic ages to assess the role of Bax in both factor-dependent and factor-independent survival of the neurons *in vitro.* To determine if these *in vitro* studies had any relevance to the situation *in vivo*, the number of neurons in the trigeminal ganglia of both *bax*$^{-/-}$ and *bax*$^{+/+}$ embryos was studied using the histological techniques described previously.

RESULTS

Response of Bax-deficient Sensory Neurons to Neurotrophins *in vitro*

Trigeminal Ganglion

At E14, there was a marginally increased level of long term survival of Bax-deficient trigeminal neurons in the presence of NGF (5ng/ml). This difference was statistically significant by 120 hours after plating (t-test, $P<0.05$). At this time, there were no surviving neurons from wild type embryos or *bax*$^{+/-}$ embryos, whereas approximately 7% of Bax-deficient trigeminal neurons were still surviving (Fig. 6.1a). In cultures that were not supplemented with NGF, there was no difference between the Bax-deficient neurons and the neurons of the other two genotypes with all neurons dying rapidly within the first 48 hours (Fig.6.1b).

By E16, Bax-deficient trigeminal neurons showed increased survival as compared to those neurons from *bax*$^{+/-}$ or bax$^{+/+}$ embryos in the presence of NGF (5ng/ml), but the difference was relatively small (Fig. 6.2a). 144 hours after plating, all except the Bax-deficient neurons had died, and only about 12% of these neurons were still surviving. In the absence of NGF, neurons from Bax-deficient embryos survived markedly better than those derived from heterozygous or wild type embryos (Fig. 6.2b). All of the neurons from the wild type embryos and those heterozygous for a null mutation in the *bax* gene were dead by 48 hours after plating. At this time, nearly 60% of trigeminal neurons from Bax-deficient embryos were still alive. This figure decreased with time, plateauing at around 7% 120 hours after plating.

At E18, the Bax-deficient trigeminal neurons showed substantially better survival in the presence of NGF (10ng/ml) than their wild type or *bax*$^{+/-}$ counterparts (Fig. 6.3a). Likewise, in the absence of NGF, bax-deficient trigeminal neurons show enhanced survival (Fig. 6.3b). After 48 hours in culture under these conditions all the wild type and *bax*$^{+/-}$ neurons were dead, whereas approximately 40% of the Bax-deficient neurons were still surviving. There was little further reduction in this number for the duration of the experiment.

By P1, there was again substantially better survival of Bax-deficient trigeminal neurons in the presence of NGF (10ng/ml) (Fig. 6.4a). However, the difference in survival between these neurons and the wild type and *bax*$^{+/-}$ neurons was not as marked as at E18. When cultured without NGF (Fig. 6.4b), Bax-deficient trigeminal neurons survive markedly better than neurons derived from neonates of the other two genotypes. 48 hours after plating, all the neurons from the wild type neonates and those from neonates heterozygous for the *bax* null mutation were dead. At this time, approximately 60% of the Bax-deficient neurons were still surviving and this figure did not decrease markedly during the remainder of the experiment.

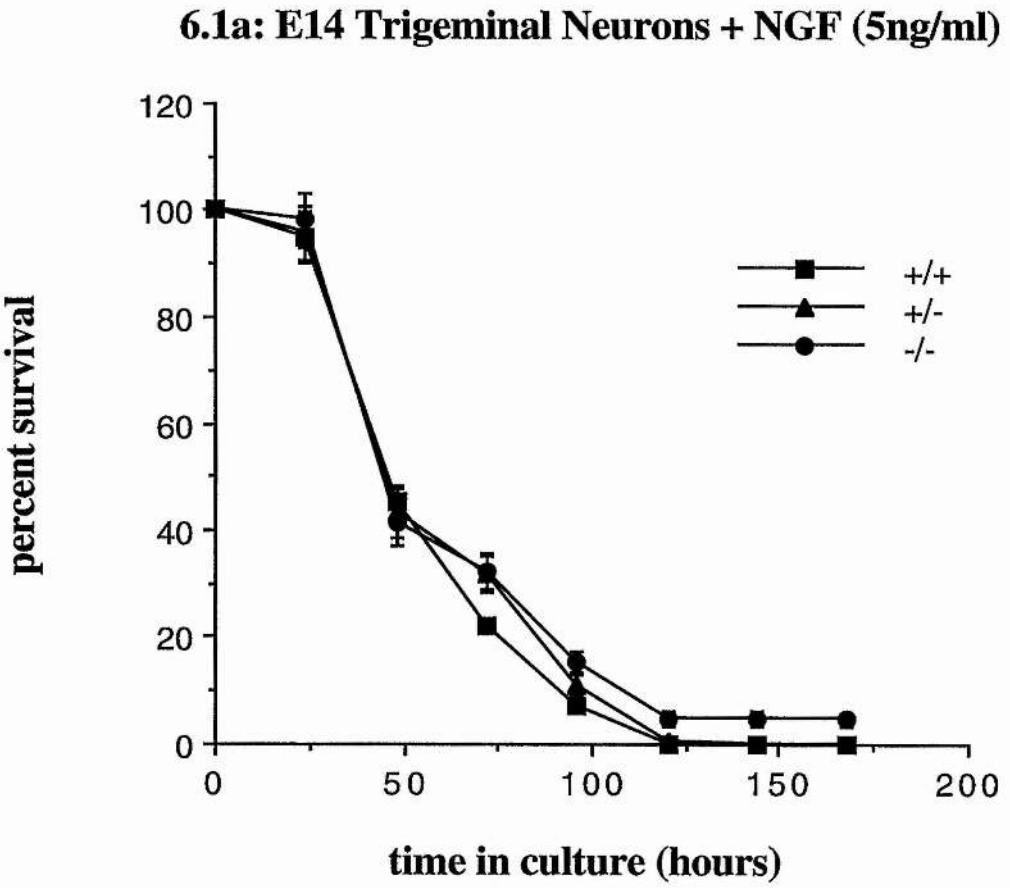


Figure 6.1a: Percent survival of E14 trigeminal neurons from *bax-/-* (n=4), *bax+/-* (n=8) and *bax +/+* (n=4) embryos grown for 168 hours in culture with NGF (5ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

6.1b: E14 Trigeminal neurons, without factors

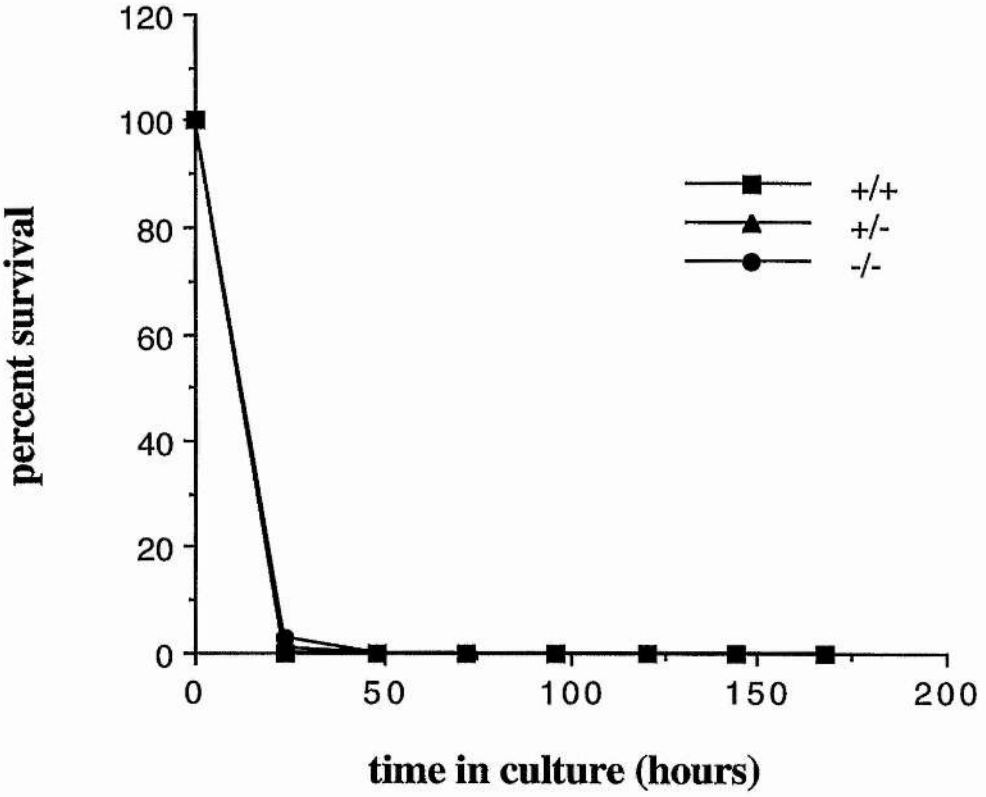


Figure 6.1b: Percent survival of E14 trigeminal neurons from *bax-/-* (n=4), *bax+/-* (n=8) and *bax +/+* (n=4) embryos grown for 168 hours in culture without NGF. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

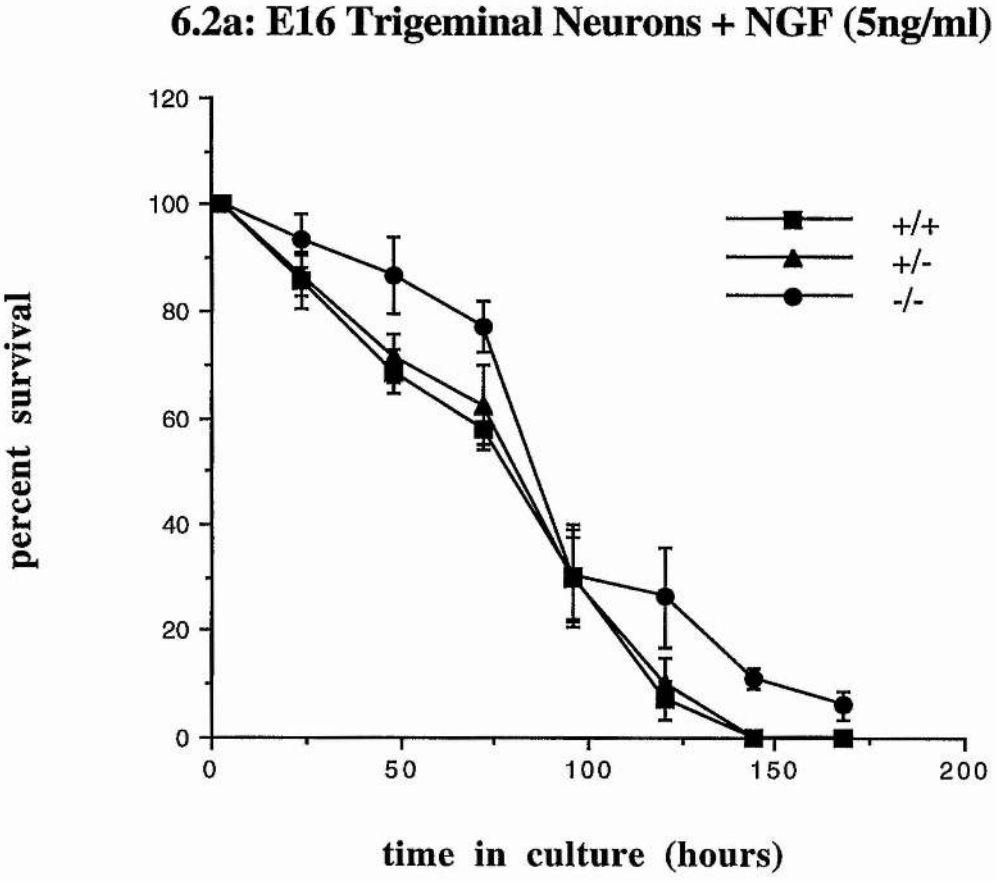


Figure 6.2a: Percent survival of E16 trigeminal neurons from *bax-/-* (n=5), *bax+/-* (n=13), and *bax +/+* (n=6) embryos grown for 168 hours in culture with NGF (5ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

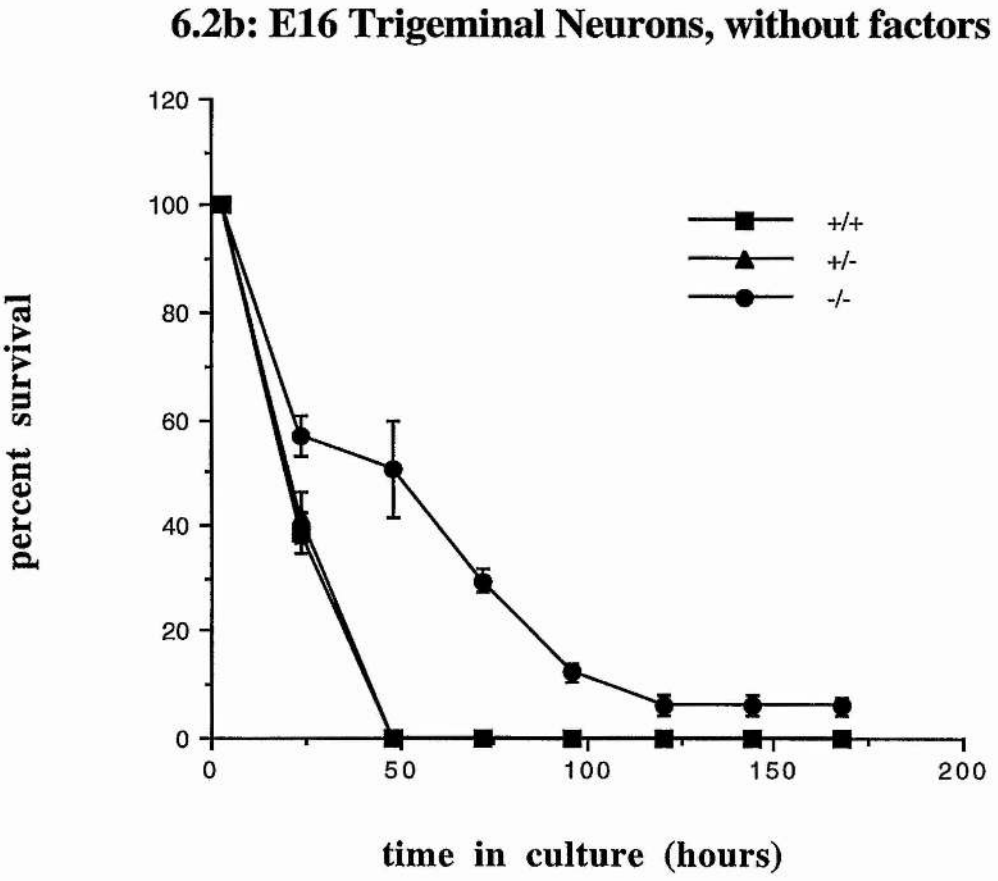


Figure 6.2b: Percent survival of E16 trigeminal neurons from *bax-/-* (n=5), *bax+/-* (n=13), and *bax +/+* (n=6) embryos grown for 168 hours in culture without NGF. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

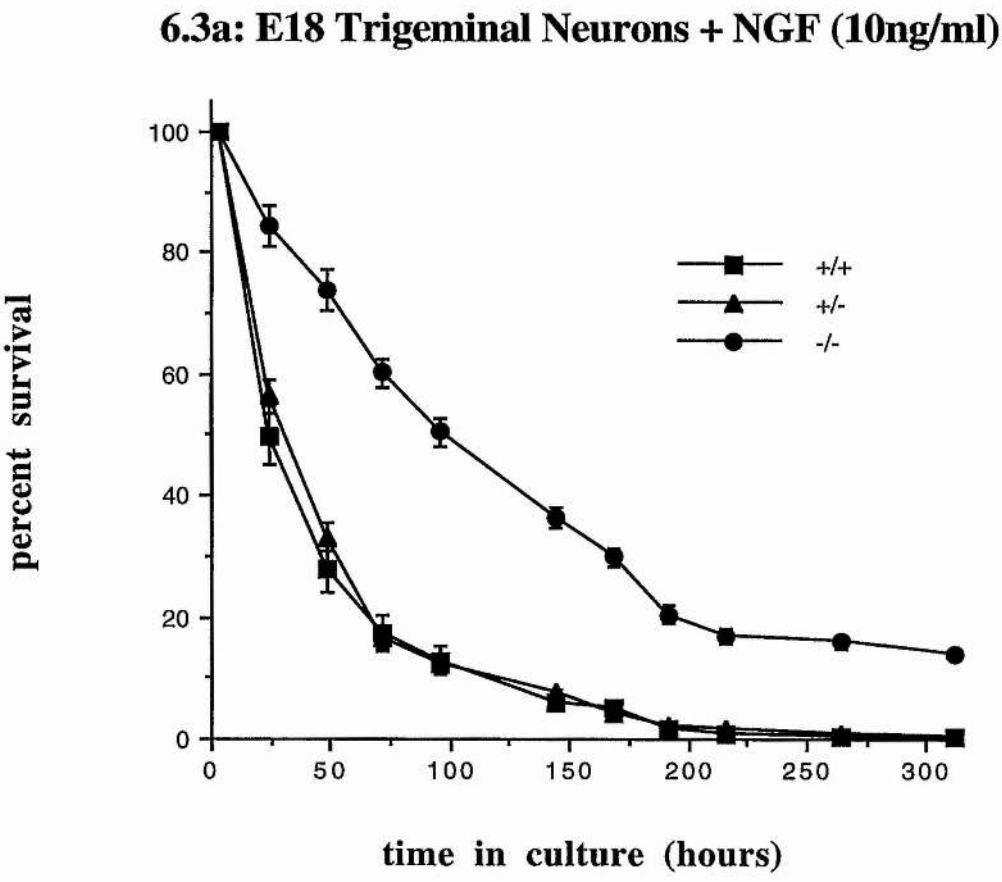


Figure 6.3a: Percent survival of E18 trigeminal neurons from *bax-/-* (n=5), *bax+/-* (n=12), and *bax +/+* (n=6) embryos grown for 312 hours in culture with NGF (10ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

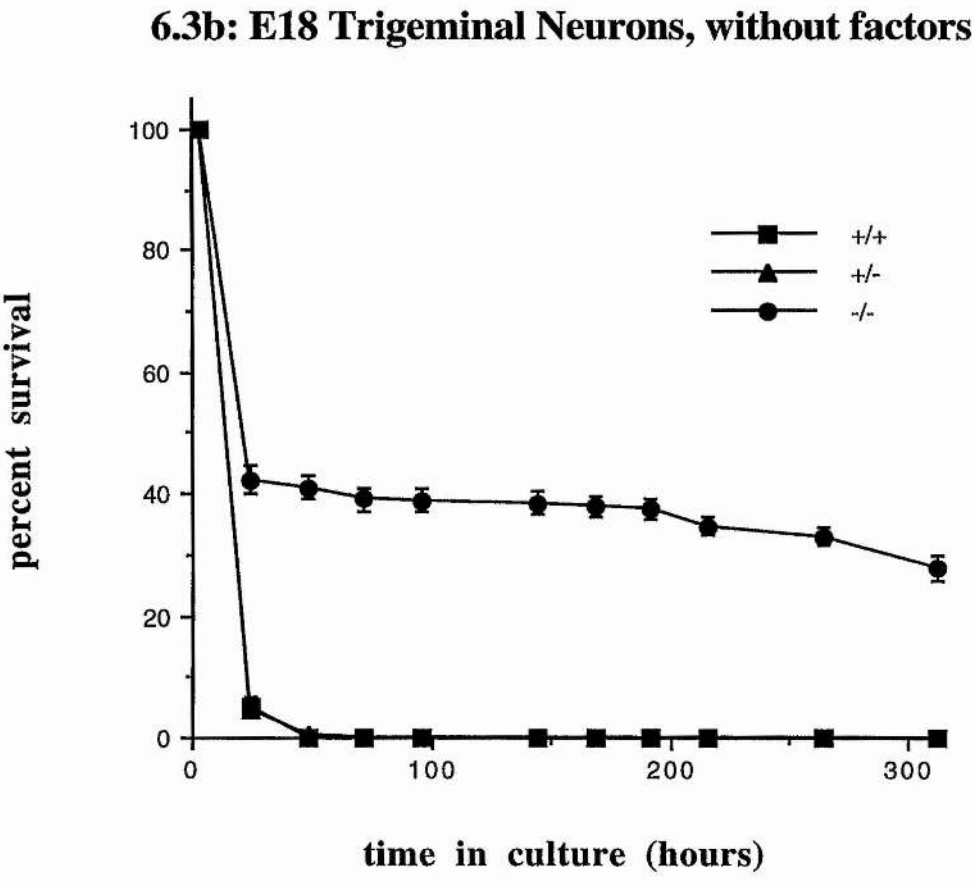


Figure 6.3b: Percent survival of E18 trigeminal neurons from *bax-/-* (n=5), *bax+/-* (n=12), and *bax +/+* (n=6) embryos grown for 312 hours in culture without NGF. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

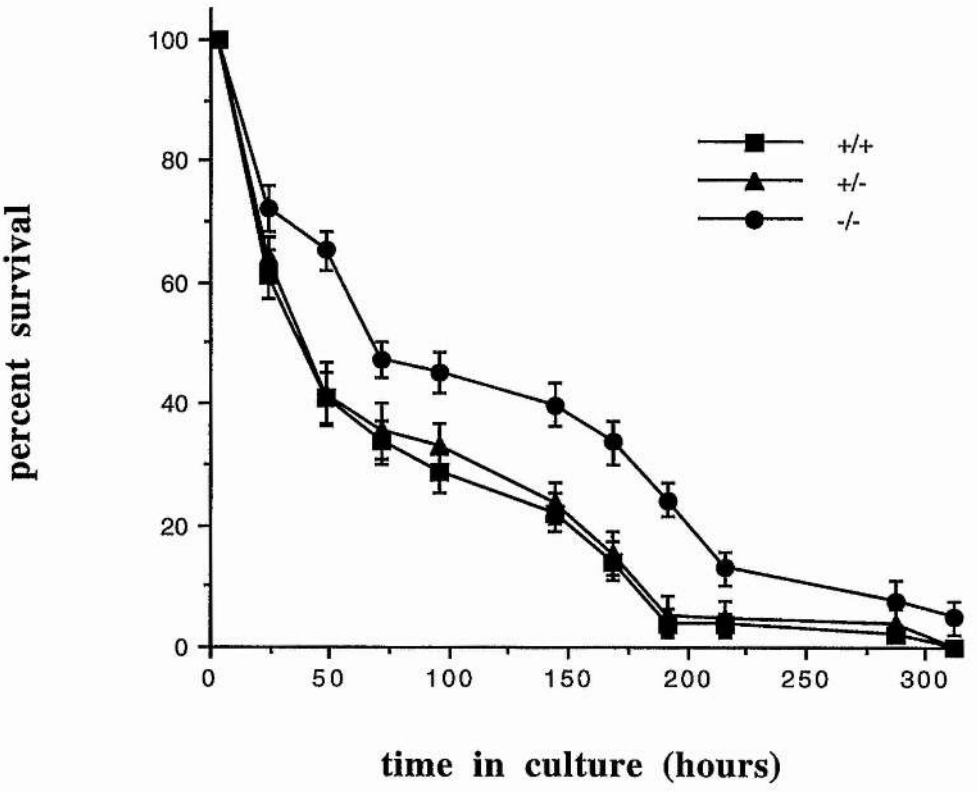


Figure 6.4a: Percent survival of P1 trigeminal neurons from *bax-/-* (n=8), *bax+/-* (n=14), and *bax +/+* (n=5) neonates grown for 312 hours in culture with NGF (10ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of neonates. For each neonate triplicate cultures were established for all conditions studied.

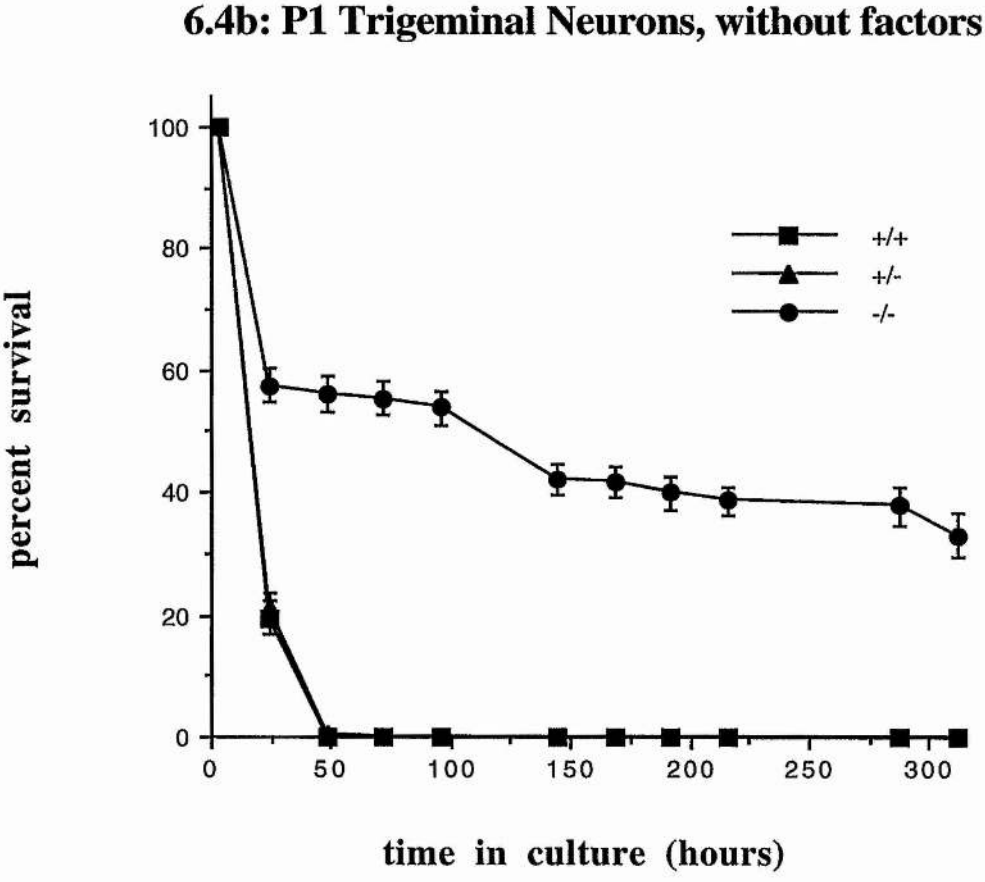


Figure 6.4b: Percent survival of P1 trigeminal neurons from *bax-/-* (n=8), *bax+/-* (n=14), and *bax +/+* (n=5) neonates grown for 312 hours in culture without NGF. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of neonates. For each neonate triplicate cultures were established for all conditions studied.

Nodose Neurons

At E14, Bax-deficient nodose neurons survived better in the presence of BDNF (5ng/ml) than neurons derived from *bax*$^{+/-}$ or wild type embryos (Fig. 6.5a). By 120 hours after plating, there were no neurons surviving from embryos of these genotypes whilst 20% of Bax-deficient neurons were still surviving. In the absence of BDNF, there was no difference between the three genotypes (Fig. 6.5b). In all cases, all neurons were dead by 24 hours after plating.

At E16, Bax-deficient nodose neurons showed a marked increase in survival in the presence of BDNF (5ng/ml) (Fig. 6.6a) as compared to wild type neurons and those derived from embryos which were heterozygous for the Bax null mutation. When cultured without BDNF, Bax-deficient neurons showed increased survival *in vitro*. 48 hours after plating, there were no nodose neurons derived from wild type or *bax*$^{+/-}$ embryos surviving (Fig. 6.6b) whilst approximately 40% of Bax-deficient neurons were still alive. This number declined with time in culture and reached a plateau at 120 hours after plating with just under 10% of the Bax-deficient neurons still alive.

At E18, Bax-deficient neurons again survived better with BDNF (10ng/ml) than neurons from wild type of heterozygous animals (Fig. 6.7a). This same pattern was seen in cultures without BDNF (Fig. 6.7b). In the absence of BDNF, all neurons derived from *bax*$^{+/-}$ and *bax*$^{+/+}$ embryos were dead 48 hours after plating. Approximately 55% of the Bax-deficient neurons were still alive. This declined slightly over the next few days, plateauing at around 40% survival.

A very similar pattern was seen for P1 nodose neurons where there was once again enhanced survival of neurons derived from Bax-deficient neonates both in the presence of BDNF (10ng/ml)(Fig. 6.8a) and in the absence of neurotrophic factors (Fig. 6.8b).

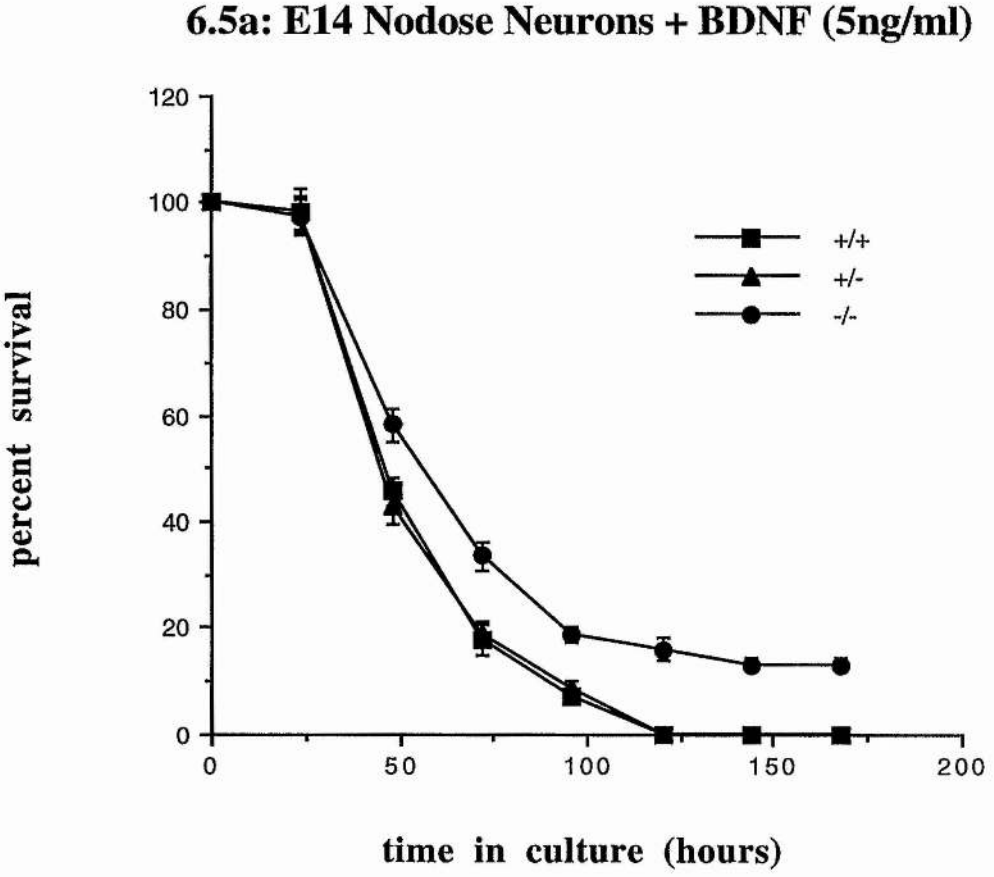


Figure 6.5a: Percent survival of E14 nodose neurons from *bax-/-* (n=4), *bax+/-* (n=8), and *bax +/+* (n=4) embryos grown for 168 hours in culture with BDNF (5ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

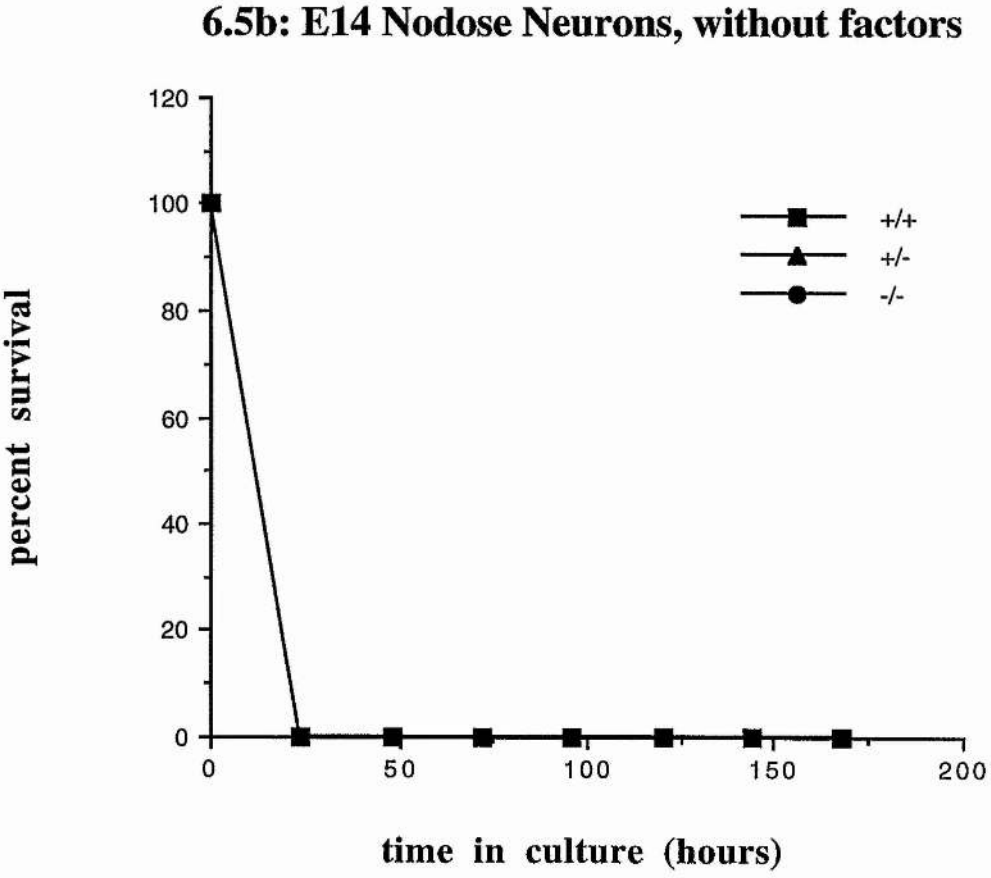


Figure 6.5b: Percent survival of E14 nodose neurons from *bax-/-* (n=4), *bax+/-* (n=8), and *bax +/+* (n=4) embryos grown for 168 hours in culture without BDNF. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

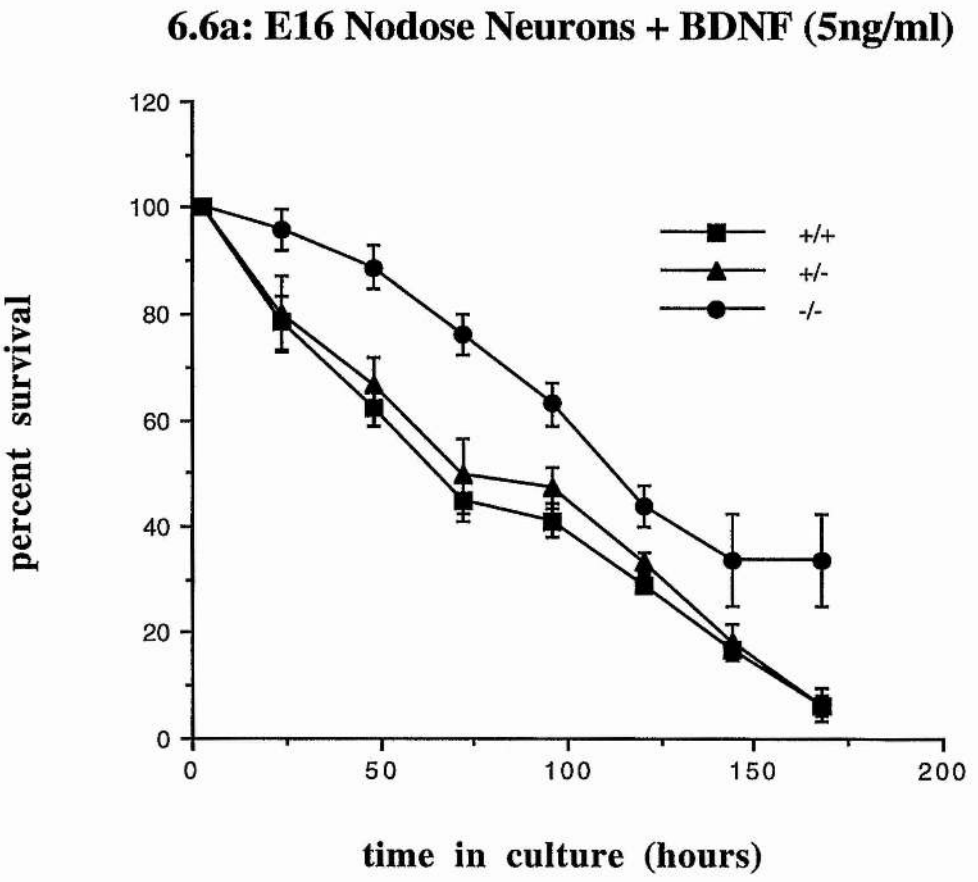


Figure 6.6a: Percent survival of E16 nodose neurons from *bax-/-* (n=5), *bax+/-* (n=13), and *bax +/+* (n=6) embryos grown for 168 hours in culture with BDNF (5ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

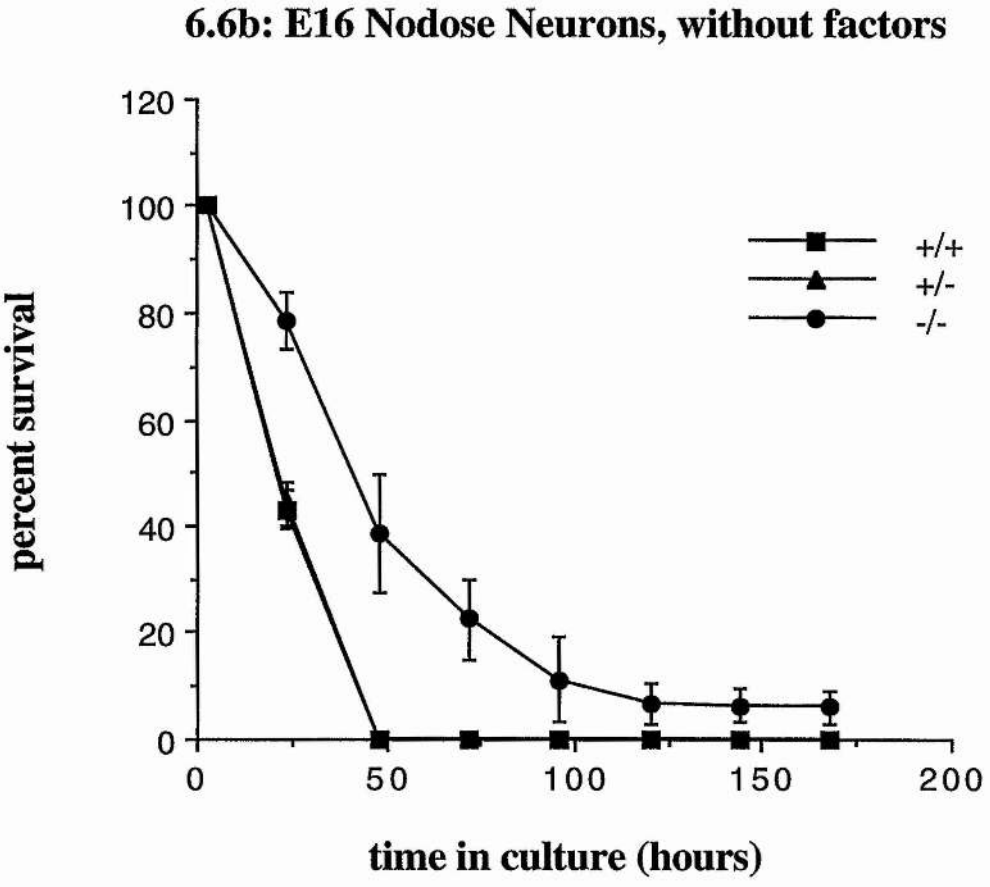


Figure 6.6b: Percent survival of E16 nodose neurons from *bax-/-* (n=5), *bax+/-* (n=13), and *bax +/+* (n=6) embryos grown for 168 hours in culture without BDNF. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

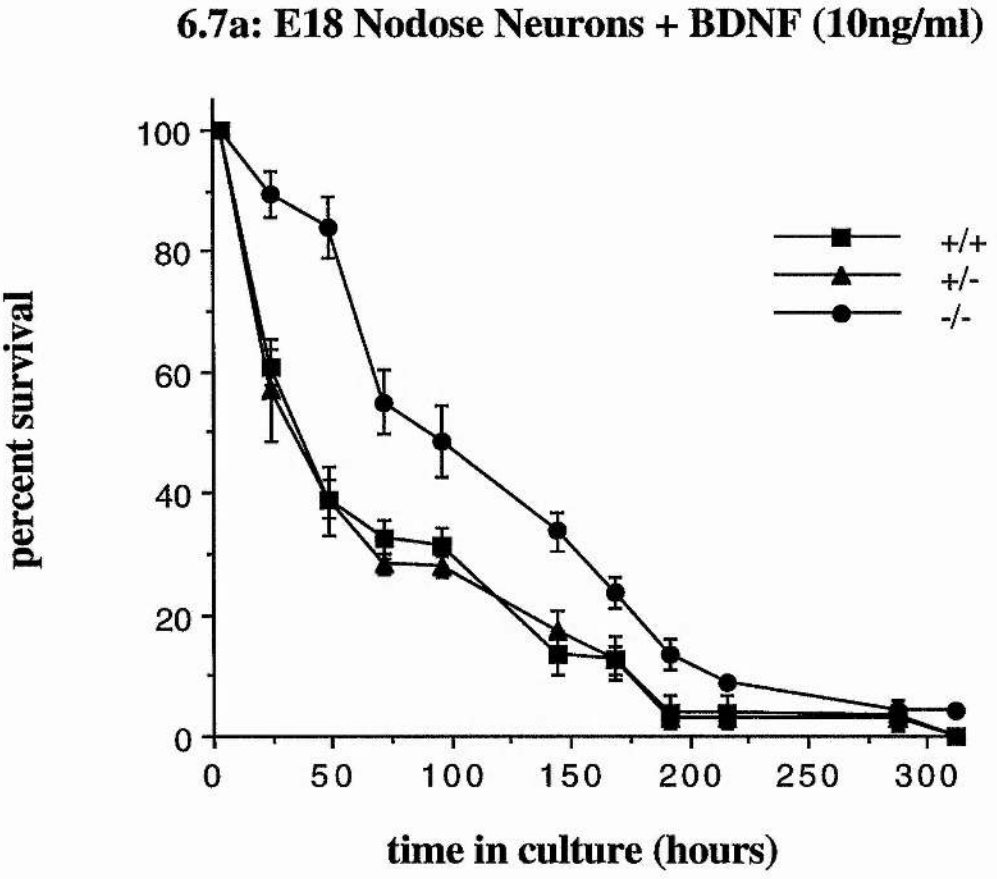


Figure 6.7a: Percent survival of E18 nodose neurons from *bax-/-* (n=5), *bax+/-* (n=12), and *bax +/+* (n=6) embryos grown for 312 hours in culture with BDNF (10ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

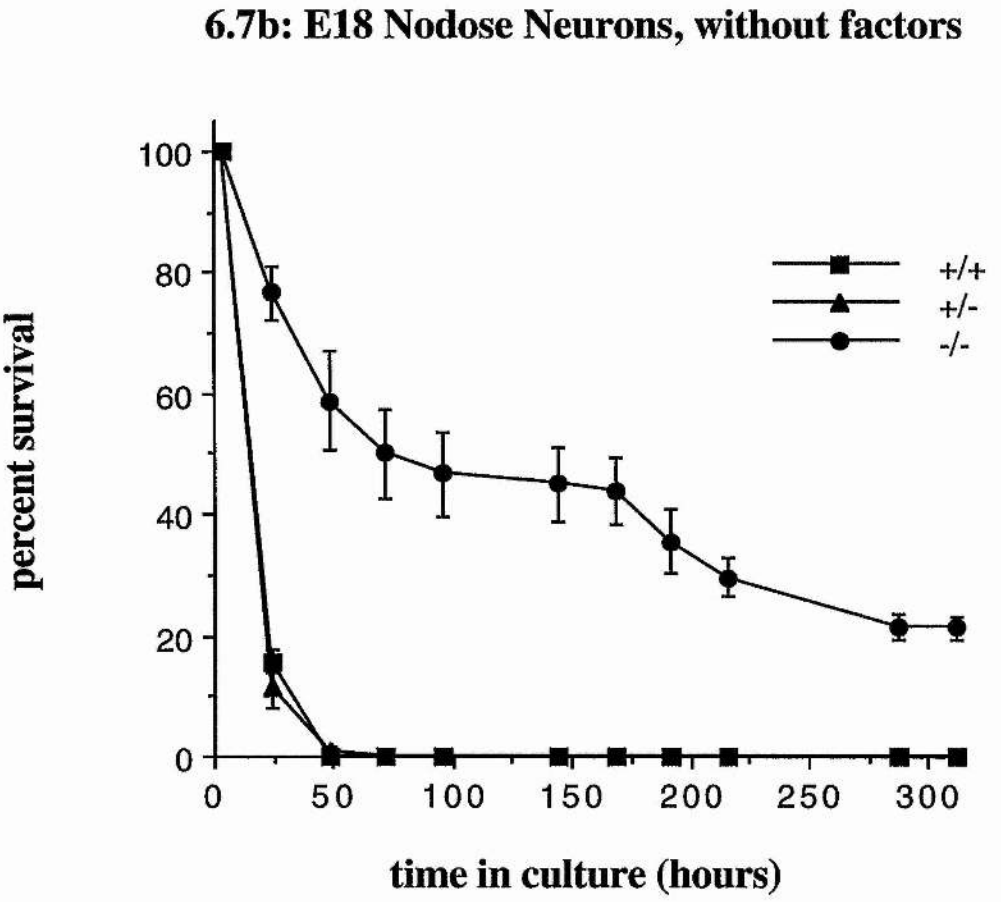


Figure 6.7b: Percent survival of E18 nodose neurons from *bax-/-* (n=5), *bax+/-* (n=12), and *bax +/+* (n=6) embryos grown for 312 hours in culture without BDNF. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

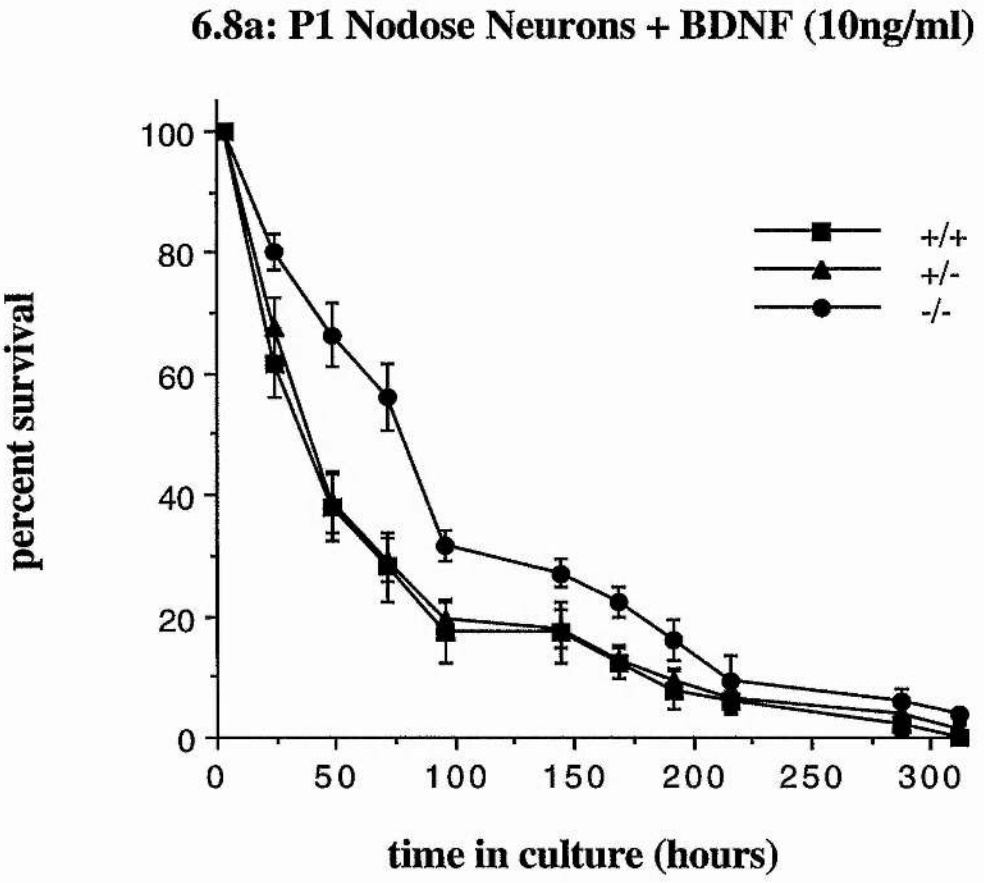


Figure 6.8a: Percent survival of P1 nodose neurons from *bax-/-* (n=7), *bax+/-* (n=14), and *bax +/+* (n=5) neonates grown for 312 hours in culture with BDNF (10ng/ml). The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of neonates. For each neonate triplicate cultures were established for all conditions studied.

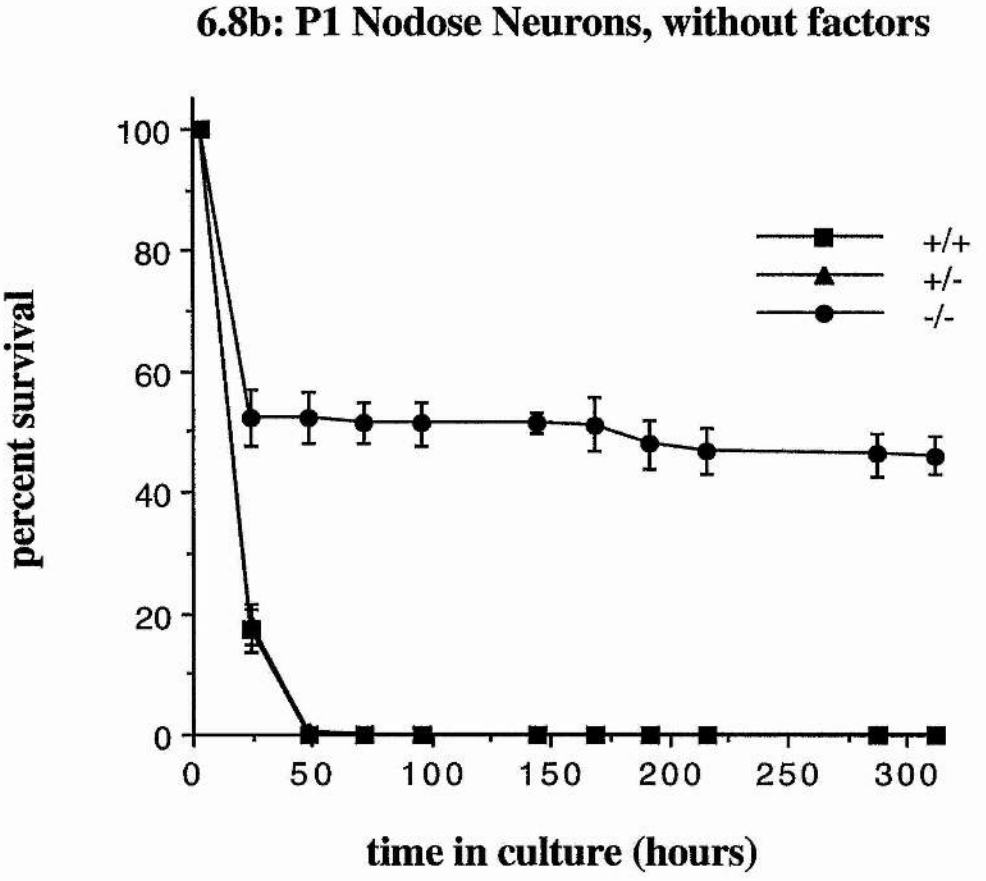


Figure 6.8b: Percent survival of P1 nodose neurons from *bax-/-* (n=7), *bax+/-* (n=14), and *bax +/+* (n=5) neonates grown for 312 hours in culture without BDNF. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of neonates. For each neonate triplicate cultures were established for all conditions studied.

Decreased Survival of Bax-deficient Neurons in Response to Neurotrophins

An interesting survival pattern was seen in both the nodose and trigeminal cranial sensory neurons derived from Bax-deficient embryos at E18 and neonates at P1. Over 312 hours in culture, the neurons that were grown without neurotrophic support survived better than those that were grown in the presence of their appropriate neurotrophic factor. This pattern was more marked in nodose neurons but was present in both cell types. Furthermore, the distinction was clearer at P1 than at E18, and again this is particularly true of the nodose neurons.

At E18, Bax-deficient trigeminal neurons (Fig. 6.9a) survived better in the absence of neurotrophins at time points after 192 hours in culture. Furthermore, when NGF (10ng/ml) was added to cultures that had been grown without factors, 48 hours after plating, the neurons in these cultures died more rapidly than those in cultures that lacked NGF. A similar pattern was seen in cultures of P1 Bax-deficient trigeminal neurons (Fig. 6.9b). Here after 168 hours in culture there was a clear difference between cells that were grown for the duration of the experiment in the absence of neurotrophins and those which were grown with NGF (10ng/ml) either from the outset or after 48 hours.

The same pattern was observed for nodose neurons. Here, at E18, the Bax-deficient neurons cultured without factors survived better at time points after 144 hours after plating compared to those in cultures containing BDNF (Fig. 6.10a). The same pattern was repeated in the data obtained from Bax-deficient P1 nodose neurons (Fig. 6.10b). After only 96 hours in culture there was a clear difference between the survival of Bax-deficient nodose neurons grown in the presence of BDNF and those that were not. At the end of the experiment, 312 hours after plating, there were virtually no cells surviving in the dishes supplemented with BDNF whilst 50% of the neurons were still alive in the cultures that lacked neurotrophins.

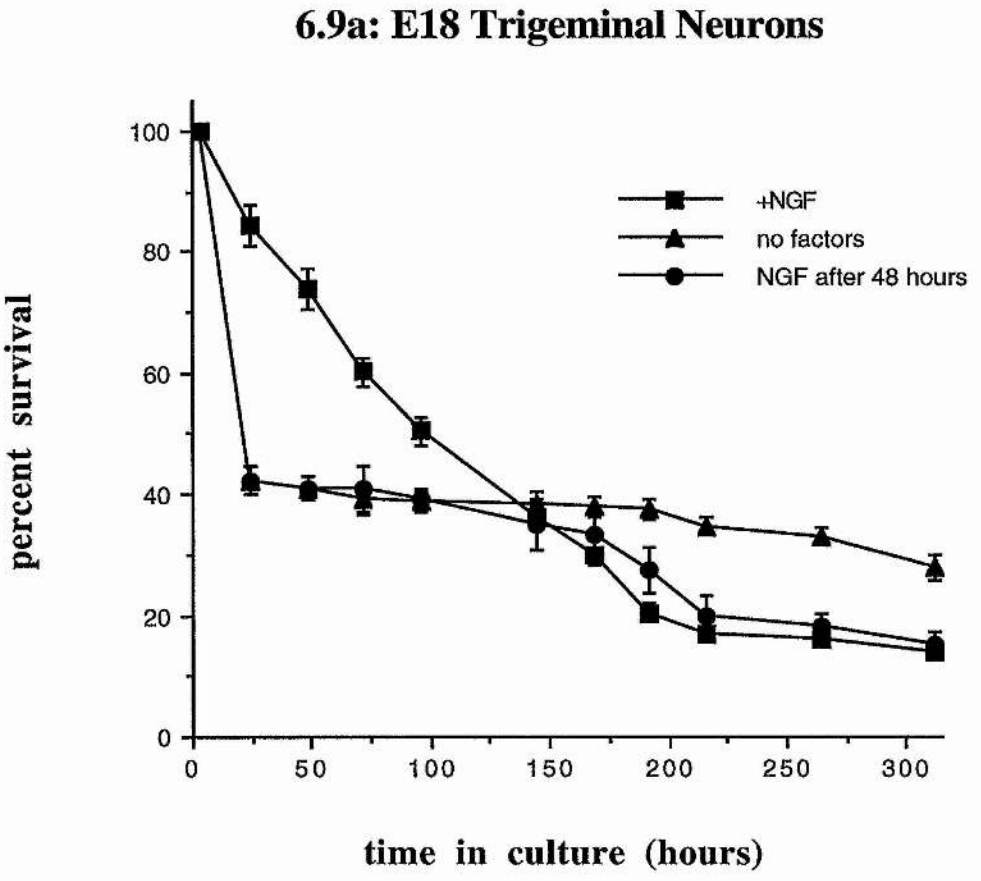


Figure 6.9a: Survival of Trigeminal Neurons derived from Bax-deficient embryos at E18 (n=5), grown for 312 hours in culture without factors, with NGF (10ng/ml) from the outset or added to the cultures after 48 hours. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

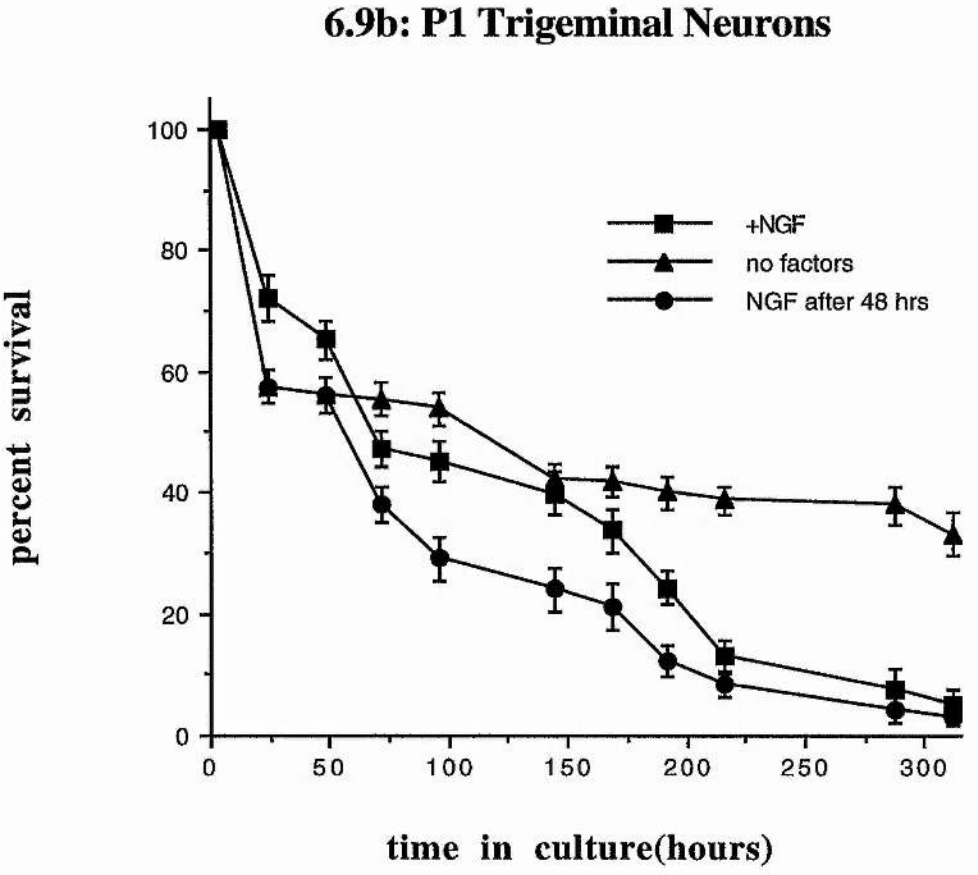


Figure 6.9b: Survival of Trigeminal Neurons derived from Bax-deficient neonates at P1 (n=7), grown for 312 hours in culture without factors, with NGF (10ng/ml) from the outset or added to the cultures after 48 hours. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of neonates. For each neonate triplicate cultures were established for all conditions studied.

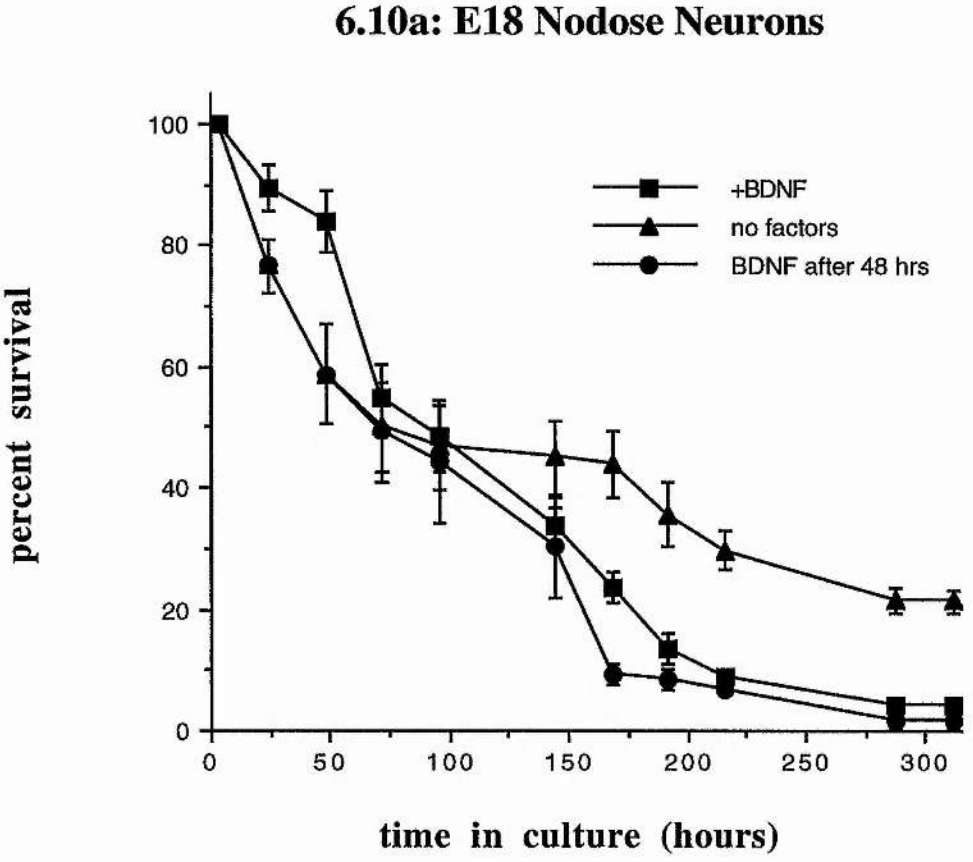


Figure 6.10a: Survival of Nodose Neurons derived from Bax-deficient embryos at E18 (n=5), grown for 312 hours in culture without factors, with BDNF (10ng/ml) from the outset or added to the cultures after 48 hours. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of embryos. For each embryo triplicate cultures were established for all conditions studied.

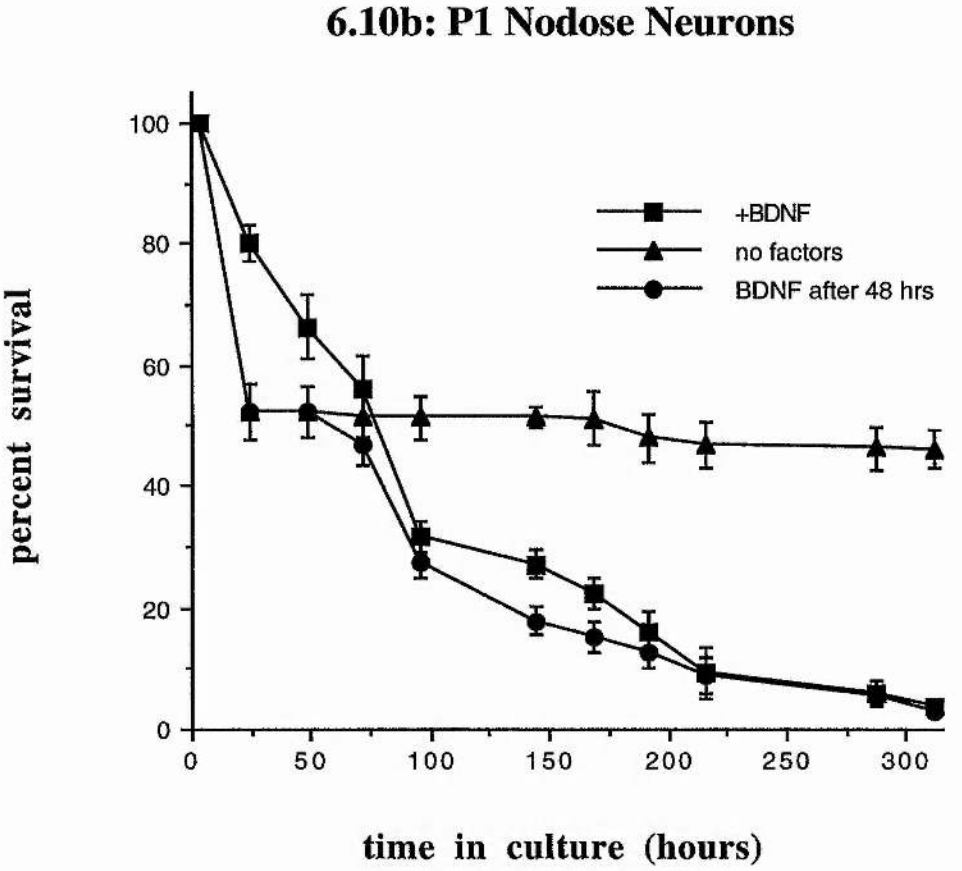


Figure 6.10b: Survival of Nodose Neurons derived from Bax-deficient neonates at P1 (n=7), grown for 312 hours in culture without factors, with BDNF (10ng/ml) from the outset or added to the cultures after 48 hours. The means and standard errors of the means are shown. Each point in the graph represents the mean of a number of neonates. For each neonate triplicate cultures were established for all conditions studied.

Effects of Neurotrophic Factors on Neurite Outgrowth of Bax-deficient Neurons

Whilst studying the cultures of Bax-deficient trigeminal and nodose neurons grown in medium not supplemented with neurotrophic factors, it was apparent that many of the neurons in these cultures did not extend long neurites compared with those cultured in the presence of neurotrophic factors. To determine whether the reduction in neurite length was a direct result of neurotrophin deficiency, the number of trigeminal and nodose neurons projecting neurites of greater than two soma diameters were counted 48, 72, and 96 hours after plating in cultures lacking neurotrophins. These data were compared to the number of trigeminal and nodose neurons projecting neurites longer than two soma diameters in cultures that lacked neurotrophic factors for the first 48 hours, but to which neurotrophins were added after this time. These data are summarised in Figures 6.11 and 6.12.

At E18, around 50% of trigeminal neurons grown without NGF for 48 hours had neurites of greater than 2 soma diameters (Fig. 6.11a). After another 48 hours in culture, this figure had increased to 60%. In cultures that were supplemented with NGF(10ng/ml) after 48 hours, over 90% of the surviving neurons had processes longer than two soma diameters by 96 hours after plating. Very similar observations were made on P1 trigeminal neurons where after 96 hours in culture without NGF less than 70% of the neurons had processes that were greater then two soma diameters (Fig. 6.11b). In cultures that were supplemented with NGF after 48 hours, 100% of the neurons had processes fulfilling this criterion by 96 hours after plating.

At E18, around 50% of nodose neurons grown without BDNF for 48 hours *in vitro* had neurites greater than two soma diameters. By 96 hours, this figure had risen to almost 70% (Fig. 6.12a). In cultures that were supplemented with BDNF (10ng/ml) after 48 hours, 100% of the neurons had processes of greater than 2 soma diameters after 96 hours incubation. P1 nodose neurons showed virtually identical

results (Fig. 6.12b).

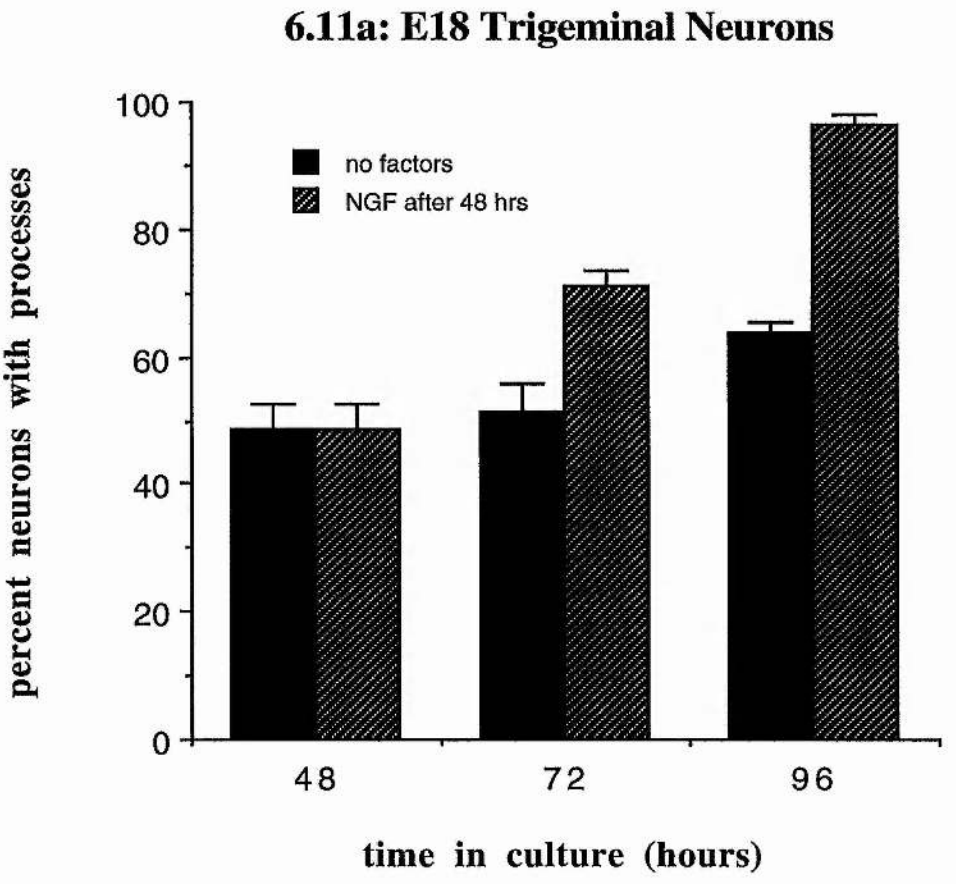


Figure 6.11a: Bar graphs of the proportion of E18 Bax-deficient trigeminal neurons bearing neurites of greater than 2 soma diameters. The means and standard errors of the means are shown. Each point in the graph represents the mean of 5 embryos. For each embryo triplicate cultures were established for all conditions studied.

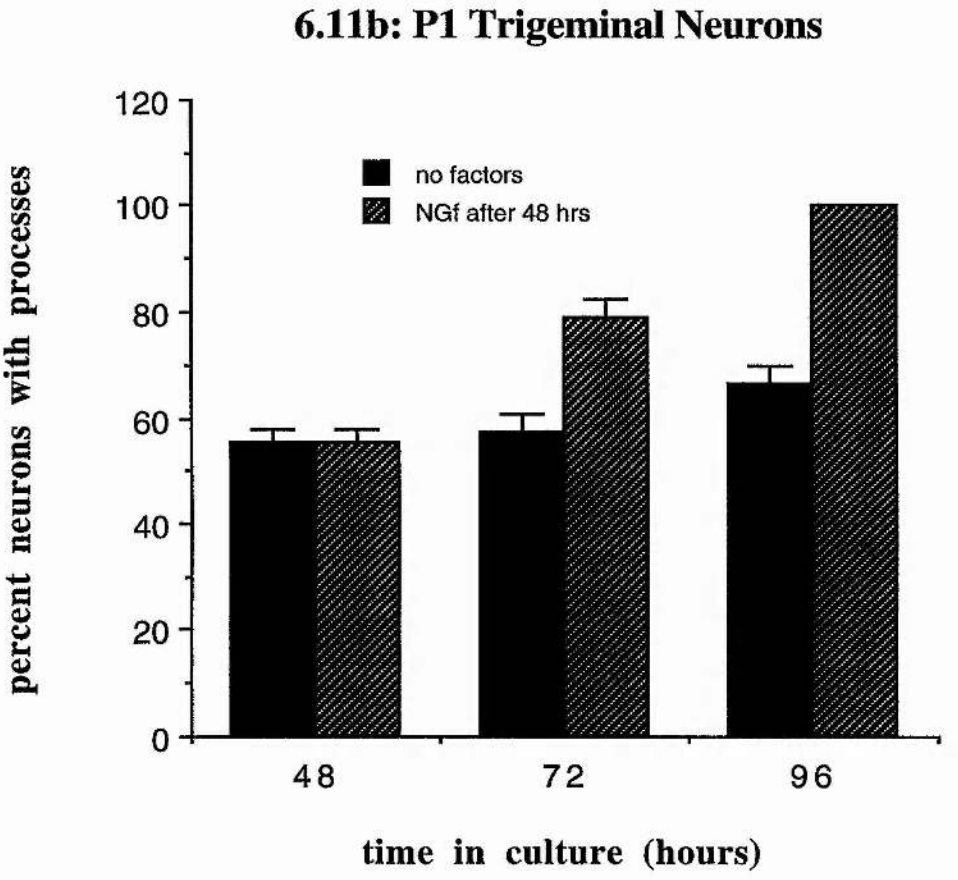


Figure 6.11b: Bar graphs of the proportion of P1 Bax-deficient trigeminal neurons bearing neurites of greater than 2 soma diameters. The means and standard errors of the means are shown. Each point in the graph represents the mean of 7 neonates. For each neonate triplicate cultures were established for all conditions studied.

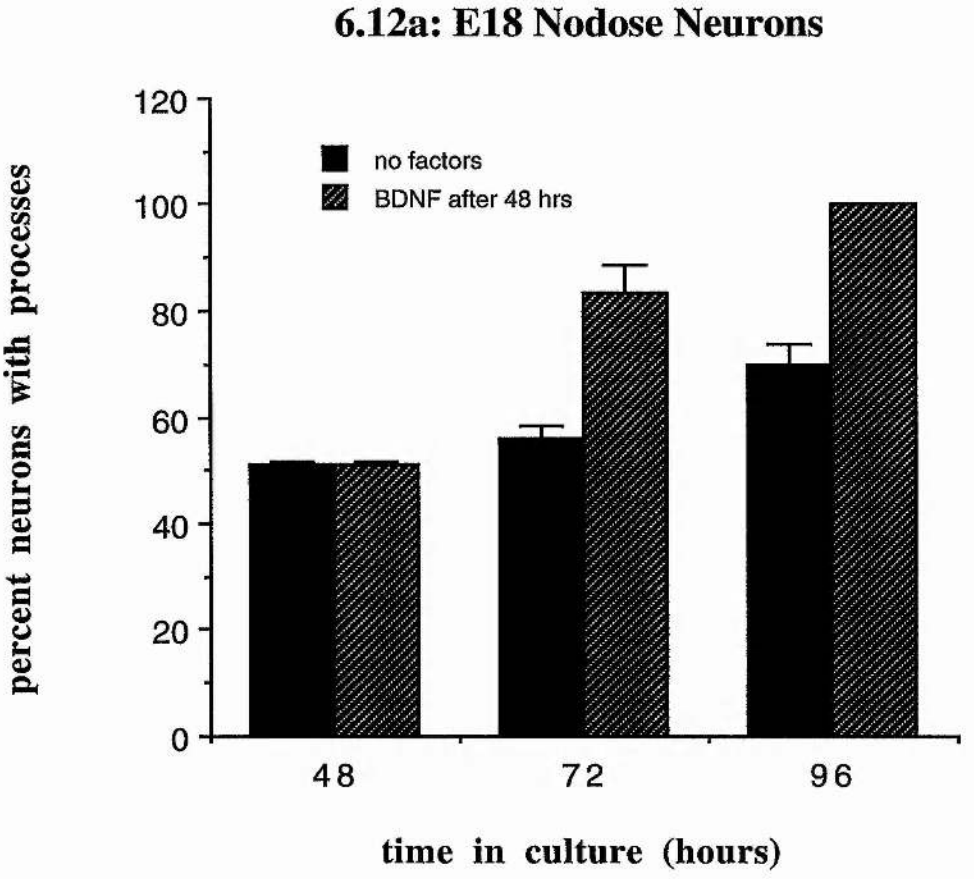


Figure 6.12a: Bar graphs of the proportion of E18 Bax-deficient nodose neurons bearing neurites of greater than 2 soma diameters. Each point in the graph represents the mean of 5 embryos. For each embryo triplicate cultures were established for all conditions studied.

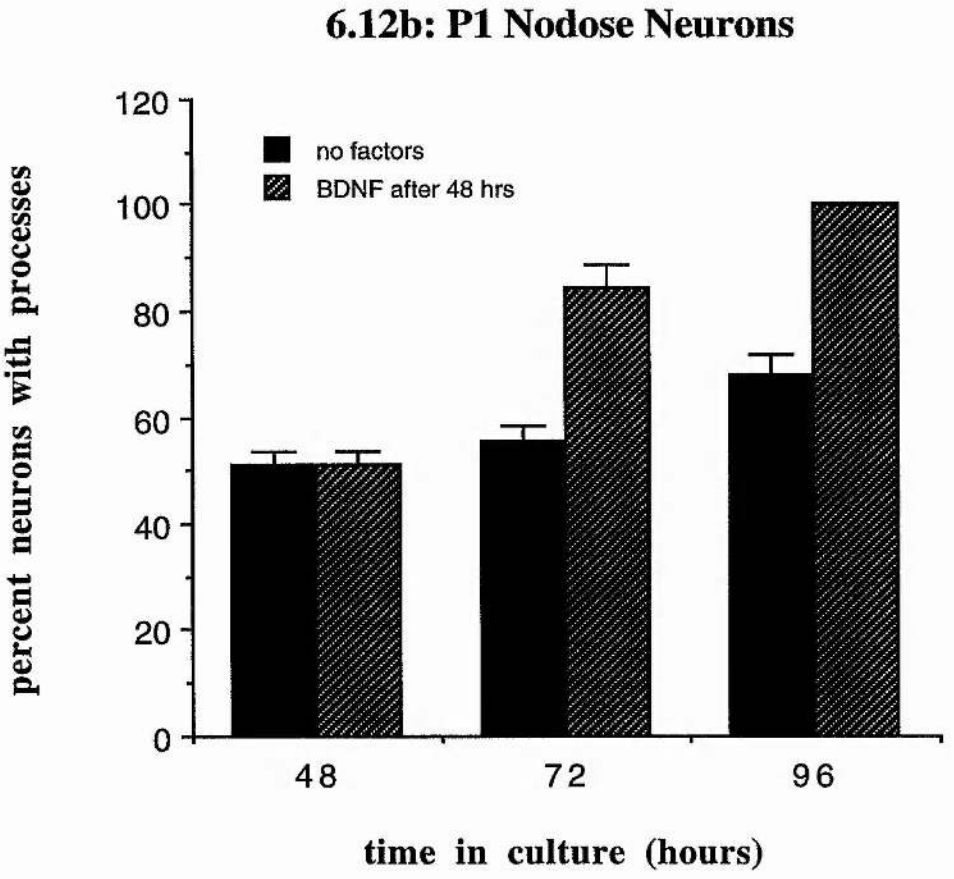


Figure 6.12b: Bar graphs of the proportion of P1 Bax-deficient nodose neurons bearing neurites of greater than 2 soma diameters. The means and standard errors of the means are shown. Each point in the graph represents the mean of 7 neonates. For each embryo triplicate cultures were established for all conditions studied.

Survival of Bax-deficient Neurons *in vivo*

Histological analysis of the number of neurons in the trigeminal ganglia of Bax-deficient embryos was carried out at E14, E16, and E18. At all ages there were significantly more neurons in the ganglia of Bax-deficient embryos (Fig. 6.13a). Furthermore, there was a marked decrease in the number of pyknotic neurons observed in Bax-deficient specimens (Fig. 6.13b). At E14, there was a 67% decrease in the number of pyknotic neurons observed in the trigeminal ganglia of Bax-deficient embryos. By E16, this difference had fallen to 48% and by E18 there was no difference observed.

This very marked decrease in pyknosis observed at E14, which is the peak of naturally occurring neuronal death in the trigeminal ganglion *in vivo,* is reflected by the fact that the greatest difference between neuronal number in the wild type and Bax-deficient trigeminal ganglia is at E16. It is perhaps worth noting that the neuronal and pyknotic counts observed were higher than those previously reported even in the wild type embryos. This does not necessarily mean that there is a problem with the quantification as this may be due to the different background strains of the mice studied.

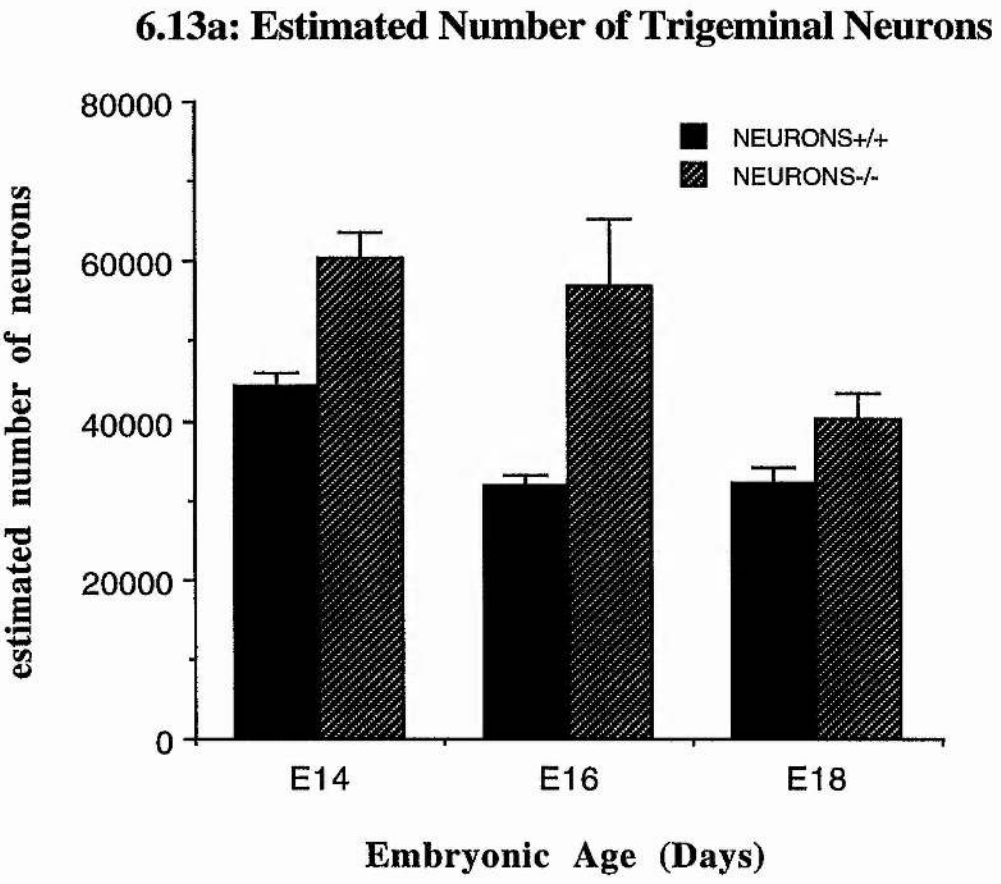


Figure 6.13a: Bar graphs showing the estimated number of neurons in the trigeminal ganglia of Bax-deficient and wild type embryos. The means and the standard errors of the means are shown. For Bax-deficient embryos, n=14 at E14, n=10 at E16, and n=17 at E18. For wild type embryos, n=18 at E14, n=10 at E16, and n=16 at E18.

6.13b: Estimated Number of Pyknotic Trigeminal Neurons

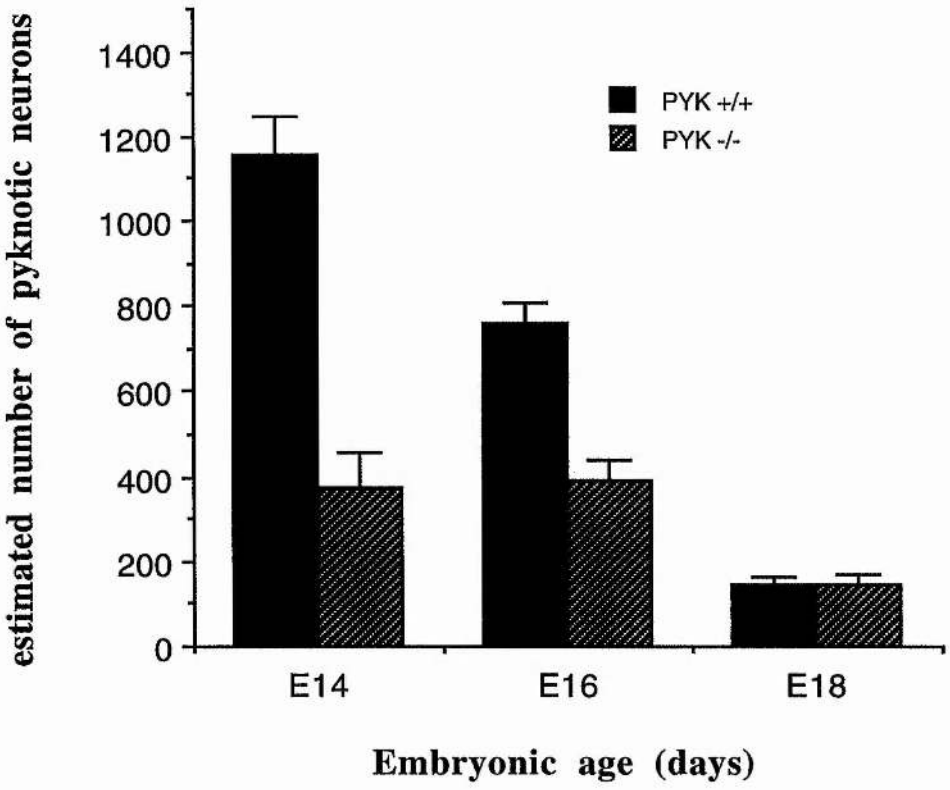


Figure 6.13b: Bar graphs showing the estimated number of pyknotic neurons in the trigeminal ganglia of Bax-deficient and wild type embryos. The means and the standard errors of the means are shown. For Bax-deficient embryos, n=14 at E14, n=10 at E16, and n=17 at E18. For wild type embryos, n=18 at E14, n=10 at E16, and n=16 at E18.

DISCUSSION

The data obtained from both *in vitro* and *in vivo* experiments using Bax null mutant mice have indicated that Bax does play a role in executing the cell death programme in at least some sensory neurons during development. However, the *in vitro* and *in vivo* data conflict with regard to the developmental period during which Bax is most important in regulating neuronal cell death. In culture, E14 Bax-deficient sensory neurons do not show significantly enhanced survival over wild type neurons, either with or without neurotrophins. However, at older ages, significantly more *bax* $^{-/-}$ sensory neurons survive in both neurotrophin supplemented and neurotrophin deficient cultures compared to wild type neurons. The importance of Bax's role in the cell death programme appears to become more pronounced with increasing age. Thus, the difference in survival between *bax*$^{+/+}$ and *bax*$^{-/-}$ neurons is greatest at P1, the oldest age studied. From the *in vitro* studies it seems likely that Bax does not play a significant part in regulating developmental neuronal cell death in the mouse trigeminal ganglion, where developmental cell death peaks at E14 and is largely over by birth (Davies & Lumsden, 1984; Pinon et al, 1996).

In contrast, it seems from the study of the developing trigeminal ganglia of *bax* $^{-/-}$ and wild type mice *in vivo* that Bax does play a significant role in the execution of developmental neuronal cell death. Large differences between the number of neurons in *bax* $^{-/-}$ and wild type trigeminal ganglia are apparent at E14 (the earliest age studied) and E16. Accordingly, the greatest reduction in the number of pyknotic nuclei in *bax*$^{-/-}$ trigeminal ganglia as compared to wild type ganglia occurs at E14, and by E18 the number of pyknotic nuclei in the ganglia of both genotypes is the same.

A number of possibilities could explain the discrepancy between the *in vitro* and *in vivo* data. For example, it is possible that the greater number of neurons in *bax* $^{-/-}$ trigeminal ganglia compared to wild type ganglia does not reflect a role for Bax in neuronal killing but rather in the programmed death of cells within the target field

that synthesise neurotrophins. Thus, in the absence of Bax, a greater number of neurotrophin synthesising cells would survive which would increase target field neurotrophin expression and this in turn, according to the neurotrophin hypothesis, would increase the number of neurons surviving the period of developmental cell death. The validity of this hypothesis could be tested by using quantitative RT-PCR to measure the relative levels of neurotrophin mRNAs in the target fields of *bax* $^{-/-}$ and wild type embryos.

A number of different stimuli apart from growth factor deficiency can induce apoptosis. Cell death induced by different stimuli may not be executed in the same way using the same death promoting molecules. It is possible that in the case of sensory neurons the process of dissection, trypsinisation, trituration and plating in defined medium activates a cell death programme that involves other pro-apoptotic members of the Bcl-2 family, such as Bcl-x_S and/or Bad, or alternatively a mechanism not involving members of the Bcl-2 family. This may explain why cell death of E14, and to a certain extent E16, trigeminal neurons appears to be Bax-independent *in vitro*, but Bax dependent *in vivo*.

The presence of non-neuronal cells in culture may provide one possible explanation to account for the increased survival of older *bax*$^{-/-}$ sensory neurons *in vitro* at a time when there is little difference in the survival of *bax*$^{-/-}$ and *bax* $^{+/+}$ neurons *in vivo*. It is possible that the absence of a functional *bax* gene not only increases the numbers of neurons within sensory ganglia but also increase numbers of certain types of non-neuronal cells within the ganglia. Because of technical difficulties it was not possible to separate neurons from non-neuronal cells in cultures established from individual embryos. As a result, there may have been higher numbers of non-neuronal cells in the cultures from Bax-deficient animals, particularly at older embryonic ages. Thus, the number and identity of non-neuronal cells in cultures from older *bax* $^{-/-}$ sensory ganglia may be markedly different to that in cultures from wild type ganglia of the same age. Ganglionic non-neuronal cells in culture

synthesise a number of growth promoting molecules including trophic factors and extracellular matrix proteins. It is therefore possible that the extended survival of a subpopulation of *bax*$^{-/-}$ neurons in the absence of added trophic factor may, in part, be due to the production of trophic molecules by a population of non-neuronal cells that are particularly augmented in *bax*$^{-/-}$ cultures.

Another curiosity of the tissue culture data presented in this chapter is that survival of *bax* $^{-/-}$ neurons is greater in the absence of neurotrophic factor than in the presence of neurotrophic factors. Most surprisingly, the addition of neurotrophic factor to cultures of *bax*$^{-/-}$ neurons that had previously been cultured without neurotrophins accelerates cell death. One possible explanation for this latter phenomenon is that during culture in the presence of neurotrophins trk receptors are down-regulated and/or p75 neurotrophin receptors are up-regulated. Much evidence now exists to suggest that binding of neurotrophins to p75 in the absence of the appropriate high affinity trk receptor can induce apoptosis in at least some cell types (Rabizeh et al., 1993; Barrett & Bartlett, 1994; Casaccia-Bonnefil et al., 1996; Farde et al., 1996). Further work, in particular to determine what happens to the expression of trk and p75 receptors in prolonged culture, both in the presence and absence of neurotrophins, will be needed to determine whether this is indeed the case.

Previously published data have suggested that the levels of Bcl-2 and Bax expressed by neurons are important in determining whether neurons live or die (Allsopp et al., 1993, 1995; Martinou et al, 1993, Garcia et al, 1992; Vekrellis et al., 1997). However, the data presented in this chapter clearly show that the Bax:Bcl-2 rheostat model (Oltvai et al., 1993) cannot entirely account for the regulation of neuronal survival in all sensory neurons at all stages of development. For example, the *in vitro* data clearly shows that older sensory ganglia contain two populations of neurons. One population shows greatly enhanced survival in the absence of Bax, as would be expected according to the Bax: Bcl-2 rheostat model. However, a second

population of neurons also exists that dies rapidly irrespective of whether Bax is expressed or not. Moreover, although *bax* $^{-/-}$ trigeminal ganglia show reduced numbers of pyknotic nuclei at E14 and E16 compared to wild type ganglia, a considerable number of pyknotic nuclei are still present, indicating that the absence of Bax does not entirely prevent developmental neuronal death. The existence of Bcl-2/Bax independent mechanisms that regulate neuronal survival is also suggested from the data presented in Chapter 4 of this thesis. Although Bcl-2 null mutant embryos contain fewer trigeminal neurons than wild type embryos at E16 and E18, the majority of neurons still survive the period of neuronal cell death. It would be interesting to determine whether the phenotype of the sensory neuronal sub-population lost in the Bcl-2 knockout is the same as that of the neuronal population whose numbers are increased in the Bax knockout. The survival of those neurons whose fate does not appear to depend upon Bcl-2 and Bax expression may be regulated by other pro- and anti-apoptotic members of the Bcl-2 family, or by another unrelated mechanism. Analysis of transgenic animals containing single and double null mutations of other Bcl-2 family members could resolve this question.

One should be cautious in interpreting the results of experiments involving transgenic animals. It is not known to what extent, if any, the expression of Bcl-2 family members in any one cell type are linked and to what extent there is overlap and redundancy amongst members of the Bcl-2 family. For example, if Bax expression is down-regulated or abolished in neurons, does the expression of other pro-apoptotic members of the Bcl-2 family increase to compensate, or does the level of Bcl-2 in neurons decrease to try and maintain the correct Bax:Bcl-2 ratio? If this were the case then it could obscure the true function and/or importance of the gene under investigation. The number of neurons that normally die by a Bax-dependent mechanism may be much greater than that suggested by the neuronal counts in trigeminal ganglia of E14 and E16 Bax knockout embryos. Perhaps in the absence of Bax other pro-apoptotic members of the Bcl-2 family take over its

function in many neurons, possibly after compensatory up-regulation of their expression, so that the density of target field innervation is maintained near normal. Further studies at the molecular level will be required to determine whether the of expression of bcl-2 family members are co-ordinately regulated.

Finally, another interesting observation to emerge from the study of sensory neurons from bax null mutant embryos is that bax-deficient neurons cultured in the absence of neurotrophic factors show reduced neurite length compared to those grown with neurotrophins. Some of the earliest studies on the prototypical neurotrophin, NGF, showed that it could enhance neurite outgrowth from sympathetic neurons. However, since sympathetic neurons are critically dependent on NGF for survival it was not entirely clear whether enhancement of neurite outgrowth is a result of general increased vitality or whether NGF specifically regulates process outgrowth. To date, little data have emerged on ability of other neurotrophins to regulate process outgrowth. Since a proportion of bax-deficient sensory neurons survive in the absence of neurotrophins, they could provide a good model system to investigate the role that neurotrophins play in regulating neurite outgrowth in this neuronal type.

REFERENCES

REFERENCES

Abe, D.S., Harada, N., Yamada, K., & Tanaka, R. (1993). Bcl-2 gene is highly expressed during neurogenesis in the central nervous system. Biochem Biophys Res Commun, 191(3): 915-21.

Adler, R., Landa, K.B., Manthorpe, M., & Varon, S. (1979). Cholinergic neuronotrophic activity for ciliary neurons. Science 1204(4400): 1434-1436.

Alberi S, Raggenbass M, de Bilbao F, & Dubois-Dauphin M (1996). Axotomize neonatal motoneurons overexpressing the bcl2 proto-oncogene retain functional electrophysiological properties. Proc Natl Acad Sci USA, 93(9): 3978-3983

Albers, K.M., Perrone, T.N., Goodness, T.P., Jones, M.E., Green, M.A.,& Davis, B.M. (1996). Cutaneous overexpression of NT-3 increases sensory and sympathetic neurons number and enhances touch dome and hair follicle innervation. J. Cell Biol., 134(2): 487-497.

Albers, K.M., Wright, D.E., & Davis, B.M. (1994). Overexpression of nerve growth factor in epidermis of transgenic mice causes hypertrophy of the peripheral nervous system. J Neurosci. 14(3): 1422-32.

Alderson, R.F., Alterman, A.L., Barde, Y.A., & Lindsay, R.M. (1990). Brain-derived neurotrophic factor increases survival and differentiated functions of rat septal cholinergic neurons in culture. Neuron, 5(3): 297-306.

Allsopp, T.E., Kiselev, S., Wyatt, S., & Davies, A.M. (1995). Role of Bcl-2 expression in the BDNF survival response. Euro. J. Neurosci.7(6): 1266-72

Allsopp, T.E., Wyatt, S., Paterson, H.F., & Davies, A.M. (1993). The proto-oncogene bcl-2 can selectively rescue neurotrophic factor-dependent neurons from apoptosis. Cell, 73(2): 295-307.

Alnemri, E.S., Robertson, N.M., Ternandes, T.F., Croce, C.M., & Litwack, G. (1992). Overexpressed full-length human BCL2 extends the survival of baculvirus-infected Sf9 insect cells. Proc. Natl. Acad. Sci. USA, 89:7295-7299.

Altman, J. (1992). Programmed cell death: the paths to suicide [news]. Trends Neurosci, 15(8): 278-80.

Angeletti, R.H., & Bradshaw, R.A. (1971). Nerve growth factor from mouse submaxillary gland: amino acid sequence. Proc. Natl. Acad. Sci. USA 68: 2417-20

Apte S.S, Mattei M.G, & Olsen B.R. (1995). Mapping of the human BAX gene to chromosome 19q13.3-q13.4 and isolation of a novel alternatively spliced transcript, BAX delta. Genomics 26(3): 592-594

Arakawa, Y., Sendtner, M., & Thoenen, H. (1990). Survival effect of ciliary neurotrophic factor (CNTF) on chick embryonic motoneurons in culture: comparison with other neurotrophic factors and cytokines. J Neurosci, 10(11): 3507-15.

Ard, M.D., & Morest, D.K. (1984). Cell death during the development of the vestibular and cochlear ganglia of the chick. Int. J. Devel. Neurosci., 2: 535-170.

Averbuch, H.L., Pruginin, M., Kahane, N., Tsoulfas, P., Parada, L., Rosenthal, A., & Kalcheim, C. (1994). Neurotrophin 3 stimulates the differentiation of motoneurons from avian neural tube progenitor cells. Proc Natl Acad Sci U S A, 91(8): 3247-51.

Barbin, G., Manthorpe, M., & Varon, S. (1984). Purification of the chick eye ciliary neuronotrophic factor. J Neurochem, 43(5): 1468-78.

Barde, Y.A. (1989). Trophic factors and neuronal survival. Neuron, 2(6):1525-34.

Barde, Y.A., Davies, A.M., Johnson, J.E., Lindsay, R.M., & Thoenen, H. (1987). Brain derived neurotrophic factor. Prog Brain Res, 71(1):185-9.

Barde, Y.A., Edgar, D., & Thoenen, H. (1982). Purification of a new neurotrophic factor from mammalian brain. EMBO J., 1:549-553.

Barker, P.A., Lomen-Hoerth, C., Gensch, E.M., Meakin, S.O., Glass, D.J., & Shooter, E.M. (1993). Tissue-specific alternative splicing generates two isoforms of the *trk*A receptor. J. Biol. Chem., 268: 15150-15157.

Barres, B.A., Raff, M.C., Gaese, F., Bartke, I., Dechant, G., & Barde, Y. (1994). A crucial role for neurotrophin-3 in oligodendrocyte development. Nature, 367: 371-375.

Barrett, G.L., & Bartlett, P.F. (1994). The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. Proc. Natl. Acad. Sci. USA, 91(14): 6501-6505.

Batistatou, A., Merry, D.E., Korsmeyer, S.J., & Greene, L.A. (1993). Bcl-2 affects survival but not neuronal differentiation of PC12 cells. J. Neurosci., 13(10): 4422-4428.

Bazan, J.F. (1991). Neuropoietic cytokines in the hematopoietic fold. Neuron, 7(2): 197-208.

Beck, K.D., Valverde, J., Alexi, T., Poulsen, K., Moffat, B., Vandlen, R.A., Rosenthal, A., & Hefti, F. (1995). Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. Nature, 373: 339-341.

Behl, C., Hovey, L.3., Krajewski, S., Schubert, D., & Reed, J. C. (1993). BCL-2 prevents killing of neuronal cells by glutamate but not by amyloid beta protein. Biochem Biophys Res Commun, 197(2):949-56.

Benedetti, M., Levi, A., & Chao, M. V. (1993). Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. Proc. Natl. Acad. Sci. U S A, 90(16): 7859-7863.

Benito A, Silva M, Grillot D, Nunez G, Fernandez-Luna JL (1996). Apoptosis induced by erythroid differentiation of human leukemia cell lines is inhibited by Bcl-XL. Blood 87(9):3837-3843

Berger & Shooter (1977). Evidence for pro-β-nerve growth factor, a biosynthetic precursor to β–nerve growth factor. Pro. Natl. Acad. Sci. USA 74:3647-3651

Berkemeier, L.R., Winslow, J.W., Kaplan, D.R., Nikolics, K., Goeddel, D.V. & Rosenthal, A. (1991). Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. Neuron 7: 857-866.

Bissonnette, R. P., Echeverri, F., Mahboubi, A., & Green, D. R. (1992). Apoptotic cell death induced by c-myc is inhibited by bcl-2. Nature, 359(6395): 552-4.

Bockman D.E. & Kirby M.L. (1984). Dependence of thymus development on derivatives of neural crest. Science 223: 498-500.

Boise, L.H., Gonzalez, G.M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A. Mao, X., Nunez, G., & Thompson, C.B. (1993). bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell, 74(4): 597-608.

Borner, C. (1996). Diminished cell proliferation associated with the death-protective activity of Bcl-2. J. Biol. Chem 271(22):12695-12698

Borner, C., Martinou, I., Mattmann, C., Irmler, M., Schaerer, E., Martinou, J.I & Tschopp, J. (1994). The protein bcl-2 alpha does not require membrane attachment, but two conserved domains to suppress apoptosis. J. Cell Biol. 126(4): 1059-1068.

Bothwell, M. (1991). Tissue localization of nerve growth factor and nerve growth factor receptors. Curr Top Microbiol Immunol, 165(1): 55-70.

Boyd, J.M, Galo, G.J, Elangovan, B, Houghton, A.B, Malstrom, S, Avery, B.J, EBB, R.G, Subramanian, T, Chittenden, T & Lutz, R.J. (1995). Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. Oncogene 11(9):1921-1928

Brady, H.J, Gil-Gomez, G, Kirberg, J & Berns, A.J. (1996). Bax alpha perturbs T cell development and affects cell cycle entry of T cells. EMBO J 15(24): 6991-7001

Brady, M.J., & Palfrey, H. C. (1993). Rapid and sustained phosphorylation of a calmodulin-binding protein (CaM-BP100) in NGF-treated PC12 cells. J Biol Chem, 268(24): 17951-8.

Buchman, V.L., & Davies, A.M. (1993). Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons. Development, 118(3): 989-1001.

Buj-Bello, A., Buchman, V.L., Horton, A., Rosenthal, A., & Davies, A.M. (1995). GDNF is an age-specific survival factor for sensory and autonomic neurons. Neuron, 15: 821-828.

Buttke, T.M., & Sandstrom, P.A. (1994). Immunol. Today, 15: 7-10.

Carr, V.M., & Simpson, S.B. (1978). Proliferative and degenerative events in the early development of chick dorsal root ganglia. II. Responses to altered peripheral fields. J. Comp. Neurol. 182: 741-756.

Casaccia-Bonnefil, P., Carter, B.D., Dobrowsky, R.T., & Chao, M.V. (1996). Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. Nature, 383: 716-719.

Chao M. (1992). Neurotrophin receptors: A Window into neuronal differentiation. Neuron 9: 583-93.

Chao, M. (1994). The p75 neurotrophin receptor. J. Neurobiol., 25: 1373-1385.

Chao, M.V., Bothwell, M.A., Ross, A.H., Koprowski, H., Lanahan, A.A., Buck, C.R., & Sehgal, A. (1986). Gene transfer and molecular cloning of the human NGF receptor. Science, 232: 518-521.

Chapman, B.S. (1995). A region of the 75 kDa neurotrophin receptor homologous to the death domains of TNFR-I and Fas. FEBS Lett, 374: 216-220.

Chen, D.F., Schneider, G.E., Martinou, J.C., & Tonegawa, S. (1997). Bcl-2 promotes regeneration of severed axons in mammalian CNS. Nature, 385: 434-439.

Cheng E.H, Levine B, Boise L.H, Thompson C.B, Hardwick J.M (1996). Bax-independent inhibition of apoptosis by Bcl-XL. Nature 379(6565):554-556

Chen-Levy. Z., & Cleary, M.L. (1990). Membrane topology of the Bcl-2 proto-oncogenic protein demonstrated in vitro. J Biol Chem, 265(9): 4929-33.

Chiou, S.K., Rao, L., & White, E. (1994). Bcl-2 blocks p53-dependent apoptosis. Mol Cell Biol, 14(4): 2556-63.

Chittenden, T., Harrington, E.A., O'Connor, R., Flemington, C., Lutz, R.J., Evan, G.I., & Guild, B.C. (1995). Induction of apoptosis by the Bcl-2 homologue Bak. Nature, 374: 733-736.

Choi S.S., Prk S.H, Kim U.J, & Shin H.S (1997). Bfl-1, a Bcl-2-related gene, is the human homolog of the murine A1, and mas to chromoscome 15q24.3 Mamm Genome 8(10):781-782

Chun, L.L., & Patterson, P.H. (1977). Role of nerve growth factor in the development of rat sympathetic neurones in vitro. II. Developmental studies. J. Cell Biol.75: 705-711.

Clarke, P.G.H. (1990) Developmental cell death: morphological diversity and multiple mechanisms. Anat. Embryol 181: 195-213.

Clary, D.O., & Reichardt, L.F. (1994). An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin-3. Proc. Natl. Acad. Sci. USA, 91: 11133-11137.

Claude P, Parada I.M, Gordon K.A, D'Amore P.A, Wagner J.A (1988). Acidic fibroblast growth factor stimulates adrenal chromaffin cells to proliferate and to extend neurites, but is not a long-term survival factor. Neuron 1(9):783-790

Cohen, S. (1960). Purification of nerve growth factor-promoting protein from the mouse salivary gland and its neurocytotoxic antiserum. Proc. Natl. Acad. Sci. 46.: 302-311.

Conover, J.C., Erickson, J.T., Katz, D.M., Bianchi, L.M., Poueymirou, W.T., McClain, J., Pan, L., Helgren, M., Ip NY, & P., B. (1995). Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. Nature 375: 235-238.

Copp, A.J., Brook P.A., & Estibeiro J.P. (1990). The Embryonic Development of Mammalian Neural Tube Defects. Progress in Neurobiology 35:363-403.

Cowan, W.M., Fawcett, J.W., & O'Leary, D.D., & Stanfield, B.B. (1984). Regressive events in neurogenesis. Science, 225(4668): 1258-65.

Crowley, C., Spencer, S.D., Nishimura, M.C., Chen, K.S., S., P.-M., Armanini, M.P., Ling, L.H., McMahon, S.B., Shelton, D.L., Levinson, A.D., & Phillips, H.S. (1994). Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell, 76(6): 1001-1011.

Cruz-Reyes J, & Tata J.R (1995). Cloning, Characterization and expression of two Xenopus bcl-2-like cell-survival genes. Gene 1995 158(2):171-179

Cuende, E., Ales, M.J., Ding, L., Gonzalez, G.M., Martinez, C., & Nunez, G. (1993). Programmed cell death by bcl-2-dependent and independent mechanisms in B lymphoma cells. Embo J, 12(4): 1555-60.

D'Amico-Martel A .(1982). Temporal patterns of neurogenesis in avian cranial sensory and autonomic ganglia. Am J Anat 163(4):351-372

Davies, A.M. (1986). The survival and growth of embryonic proprioceptive neurons is promoted by a factor present in skeletal muscle. Dev Biol, 115(1): 56-67.

Davies, A.M. (1987a). Molecular and cellular aspects of patterning sensory neurone connections in the vertebrate nervous system. Development, 101(2): 185-208.

Davies, A.M. (1987b). The growth rate of sensory nerve fibres in the mammalian embryo. Development, 100(2): 307-311.

Davies, A.M. (1988a). Neurotrophic factor bioassay using dissociated neurons. In R. Rush (Ed.), Nerve Growth Factors (pp. 95-109).

Davies, A.M. (1988b). Role of neurotrophic factors in development. Trends Genet, 4(5): 139-43.

Davies, A.M. (1988c). The emerging generality of the neurotrophic hypothesis Trends Neurosci, 11(6): 243-4.

Davies, A.M. (1988d). The trigeminal system: an advantageous experimental model for studying neuronal development. Development, 103(1): 175-83.

Davies, A.M. (1994a). Intrinsic programmes of growth and survival in developing vertebrate neurons. Rends Neurosci.17: 195-199.

Davies, A.M. (1994b). Switching neurotrophin dependence. Curr. Biol.4: 273-276.

Davies, A.M. (1994c). The role of neurotrophins during successive stages of sensory neuron development. Prog. Growth Factor Res.5: 263-289.

Davies, A.M. (1995). The Bcl-2 family of proteins and the regulation of neuronal survival. Trends Neurosci.18: 355-358.

Davies, A.M., Bandtlow, C., Heumann, R., Korsching, S., Rohrer, H., & Thoenen, H, (1987b). Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. Nature, 326: 353-358.

Davies, A.M., Lee, K.F., & Jaenisch, R. (1993). p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. Neuron, 11 (4): 565-74.

Davies, A. M., & Lindsay, R. M. (1985). The cranial sensory ganglia in culture: Differences in the response of placode-derived and neural crest-derived neurons to nerve growth factor. Dev. Biol.111: 62-72.

Davies, A.M., & Lumsden, A.G. (1986). Fasciculation in the early mouse trigeminal nerve is not ordered in relation to the emerging pattern of whisker follicles. J. Comp. Neurol.253(1): 13-24.

Davies, A.M. & Lumsden, A.G.S. (1984). Relation of target encounter and neuronal death to nerve growth factor responsiveness in the developing mouse trigeminal ganglion. J. Comp. Neurol.,223(1): 124-137.

Davies, A.M., Lumsden, A.G., & Rohrer, H. (1987a). Neural crest-derived proprioceptive neurons express nerve growth factor receptors but are not supported by nerve growth factor in culture. Neuroscience, 20(1): 37-46.

Davies, A.M., Thoenen, H., & Barde, Y.A. (1986a). Different factors from the central nervous system and periphery regulate the survival of sensory neurones. Nature, 319(6053): 497-499.

Davies, A.M., Thoenen, H., & Barde, Y.A. (1986b). The response of chick sensory neurons to brain-derived neurotrophic factor. J. Neurosci.6(7): 1897-1904.

Davies, A.M, Wyatt, S, Nishimura & M, Phillips, H (1995). NGF receptor expression in sensory neurons develops normally in embryos lacking NGF. Dev Biol 171(2): 434-438

Davis, S., & Yancopoulos, G.D. (1993). The molecular biology of the CNTF receptor. Curr Opin Neurobiol,3(1): 20-4.

Deckwerth, T.L, Elliott, J.L, Knudson, C.M, Johnson, E.M Jr, Snider, W.D & Korsmeyer, S.J. (1996). BAX is required for neuronal death after trophic factor deprivation and during development. Neuron 17(3) 401-411

DeGregori, J, Leone, G, Ohtani, K, Miron, A & Nevins, J.R. (1995). E2F-1 accumulation bypasses a G1 arrest resulting from the inhibition of G1 cyclin-dependent kinase activity. Genes Dev. 9(23): 2873-2887

DiCicco-Bloom, E., Friedman, W.J., & Black, I.B. (1993). NT-3 stimulates sympathetic neuroblast proliferation by promoting precursor cell survival. Neuron,11: 1101-1111.

DiStefano, P.S., & Johnson, E.M.J. (1988). Identification of a truncated form of the nerve growth factor receptor. Proc Natl Acad Sci U S A, 85(1): 270-274.

Dobrowsky, R.T., Werner, M.H., Castellino, A.M., Chao, M.V. & Hannun, Y.A. (1994). Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science 265: 1596-9.

Dole, M, Nunez, G, Merchant, A.K, Maybaum, J, Rode, C.K, Bloch, C.A, Castle, V.P (1994). Bcl-2 inhibits chemotherapy-induced apoptosis in neuroblastoma. Cancer Res. 54(12): 3253-3259

D'Sa-Eipper, C, Subramanian, T & Chinnadurai, G (1996). bfl-1, a bcl-2 homologue, suppresses p53-induced apoptosis and exhibits potent cooperative transforming activity. Cancer Res. 56(17): 3879-3882.

Duan H, Chinnaiyan A.M, Hudson P.L, Wing J.P, He W.W. & Dixit V.M (1996). ICE-LAP3, a novel mammalian homologue of the Caenorhabditis elegans cell death protein Ced-3 is activated during Fas- and tumor necrosis factor-induced apoptosis. J. Biol. Chem. 271(3):1621-1625

Dubois-Dauphin, M., Frankowski, H., Tsujimoto, Y., Huarte, J., & Martinou, J.C. (1994). Neonatal motoneurons overexpressing the bcl-2 protooncogene in transgenic mice are protected from axotomy-induced cell death. Proc. Natl. Acad. Sci. USA, 91(8): 3309-3313.

Dwyer, J. (1993). The Body at War. How our Immune System Works. The Orion Publishing Group.

Ebendal, T. (1992). Function and evolution in the NGF family and its receptors J Neurosci Res, 32(4): 461-70.

Ebendal, T., Larhammar, D., & Persson, H. (1986). Structure and expression of the chicken beta nerve growth factor gene. Embo J, 5(7): 1483-7.

Eckenstein, F.P., Esch, F., Holbert, T., Blacher, R.W., & Nishi, R. (1990). Purification and characterization of a trophic factor for embryonic peripheral neurons: comparison with fibroblast growth factors. Neuron, 4(4): 623-31.

Edwards, R.H., Selby, M.J., Mobley, W.C., Weinrich, S.L., Hruby, D.E., & Rutter, W.J. (1988). Processing and secretion of nerve growth factor: expression in mammalian cells with a vaccinia virus vector. Mol Cell Biol, 8(6): 2456-64.

Eguchi, Y., Ewert, D.L., & Tsujimoto, Y. (1992). Isolation and characterization of the chicken bcl-2 gene: expression in a variety of tissues including lymphoid and neuronal organs in adult and embryo. Nucleic Acids Res, 20(16): 4187-92.

Ellis, R.E., & Horvitz, H.R. (1991). Two C. elegans genes control the programmed deaths of specific cells in the pharynx. Development, 112(2): 591-603.

Ellis R.E., Yuan J.Y, & Horvitz H.R .(1991). Mechanisms and functions of cell death. Annu. Rev. Cell Biol. 7: 663-698.

Erickson, J.T., Conover, J.C., Borday, V., Champagnat, J., Barbacid, M., Yancopoulos, G. & Katz, D.M. (1996). Mice lacking brain-derived neurotrophic factor exhibit visceral sensory neuron losses distinct from mice lacking NT4 and display a severe developmental deficit in control of breathing. J. Neurosci. 16: 5361-5371.

Ernfors, P., Lee, K.F., & Jaenisch, R. (1994). Mice lacking brain-derived neurotrophic factor develop with sensory deficits. Nature, 368(6467): 147-150.

Ernfors, P., Merlio, J., & Persson, H. (1992). Cells expressing mRNA for neurotrophins and their receptors during embryonic rat development. Euro. J. Neurosci., 4: 1140-1158.

Ernfors, P., Wetmore, C., Olson, L., & Persson, H. (1990). Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. Neuron, 5(4): 511-26.

Esch F, Ueno N, Baird A, Hill F, Denoroy L, Ling N, Gospodarowicz D & Guillemin R (1985). Primary structure of bovine brain acidic fibroblast growth factor (FGF). Biochem. Biophys Res Commun 133(2):554-562.

Evan, G.I., Wyllie A.H., Gilbert C.S. (1992). Induction of apoptosis in fibroblasts by c-myc protein. Cell 69:119-28.

Fang W, Rivard J.J, Mueller D.L, Behrens T.W (1994). Cloning and molecular characterization of mouse bcl-x in B and T lymphocytes. J. Immunol 153(10): 4388-4398.

Fanidi, A., Harrington, E. .A., & Evan, G. I.. (1992). Cooperative interaction between c-myc and bcl-2 proto-oncogenes. Nature, 359(6395): 554-6.

Farde, J.M., Rodriguez-Tebar, A. & Barde, Y.A. (1996). Induction of cell death by endogenous nerve growth factor through its p75 receptor. Nature 383: 166-168.

Farinas, I., Jones, K. R., Backus, C., Wang, X. Y., & Reichardt, L. F. (1994). Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. Nature, 369(6482): 658-661.

Farlie, P.G., Dringen, R., Rees, S.M., Kannourakis, G., & Bernard, O. (1995). bcl-2 transgene expression can protect neurons against developmental and induced cell death. Proc. Natl. Acad. Sci. USA, 92: 4397-4401.

Farrow, S.N., White, J.H. M., Marinou, I., Raven, T., Pun, K.-T., Grinham, C.J., Martinou, J.-C., & Brown, R. (1995). Cloning of a *bcl-2* homologue by interaction with adenvirus E1B 19K. Nature, 374: 713-733.

Faucheu, C., Diu, A., Chan, A.W, Blanchet, A.M., Miossec, C., Herve, F, Collard-Dutilleul, V., Gu, Y., Aldape, R.A., Lippke J.A. (1995). A novel human protease similar to the interleukin-1 beta converting enzyme induces apoptosis in transfected cells. EMBO J 14(9): 1914-1922.

Fernandez-Alnemri, T., Litwack, G., & Alnemri, E.S. (1994). CPP32, a novel human apoptotic protein with homology to Caenorhabditis elegans cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. J. Biol. Chem. 269: 30761-64.

Fernandez-Alnemri, T., Litwack, G. & Alnemri, E.S. (1995a). Mch-2, a new member of the apoptotic CEO-3/ICE cysteine protease gene family. Cancer Res. SS: 2737-42.

Fernandez-Alnemri,T., Takahashi, A., Armstrong, R., Krebs, J., Fritz, L, Tomaselli, K.J., Wang, L., Yu, Z., Croce, C.M., Salveson, G., Earnshaw, W.C., Litwack, G. & Alnemri E.S. (1995b). Mch-3, a novel human apoptotic cysteine protease highly related to cpp-32. Cancer Res. SS:6045-52.

Fernadez-Sarabia, M., & Bischoff, J.R. (1993). Bcl-2 associates with the *ras*-related protein R-*ras* p23. Nature, 366: 274-275.

Field, S.J, Tsai, F.Y, Kuo, F., Zubiaga, A.M, Kaelin, W.G Jr, Livingston, D.M, Orkin, S.H. & Greenberg, M.E., (1996). E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell 85(4): 549-561.

Folkman, J. & Klagsburn, M. (1987). Angiogenic factors. Science 235(4787): 442-447.

Friedman, W.J., Ibanez, C.F., Hallbook, F., Persson, H., Cain, L.D., Dreyfus, C.F., & Black, I.B. (1993). Differential actions of neurotrophins in the locus coeruleus and basal forebrain. Exp Neurol, 119(1): 72-8.

Garcia, I., Martinou, I., Tsujimoto, Y., & Martinou, J.C. (1992). Prevention of programmed cell death of sympathetic neurons by the bcl-2 proto-oncogene. Science, 258(5080): 302-304.

Gauthier, E.R., Piche, L., Lemieux, G. & Lemieux, R. (1996) Role of bcl-X(L) in the control of apoptosis in murine myeloma cells. Cancer Res. 56(6):1451-1456.

Gillardon, F., Klimaschewski, L., Wickert, H., Krajewski, S., Reed, J.C., & Zimmermann, M. (1996). Expression pattern of candidate cell death effector proteins Bax, Bcl-2, Bcl-X, and c-Jun in sensory and motor neurons following sciatic nerve transection in the rat. Brain Res. 739(1-2): 244-250.

Gillardon, F., Wickert, H. & Zimmermann, M. (1995). Up-regulation of bax and down-regulation of bcl-2 is associated with kainate-induced apoptosis in mouse brain. Neurosci. Lett. 192(2): 85-88.

Glass, D.J., Nye, S.H., Hantzopoulos, P., Macchi, M.J., Squinto, S.P., Goldfarb, M., & Yancopoulos, G.D. (1991). TrkB mediates BDNF/NT-3-dependent survival and proliferation in fibroblasts lacking the low affinity NGF receptor. Cell,66(2): 405-413.

Gonzalez-Garcia, M., Perez-Ballestero, R., Ding, L., Duan, L., Boise, L.H., Thompson, C.B., & Nunez, G. (1994). *bcl-xL* is the major *bcl-x* mRNA form expressed during murine development and its product localizes to mitochondria. Development, 120: 3033-3042.

Gonzalez-Garcia, M., Garcia, I., Ding, L., O'Shea, S., Boise, L.H., Thompson, C.B & Nunez, G. (1995). bcl-x is expressed in embryonic and postnatal neural tissues and functions to prevent neuronal cell death. Proc. Natl. Acad. Sci USA 92(10):4304-4308.

Gospodarowicz, D. (1990). Fibroblast growth factor and its involvement in developmental processes. Curr Top Develop Biol, 24: 57-93.

Gotz, R., Koster, R., Winkler, C., Raulf, F., Lottspeich, F., Schartl, M., & Thoenen, H. (1994). Neurotrophin-6 is a new member of the nerve growth factor family. Nature, 372: 266-269.

Greenlund, L.J., Korsmeyer, S.J., & Johnson, E.M. (1995). Role of BCL-2 in the survival and function of developing and mature sympathetic neurons. Neuron, 15: 649-661.

Haldar, S., Beatty, C,. Tsujimoto, Y. & Croce, C.M. (1989). The Bcl-2 gene encodes a novel G protein. Nature 342: 195-98

Hallbook, F., Ibanez, C.F., Ebendal, T., & Persson, H. (1993). Cellular localization of brain-derived neurotrophic factor and neurotrophin-3 mRNA expression in the early chicken embryo. Eur. J. Neurosci., 5(1): 1-14.

Hallbook, F., Ibanez, C.F., & Persson, H. (1991). Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron, 6(5): 845-858.

Hamburger, V., Brunso-Bechtold, J.K., & Kip, J.W. (1981). Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. J. Neurosci. 1: 60-71.

Hamburger, V. & Levi.Montalcini, R. (1949). Proliferation, differentiation, and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. J. Exp. Zool. 162: 133-160.

Hamburger, V., & Yip, J. W. (1984). Reduction of experimentally induced neuronal death in spinal ganglia of the chick embryo by nerve growth factor. J Neurosci,4(3): 767-74.

Hanada, M., Krajewski, S., Tanaka, S., Cazals-Hatem, D., Spengler, B.A, Ross, R.A, Biedler, J.L, Reed & J.C. (1993). Regulation of Bcl-2 oncoprotein levels with differentiation of human neuroblastoma cells. Cancer Res. 53(20): 4978-4986.

Harper, S., & Davies, A. M. (1990). NGF mRNA expression in developing cutaneous epithelium related to innervation density. Development, 110(2): 515-519.

Hartikka, J., & Hefti, F. (1988). Development of septal cholinergic neurons in culture: plating density and glial cells modulate effects of NGF on survival, fiber growth, and expression of transmitter-specific enzymes. J. Neurosci., 8(8): 2967-2985.

Heller, S., Huber, J., Finn, T.P., Nishi, R. & Rohrer, H. (1993). GPA and CNTF produce similar effects in sympathetic neurones but differ in receptor binding. Neuroreport 5: 357-60.

Hempstead, B.L., Schleifer, L.S., & Chao, M.V. (1989). Expression of functional nerve growth factor receptors after gene transfer. Science, 243(4889): 373-5.

Henderson, C.E., Camu, W., Mettling, C., Gouin, A., Poulsen, K., Karlaloo, M., Rullamas, J., Evans, T., McMahon, S.B., Armanini, M. P., Berkemeier, L., Phillips, H.S., & Rosenthal, A. (1993). Neurotrophins promote motor neuron survival and are present in embryonic limb bud. Nature, 363: 266-270.

Henderson, C.E., Phillips, H.S., Pollock, R.A., Davies, A.M., Lemeulle, C., Armanini, M.P., Simpson, L.C., Moffet, B., Vandlen, R.A., Koliatsos, V.E & Rosenthal, A. (1994). GDNF: A potent survival factor for motoneurons present in peripheral nerve and muscle. Science, 266: 1062-1064.

Hengartner, M.O., & Horvitz, H.R. (1994a). Activation of C. elegans cell death protein CED-9 by an amino-acid substitution in a domain conserved in Bcl-2 [see comments]. Nature, 369(6478): 318-20.

Hengartner, M.O., & Horvitz, H.R. (1994b). C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. Cell, 76(4): 665-76.

Heumann, R., Korsching, S., Scott, J., & Thoenen, H. (1984). Relationship between levels of nerve growth factor (NGF) and its messenger RNA in sympathetic ganglia and peripheral target tissues. Embo J, 3(13): 3183-9.

Hirose K, Longo D.L., Oppenheim J.J., Matsushima K. (1993). Overexpression of mitochondrial manganese superoxide dismutase provides the survival of tumor cells exposed to interleukin-1, tumor nearosis factor, selected anti-cancer drugs and ionizing radiation. FASEBJ. 7: 361-8.

Hockenbery DM (1995). bcl-2, a novel regulator of cell death. Bioessays 17(7): 631-638

Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.L., & Korsmeyer, S.J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell, 75(2): 241-251.

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R.D., & Korsmeyer, S.J. (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature, 348(6299): 334-6.

Hockenbery, D. M., Zutter, M., Hickey, W., Nahm, M., & Korsmeyer, S. J. (1991). BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proc Natl Acad Sci U S A, 88(16): 6961-5.

Hofer, M.M. & Barde, Y.A. (1988). Brain-derived neurotrophic factor prevents neuronal death in vivo. Nature 331: 261-2.

Hofer, M., Pagliusi, S.R., Hohn, A., Leibrock, J., & Barde, Y.A. (1990). Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. Embo J, 9(8): 2459-64.

Hohn, A., Leibrock, J., Bailey, K., & Barde, Y.A. (1990). Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. Nature, 344(6264): 339-341.

Hollstein, M., Sidransky, D., Vogelstein, R., & Harris, C.C. (1991). Science,253: 49-53.

Horigome K, Pryor J.C., Bullock E.D. & Johnson E.M. (1993) Mediator release from mast cells by nerve growth factor - neurotrophin specificity and receptor mediation. J. Biol. Chem. 268(20): 14881-87.

Hughes, P., Dragunow, M., Beilharz, E., Lawlor, P., & Gluckman, P. (1993). MK801 induces immediate-early gene proteins and BDNF mRNA in rat cerebrocortical neurones. Neuroreport, 4(2): 183-6.

Ip, N.Y., Li, Y., Yancopoulos, G.D., & Lindsay, R.M. (1993). Cultured hippocampal neurons show responses to BDNF, NT-3, and NT-4, but not NGF. J. Neurosci, 13(8), 3394-405.

Ip, N.Y., Li, Y.P., van, d. S.I., Panayotatos, N., Alderson, R.F., & Lindsay, R.M (1991). Ciliary neurotrophic factor enhances neuronal survival in embryonic rat hippocampal cultures. J Neurosci, 11(10): 3124-34.

Ip, N.Y., Nye, S.H., Boulton, T.G., Davis, S., Taga, T., Li, Y., Birren, S.J., Yasukawa, K., Kishimoto, T., Anderson, D.J., & et, a. l. (1992). CNTF and LIF act on neuronal cells via shared signalling pathways that involve the IL-6 signal transducing receptor component gp130. Cell, 69(7),: 1121-32.

Jacobson, M.D., Burne, J.F., & Raff, M.C. (1994). Programmed cell death and Bcl-2 protection in the absence of a nucleus. Embo J, 13(8): 1899-910.

Jacobson M.D., Raff, M.C. (1995). Programmed cell death and Bcl-2 protection in very low oxygen. Nature 374: 814-6.

Jing, S., Tapley, P., & Barbacid, M. (1992). Nerve growth factor mediates signal transduction through trk homodimer receptors. Neuron, 9(6): 1067-79.

Johnson, D., Lanahan, A., Buck, C.R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M., & Chao, M. (1986). Expression and structure of the human NGF receptor. Cell, 47(4): 545-54.

Johnson, J.E., Barde, Y.A., Schwab, M., & Thoenen, H. (1986). Brain-derived neurotrophic factor supports the survival of cultured rat retinal ganglion cells. J Neurosci, 6(10): 3031-8.

Jones, K.R., Farinas, I., Backus, C., & Reichardt, L.F. (1994). Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. Cell, 76(6): 989-99.

Jones, K.R., & Reichardt, L.F. (1990). Molecular cloning of a human gene that is a member of the nerve growth factor family. Proc. Natl. Acad. Sci. U S A, 87(20): 8060-8064.

Kalcheim, C., & Gendreau, M. (1988). Brain-derived neurotrophic factor stimulates survival and neuronal differentiation in cultured avian neural crest. Brain Res.469(1-2): 79-86.

Kamada, S., Shimono, A., Shinto, Y., Tsujimura, T., Takahashi, T., Noda, T., Kitamura, Y., Kondoh, H. & Tsujimoto, Y. (1995). bcl-2 deficiency in mice leads to pleiotropic abnormalities: accelerated lymphoid cell death in thymus and spleen, polycystic kidney, hair hypopigmentation, and distorted small intestine. Cancer Res. 55(2): 354-359.

Kamens, J., Paskind, M., Hugunin, M., Talanian, R.V., Allen, H., Banach, D., Bump, N., Hackett, M., Johnston, C.G., Li, P. (1995). Identification and characterization of ICH-2, a novel member of the interleukin-1 beta-converting enzyme family of cysteine proteases. J. biol. Chem. 270(25): 15250-15256.

Kamesaki, S., Kamesaki, H., Jorgensen, T.J., Tanizawa, A., Pommier, Y. & Cossman, J. (1993). bcl-2 protein inhibits etoposide-induced apoptosis through its effects on events subsequent to topoisomerase II-induced DNA strand breaks and their repair. Cancer Res. 53(18): 4251-4256.

Kaplan, D.R., Hempstead, B.L., Martin, Z.D., Chao, M.V., & Parada, L.F. (1991a). The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science, 252(5005): 554-8.

Kaplan, D.R., Martin, Z.D., & Parada, L.F. (1991b). Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. Nature,350(6314): 158-160.

Kaupmann, K., Sendtner M, Stocki, K.A. & Jockusch H (1991). The gene for CNTF maps to murine chromosome 19 and its expression is not affected in the hereditary motoneuron disease "wobbler" of the mouse. Eur.J. Neurosci. 3: 1182-86.

Kiefer, M.C., Brauer, M.J., Powers, V.C., Wu, J.J., Umansky, S.R., Tomei, L.D. & Barr, P.J. (1995) Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. Nature 374: 736-739.

Klein, G. (1991). Comparative action of myc and bcl-2 in B-cell malignancy. Cancer Cells, 3(4): 141-3.

Klein, R. (1994). Role of neurotrophins in mouse neuronal development. FASEB J.,8(10): 738-744.

Klein, R., Conway, D., Parada, L.F., & Barbacid, M. (1990b). The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. Cell, 61(4): 647-656.

Klein, R., Jing, S.Q., Nanduri, V., O'Rourke, E., & Barbacid, M. (1991b). The trl proto-oncogene encodes a receptor for nerve growth factor. Cell, 65(1): 189-197.

Klein, R., Lamballe, F., Bryant, S., & Barbacid, M. (1992). The trkB tyrosine protein kinase is a receptor for neurotrophin-4. Neuron, 8(5): 947-956.

Klein, R., Martin, Z.D., Barbacid, M., & Parada, L.F. (1990a). Expression of the tyrosine kinase receptor gene trkB is confined to the murine embryonic and adult nervous system. Development, 109(4): 845-850.

Klein, R., Nanduri, V., Jing, S.A., Lamballe, F., Tapley, P., Bryant, S., Cordon, C.C., Jones, K.R., Reichardt, L.F., & Barbacid, M. (1991a). The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. Cell, 66(2): 395-403.

Klein, R., Silos, S.I., Smeyne, R.J., Lira, S.A., Brambilla, R., Bryant, S., Zhang, L., Snider, W.D., & Barbacid, M. (1994). Disruption of the neurotrophin-3 receptor gene trkC eliminates la muscle afferents and results in abnormal movements. Nature, 368(6468): 249-251.

Klein, R., Smeyne, R.J., Wurst, W., Long, L.K., Auerbach, B.A., Joyner, A.L., & Barbacid, M. (1993). Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. Cell, 75: 113-122.

Knudson, C.M., Tung, K.S.K., Tourtellotte, W.G., Brown, G.A.J., & Korsmeyer, S.J. (1995). Bax deficient mice with lymphoid hyperplasia and male germ cell death. Science, 270: 96-99.

Knusel, B., Winslow, J.W., Rosenthal, A., Burton, L.E., Seid, D.P., Nikolics, K., & Hefti, F. (1991). Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. Proc Natl Acad Sci U S A, 88(3): 961-5.

Korsching, S. (1993). The neurotrophic factor concept: a reexamination. J Neurosci, 13(7): 2739-48.

Korsching, S., Auburger, G., Heumann, R., Scott, J., & Thoenen, H. (1985). Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. Embo J. 4(6):1389-93.

Korsching, S., & Thoenen, H. (1983). Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: correlation with density of sympathetic innervation. Proc. Natl. Acad. Sci. USA, 80(11): 3513-3516.

Korsching, S., & Thoenen, H. (1988). Developmental changes of nerve growth factor levels in sympathetic ganglia and their target organs. Devel. Biol., 126(1): 40-46.

Korsmeyer, S.J. (1995). Regulators of cell death. Trends Genetics 11:101-105.

Korsmeyer, S.J., Shutter, J.R., Veis, D.J., Merry, D.E., & Oltvai, Z.N. (1993). Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death. Semin Cancer Biol, 4(6): 327-32.

Kotzbauer, P.T., Lampe, P.A., Heuckeroth, R.O., Golden, J.P., Creedon, D.J. Johnson, E.M.J. and Milbrandt, J. (1996). Neurturin, a relative of glial-cell-line-derived neurotrophic factor. Nature, 384: 467-470.

Kozopas. K.M., Yang T, Buchan H.L., Zhou P, Craig R.W (1993). MCL-1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to Bcl-2. Proc. Natl. Acad. Sci. USA 90:3516-20.

Krajewska, M., Fenoglio-Preiser, C.M., Krajewski, S., Song, K., Macdonald, J.S., Stemmerman, G., Reed, J.C. (1996). Immunohistochemical analysis of Bcl-2 family proteins in adenocarcinomas of the stomach. Am J. Pathol. 149(5):1449-1457.

Krajewski, S., Bodrug, S., Krajewska, M., Shabaik, A,. Gascoyne, R., Berean, K. & Reed, J.C. (1995). Immunohistochemical analysis of Mcl-1 protein in human tissues. Differential regulation of Mcl-1 and Bcl-2 protein production suggests a unique role for Mcl-1 in control of programmed cell death in vivo. Am J. Pathol 146(6):1309-1319.

Krajewski, S., Krajewski, M. & Reed, J.C. (1996). Immunohistochemical analysis of in vivo patterns of Bak expression, a proapoptotic member of the Bcl-2 protein family. Cancer Res. 56(12):2849-2855.

Krajewski, S., Krajewska, M., Shabaik, A., Miyashita, T., Wang, H.G. & Reed, J.C. (1994). Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. Am. J. Pathol 145(6):1323-1336,

Krajewski, S., Krajewska, M.. Shabaik, A., Wang, H.G., Irie, S., Fong, L. & Reed, J.C. (1994). Immunohistochemical analysis of in vivo patterns of Bcl-X expression. Cancer Res. 54(21):5501-5507.

Krajewski, S., Tanaka, S., Takayama, S., Schibler, M.J., Fenton, W., & Reed, J.C. (1993). Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res., 53(19): 4701-4714.

Kumar, S., Kinoshita, M., Noda, M., Copeland N.G. & Jenkins, N.A (1994). Induction of apoptosis by the mouse Nedd2 gene, which encodes a protein similar to the product of the Caenorhabditis elegans cell death gene ced-3 and the mammalian IL-1 beta-converting enzyme. Genes. Dev. 8(14):1613-1626.

Lam, A., Fuller, F., Miller, J., Kloss, J., Manthorpe, M., Varon, S., & Cordell, (1991). Sequence and structural organization of the human gene encoding ciliary neurotrophic factor. Gene, 102(2): 271-6.

Lam, M., Dubyak, G., Chen, L., Nunez, G., Miesfeld, R. L., & Distelhorst, C. W. (1994). Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca2+ fluxes. Proc Natl Acad Sci U S A, 91(14): 6569-73.

Lamballe, F., Klein, R., & Barbacid, M. (1991). The trk family of oncogenes and neurotrophin receptors. Princess Takamatsu Symp, 22(153): 153-70.

Large, T.H., Weskamp, G., Helder, J.C., Radeke, M.J., Misko, T.P., Shooter, E.M., & Reichardt, L.F. (1989). Structure and developmental expression of the nerve growth factor receptor in the chicken central nervous system. Neuron, 2(2): 1123-1134.

Larkfors, L., Lindsay, R.M., & Alderson, R.F. (1994). Ciliary neurotrophic factor enhances the survival of Purkinje cells in vitro. Eur J Neurosci, 6(6): 1015-1025.

Lasorella, A., Lavarone, A. & Israel, M.A. (1995). Differentiation of neuroblastoma enhances Bcl-2 expression and induces alterations of apoptosis and drug resistance. Cancer Res. 55(20):4711-4716.

Le Douarin, N. (1973). A biological cell labelling technique and its use in experimental embryology. Dev. Biol., 30(1): 217-222.

Lee, K.F., Bachman, K., Landis, S., & Jaenisch, R. (1994a). Dependence on p75 for innervation of some sympathetic targets. Science, 263(5152):1447-1449.

Lee, K.F., Davies, A.M., & Jaenisch, R. (1994b). p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. Development, 120: 1027-1033.

Lee, K.F., Li, E., Huber, L.J., Landis, S.C., Sharpe, A H., Chao, M.V., & Jaenisch, R. (1992). Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. Cell, 69(5): 737-749.

Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Thoenen, H., & Barde, Y.A. (1989). Molecular cloning and expression of brain-derived neurotrophic factor. Nature, 341(6238): 149-152.

Leung, D.W., Parent, A.S., Cachianes, G., Esch, F., Coulombe, J.N., Nikolics, K., Eckenstein, F.P., & Nishi, R. (1992). Cloning, expression during development, and evidence for release of a trophic factor for ciliary ganglion. Neuron 8 : 1045-1053.

Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. Science, 237: 1154-1162.

Lillie (1908). The Development of the Chick. Henry Holt and Company.

Lin, E.Y., Orlofsky, A., Berger, M.S., Prystowsky, M.B. (1993). Characterization of A1, a novel hemopoietic-specific early response gene with sequence similarity to bcl-2. J. Immunol 151(4):1979-1988.

Lin, E.Y., Orlofsky, A., Wang, H.G., Reed, J.C., Prystowsky, M.B. (1996). A1, a Bcl-2 family member, prolongs cell survival and permits myeloid differentiation. Blood 87(3):983-992

Lin, L.H., Doherty, D.H., Lile, J.D., Bektesh, S., & Collins, F. (1993). GDNF: A glial cell-derived neurotrophic factor for midbrain dopaminergic neurons. Science, 260: 1130-1132.

Lin, L.F., Mismer, D., Lile, J.D., Armes, L.G., Butler, E., Vannice, J L., & Collins, F. (1989). Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). Science, 246(4933): 1023-1025.

Lindsay, R.M., Thoenen, H., & Barde, Y.A. (1985). Placode and neural crest-derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor. Devel. Biol., 112(2): 319-328.

Linette, G.P., & Korsmeyer, S.J. (1994). Differentiation and cell death: lessons from the immune system. Curr. Opin. Cell Biol., 6: 809-815.

Lippke, J.A., Gu, Y., Sarneki, C., Caron, P.R. & Su, MS-S (1996). Identification and characterization of CPP-32/Mch-2 homolog 1, a novel cysteine protease similar to CPP-32. J.Biol. Chem. 271:1825-28.

Lipton, S.A., Wagner, J.A., Madison, R.D. & D'Amore, P.A. (1988). Acidic fibroblast growth factor enhances regeneration of processes by postnatal mammalian retinal ganglion cells in culture. Proc Natl. Acad. Sci USA 85(7): 2388-2392.

Liu, X., Ernfors, P., Wu, H., & Jaenisch, R. (1995). Sensory but not motor neuron deficits in mice lacking NT4 and BDNF. Nature, 375: 238-241.

Lu, P.J., Lu, Q.L, Rughetti, A. & Taylor-Papadimitriou, J. (1995). bcl-2 overexpression inhibits cell death and promotes the morphogenesis, but not tumorigenesis of human mammary epithelial cells. J. Cell Biol. 129(5):1363-1378.

Lu, Q.L., Hanby, A.M., Nasser, Hajibagheri, M.A,. Gschmeissner, S.E, Lu, P.J., Taylor-Papadimitriou, J., Krajewski, S., Reed, J.C. & Wright, N.A. (1994). Bcl-2 protein localizes to the chromosomes of mitotic nuclei and is correlated with the cell cycle in cultured epithelial cell lines. J. Cell. Sci.107(Pt2): 363-371.

Lu, Q.L., Poulsom, R., Wong, L., & Hanby, A.M. (1993). Bcl-2 expression in adult and embryonic non-haematopoietic tissues. J Pathol, 169(4): 431-7.

Ma, M., Pena, J.C., Chang, B., Margosian, E., Davidson, L., Alt F.W.,& Thompson, C.B. (1995). Bclx regulates the survival of double-positive thymocytes. Proc. Natl. Acad. Sci USA 92(11):4763-4767.

Maisonpierre, P.C., Belluscio, L., Friedman, B., Alderson, R.F., Wiegand, S.J., Furth, M.E., Lindsay, R.M., & Yancopoulos, G.D. (1990). NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. Neuron, 5(4): 501-9.

Maisonpierre, P.C., Belluscio, L., Squinto, S., Ip, N.Y., Furth, M.E., Lindsay, R.M., & Yancopoulos, G.D. (1990). Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science, 247: 1446-1451.

Maisonpierre, P.C., Le, B.M., Espinosa, R., Ip, N.Y., Belluscio, L., de la Monte, S.,Squinto, S., Furth, M.E., & Yancopoulos, G.D. (1991). Human and rat brain-derived neurotrophic factor and neurotrophin-3: gene structures, distributions, and chromosomal localizations. Genomics, 10(3): 558-68.

Manthorpe, M., Skaper, S.D., Barbin, G., & Varon, S. (1982). Cholinergic neuronotrophic factors. Concurrent activities on certain nerve growth factor-responsive neurons. J Neurochem, 38(2): 415-421.

Manthorpe, M., Skaper, S.D., Williams, L.R., & Varon, S. (1986). Purification of adult rat sciatic nerve ciliary neuronotrophic factor. Brain Res, 367(1-2): 282-6.

Manthorpe, M., & Varon, S. (1985). Regulation of neuronal survival and neurite growth in the avian ciliary ganglion by trophic factors. In G. Guroff (Ed.), Growth and maturation factors: (pp. 77-117). New York: Wiley.

Martin, D.P., Schmidt, R.E., P.D., Lowry, O.H., Carter, J.G., & Johnson, E.M.J. (1988). Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J Cell Biol, 106(3): 829-844.

Martin-Zanca, D., Barbacid, M., & Parada, L.F. (1990). Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. Genes Dev, 4(5): 683-94.

Martin-Zanca, D., Mitra, G., Long, L.K., & Barbacid, M. (1986). Molecular characterization of the human trk oncogene. 51, : 983-992.

Martin-Zanca, D., Oskam, R., Mitra, C., Copeland, T., & Barbacid, M. (1989). Molecular and biochemical characterisation of the human *trk* proto-oncogene. Mol. Cell. Biol., 9: 24-33.

Martinou, J.C., Dubois-Dauphin, M., Staple, J.K., Rodriguez, I., Frankowsky, H., Missotten, M., Albertini, P., Talabot, D., Catsicas, S., Pietra, C., & Huarte, J. (1994). Overexpression of bcl-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischaemia. Neuron, 13,:1017-1030.

Massague, J., Attisano, L., & Wrana, J.L. (1994). The TGF-β Family and its composite receptors. Trends in Cell Biology, 4:172-177.

Mazel, S, Burtrum, D. & Petrie, H.T. (1996). Regulation of cell division cycle progression by bcl-2 expression: a potential mechanism for inhibition of programmed cell death. J. Exp. Med. 183(5):2219-2226.

McDonald, J.R., Ko, C., Mismer, D., Smith, D.J., & Collins, F. (1991). Expression and characterization of recombinant human ciliary neurotrophic factor from Escherichia coli. Biochim Biophys Acta, 1090(1): 70-80.

McDonnell, T.J., Deane, N., Platt, F.M., Nunez, G., Jaeger, U., McKearn, J. P., & Korsmeyer, S. J. (1989). bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell, 57(1): 79-88.

McDonnell, T.J., Nunez, G., Platt, F.M., Hockenberry, D., London, L., McKearn, J.P., & Korsmeyer, S.J. (1990). Deregulated Bcl-2-immunoglobulin transgene expands a resting but responsive immunoglobulin M and D-expressing B-cell population. Mol Cell Biol, 10(5): 1901-7.

Meakin, S.O., & Shooter, E.M. (1992). The nerve growth factor family of receptors. Trends Neurosci, 15(9): 323-31.

Meakin, S.O., Suter, U., Drinkwater, C.C., Welcher, A.A., & Shooter, E.M. (1992). The rat trk protooncogene product exhibits properties characteristic of the slow nerve growth factor receptor. Proc Natl Acad Sci U S A, 89(6): 2374-8.

Meier, R., Becker, A.M., Gotz, R., Heumann, R., Shaw, A., & Thoenen, H. (1986). Molecular cloning of bovine and chick nerve growth factor (NGF): delineation of conserved and unconserved domains and their relationship to the biological activity and antigenicity of NGF. Embo J, 5(7): 1489-93.

Merry, D.E. & Korsmeyer, S.J. (1997). Bcl-2 gene family in the nervous system. Annu.Rev. Neurosci. 20:245-267.

Merry, D.E., Veis, D.J., Hickey, W.F., & Korsmeyer, S.J. (1994). bcl-2 protein expression is widespread in the developing nervous system and r retained in the adult PNS. Development, 120(2): 301-11.

Michaelidis, T.M., Sendtner, M., Cooper, J.D., Airaksinen, M.S., Holtmann, B., Meyer, M., & Thoenen, H. (1996). Inactivation of *bcl-2* results in progressive degeneration of motoneurons, sympathetic neurons and sensory neurons during the early postnatal development. Neuron, 17: 75-89.

Middlemas, D.S., Lindberg, R.A., & Hunter, T. (1991). trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. Mol Cell Biol, 11(1): 143-53.

Middleton, G., Nunez, G., & Davies, A.M. (1996). Bax promotes neuronal survival and antogonises the survival effects of trophic factors. Development, 122: 695-701.

Middleton, G., Pinon, L.G., Wyatt S. & Davies A.M. (1998). Bcl-2 Accelerates the Maturation of Early Sensory Neurons. J. Neuroscience 18(9):3344-3350.

Minichiello, L.F.P., Vazquez, E., Schimmang, T., Hokfelt, T., Represa, J. & Klein, R. (1995). Differential effects of combined trk receptor mutations on dorsal root ganglion and inner ear sensory neurons. Development, 121: 4067-4075.

Minn, A.J., Velez, P., Schendel, S.L., Liang, H., Muchmore, S.W., Fesik, S.W., Fill, M. & Thompson, C.B. (1997). Bcl-xL forms an ion channel in synthetic lipid membranes. Nature 385: 353-357.

Miura, M., Zhu, H., Rotello, R., Hartwieg, E.A & Yuan, J. (1993). Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. Cell 75(4):653-660

Miyashita, T., Harigai, M., Hanada, M., & Reed, J.C (1994). Identification of a p53-dependent negative response element in the bcl-2 gene. Cancer Res, 54(12): 3131-5.

Miyashita, T. & Reed, J.C. (1993). Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. Blood 81(1):151-157.

Miyashita, T. & Reed, J.C. (1995) Tumour suppresser p53 is a direct transcriptional activator of the human Bax gene. Cell 80: 293-299

Moss, S.F., Agarwal, B., Arber, N., Guan, R.J., Krajewska, M., Krajewski, S., Reed, J.C. & Holt, P.R. (1996). Increased intestinal Bak expression results in apoptosis. Biochem. Biophys. Res. Commun. 223(1):199-203.

Motoyama, N., Wang, F., Roth, K. A., Sawa, H., Nakayama, K., Nakayama, K., Negishi, I., Senju, S., Zhang, Q., Fujii, S., & Loh, D. Y. (1995). Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science, 267: 1506-1510.

Muchmore, S.W., Sattler, M., Liang, H., Meadows, R.P., Harlan, J.E, Yoon, H.S, Nettesheim, D., Chang, B.S., Thompson, C.B., Wong ,S.L., Ng, S.L. & Fesik, S.W. (1996). X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature 381(6580):335-341.

Munday, N.A., Vaillancourt, J.P., Ali, A., Casano, F.J., Miller, D.K,. Molineaux, S.M., Yamin, T.T., Yu, V.L. & Nicholson, D.W. (1995). Molecular cloning and pro-apoptotic activity of ICErelII and ICErelIII, members of the ICE/CED-3 family of cysteine proteases. J. Biol. Chem. 270(26):15870-15876.

Muzio, M., Salvesen, G.S. & Dixit, V.M .(1997). FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens. J. Biol. Chem. 272(5):2952-2956.

Nagata, S. & Golstein, P. (1995). The Fas death factor. Science 267: 1449-1456.

Nakai, M., Takeda, A., Cleary, M.L., & Endo, T. (1993). The bcl-2 protein is inserted into the outer membrane but not into the inner membrane of rat liver mitochondria in vitro. Biochem Biophys Res Commun, 196(1),:233-9.

Nakayama, K., Nakayama, K., Negishi, I., Kuida, K., Sawa, H., & Loh, D.Y. (1994). Targeted disruption of Bcl-2 alpha beta in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. Proc. Natl. Acad. Sci. USA, 91(9): 3700-3704.

Nakayama, K., Nakayama, K., Negishi, I., Kuida, K., Shinkai, Y., Louie, M.C., Fields, L.E., Lucas, P.J., Stewart, V., Alt, F.W., & Loh, D.Y. (1993). Disappearance of the lymphoid system in Bcl-2 homozygous mutant chimeric mice. Science, 261(5128): 1584-1588.

Negrini, M., Silini, E., Kozak, C., Tsujimoto, Y. & Croce, C.M. (1987). Molecular analysis of mbcl-2: structure and expression of the murine gene homologous to the human gene involved in follicular lymphoma. Cell 1987 49(4):455-463.

Neufeld, G., & Gaspoclavowicz, D. (1986). Basic and acidic fibroblast growth factors interact with the same cell surface receptors. J. Biol. Chem. 261: 5631-37.

Newton, K. & Strasser, A. (1998). The Bcl-2 family and cell death regulation. Curr Opin Genet Dev. 8(1):68-75

Nguyen, M., Millar, D.G., Yong, V.W., Korsmeyer, S.J., & Shore, G.C. (1993). Targeting of Bcl-2 to the mitochondrial outer membrane by a COOH-terminal signal anchor sequence. J. Biol. Chem., 268(34): 25265-25268.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K, Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M. & Lazebnik, Y.A (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376(6535):37-43.

Ninkina, N., Grashchuck, M., Buchman, V. L., & Davies, A. M. (1997). TrkB variants with deletions in the leucine-rich motifs of the extracellular domain. J. Biol. Chem 272(20):13019-13025.

Nunez, G., & Clarke, M.F. (1994). The Bcl-2 family of proteins: regulators of cell death and survival. Trends Cell Biol., 4: 399-403.

Nunez, G., London, L., Hockenbery, D., Alexander, M., McKearn, J.P., & Korsmeyer, S.J. (1990). Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. J Immunol, 144(9): 3602-10.

Oh, Y.J., Swarzenski, B.C., & O'Malley, K.L. (1996). Overexpression of Bcl-2 in a murine dopaminergic neuronal cell line leads to neurite outgrowth. Neurosci. Lett., 202: 161-164.

Ohga, Y., Zirrgiebel, U., Hamner, S., Michaelidis, T.M., Cooper, J., Thoenen, H. & Lindholm D. (1996) Cell-density increases Bcl-2 and Bcl-x expression in addition to survival of cultured cerebellar granule neurons. Neuroscience 73(4): 913-17.

Okazawa, H, Shimizu, J, Masaki ,K, Imafuku, I, Hamada, H, Kamazawa, I. (1996). Bcl-2 Induces Retinoic Acid-induced apoptosis during the neural differentiation of embryonic stem cells. J. Cell. Biol. 132(5):955-68.

Oltvai, Z. N., & Korsmeyer, S. J. (1994). Checkpoints of dueling dimers foil death wishes. Cell, 79: 189-192.

Oltvai, Z. N., Milliman, C.L., & Korsmeyer, S.J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell, 74(4): 609-619.

Oppenheim, R.W. (1991). Cell death during development of the nervous system. . Ann. Rev. Neurosci., 14(453): 453-501.

Oppenheim, R.W., Houenou, L.J., Johnson, J.E., Lin, L.H., Li, L., Lo, A.C., Newsome, A.L., Prevette, D.M., & Wang, S. (1995). Developing motoneurons rescued from programmed and axotomy-induced cell death by GDNF. Nature, 373,:344-346.

Oppenheim, R.W., Prevette, D., & Fuller, F. (1992). The lack of effect of basic and acidic fibroblast growth factors on the naturally occurring death of neurons in the chick embryo. J Neurosci, 12(7): 2726-34.

Osen-Sand, A., Catsicas, M., Staple, J.K., Jones, K.A., Ayala, G., Knowles, J. Grenningloh, G. & Catsicas, S. (1993). Inhibition of axonal growth by SNAP-25 antisense oligonucleoticles in vitro and in vivo. Nature 364: 445-48.

Otani, H., Erdos, M., & Leonard, W.J. (1993). Tyrosine kinase(s) regulate apoptosis and bcl-2 expression in a growth factor-dependent cell line. J Biol Chem, 268(30): 22733-6.

Pautou, M.P. (1974). Comparative development of interdigital morphogenetic necrosis in the foot of chick and duck embryos. C.R. Acad. Sci. Hebd. Seances Acad. Sci. D. 278(17): 2209-2212.

Pinon, L., Middleton, G. & Davies, A.M. (1997) Bcl-2 is required for cranial sensory neuron survival at defined stages of embryonic development. Development 124: 4173-4178.

Pinon, L.G.P., Minichiello, L., Klein, R., & Davies, A.M. (1996). Timing of neuronal death in *trkA*, *trkB* and *trkC* mutant embryos reveals developmental changes in sensory neuron dependence on Trk signalling. Development, 122,: 3255-3261.

Pirvola, U., Ylikoski, J., Palgi, J., Lehtonen, E., Arumae, U., & Saarma, M. (1992). Brain-derived neurotrophic factor and neurotrophin 3 mRNAs in the peripheral target fields of developing inner ear ganglia. Proc. Natl. Acad. Sci. U S A, 89(20): 9915-9919.

Pommerville, J. & Kochert, G. (1982). Effects of senescence on somatic cell physiology in the green alga Volvox carteri. Exp. Cell. Res. 140(1):39-45.

Prestige, M.C. (1967). The control of cell number in the ventral horns during the development of Xenopus laevis tadpoles. J. Embryol. Exp. Morphal 19: 359-387.

Purves, D., Snider, W.D., & Voyvodic, J.T. (1988). Trophic regulation of nerve cell morphology and innervation in the autonomic nervous system. Nature, 336: 123-128.

Rabizadeh, S., LaCount, D.J., Friesen, P.D., & Bredesen, D.E. (1993). Expression of the baculovirus p35 gene inhibits mammalian neural cell death. J Neurochem, 61(6): 2318-21.

Radeke, M.J., Misko, T.P., Hsu, C., Herzenberg, L.A., & Shooter, E.M. (1987). Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature, 325: 593-597.

Radziejewski, C., Robinson, R.C., Di, S.P., & Taylor, J.W. (1992). Dimeric structure and conformational stability of brain-derived neurotrophic factor and neurotrophin-3. Biochemistry, 31(18):4431-6.

Rao, L., Debbas, M., Sabbatini, P., Hockenbery, D., Korsmeyer, S. & White, I (1992a). The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. Proc. Natl. Acad. Sci USA 89(16):7742-7746.

Rao, M.S., Patterson, P.H., & Landis, S.C. (1992b). Multiple cholinergic differentiation factors are present in footpad extracts: comparison with known cholinergic factors. Development, 116(3): 731-44.

Ratts, V.S., Flaws, J.A., Kolp, R., Sorenson, C.M. & Tilly, J.L. (1995). Ablation of bcl-2 gene expression decreases the numbers of oocytes and primordial follicles established in the post-natal female mouse gonad. Endocrinology. 136(8):3665-3668.

Reed, J.C. (1997). Double identity for proteins of the Bcl-2 family. Nature 387(6635):773-776.

Reed, J.C., Tanaka, S., & Cuddy, M. (1992). Cell cycle analysis of p26-BCL-2 protein levels in proliferating lymphoma and leukemia cell lines. Cancer Res, 52(10): 2802-5.

Reynolds, J.E., Yang, T., Qian, L., Jenkinson, J.D., Zhou, P., Eastman, A. & Craig, R.W. (1994) Mcl-1, a member of the Bcl-2 family, delays apoptosis induced by c-Myc overexpression in Chinese hamster ovary cells. Cancer Res. 54(24):6348-6352.

Risau W, Gautschi-Sova P & Bohlen P (1988). Endothelial cell growth factors in embryonic and adult chick brain are related to human acidic fibroblast growth factor. EMBO J. 7(4):959-962.

Robinson, M., Adu, J., & Davies, A.M. (1996). Timing and regulation of *trkB* and *BDNF* mRNA expression in placode-derived sensory neurons and their targets. Euro. J. Neurosci., (in press).

Rogers, L.A., & Cowen, W.M. (1974). The development of the mesencephalic nucleus of the trigeminal nerve in the chick. J. Comp. Neurol., 147: 291-320.

Rogers, S., Wells, R. & Richsteimer, M. (1986). Animo acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science 234: 364-8.

Rosenthal, A., Goeddel, D.V., Nguyen, T., Lewis, M., Shih, A., Laramee, G.R., Nikolics, K., & Winslow, J.W. (1990). Primary structure and biological activity of a novel human neurotrophic factor. Neuron, 4(5): 767-773.

Sato, T., Irie, S., Krajewski, S., & Reed, J.C. (1994). Cloning and sequencing of a cDNA encoding the rat Bcl-2 protein. Gene, 140(2): 291-2.

Saunders J.W., Jr. & Fallon J.F. (1966). Cell death in morphogenesis in M.Locke (ed), Major Problems of Developmental Biology. Academic Press, New York: pp. 289-314.

Schecterson, L.C., & Bothwell, M. (1992). Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. Neuron, 9(3): 449-463.

Scheid, M.P. & Duronio, V. (1998). Dissociation of cytokine-induced phosphorylation of Bad and activation of PKB/akt: Involvement of MEK upstream of Bad phosphorylation. Proc. Natl. Acad. Sci. USA 95(13):7439-7444.

Schendel, S.L., Xie, Z., Montal, M.O., Matsuyama, S., Montal, M. & Reed, J.C. (1997). Channel formation by antiapoptotic protein Bcl-2. Proc. Natl. Acad. Sci USA 94(10):5113-5118.

Schimmang, T., Minichiello, L., Vazquez, E., Joac, I.S., Giraldez, F., Klein, R., & Represa, J. (1995). Developing inner ear sensory neurons require TrkB and TrkC receptors for innervation of their peripheral targets. Development, 121: 3381-3391.

Schoenwolf, G.C., & Sheard, P. (1990). Fate mapping the avian epiblast with focal injections of a fluorescent-histochemical marker: ectodermal derivatives. J Exp Zool, 255(3),:323-39.

Schoenwolf, G.C. & Smith, J.L.(1990). Mechanisms of neurulation: traditional viewpoint and recent advances. Development 109(2):243-270.

Schott, A.F., Apel, I.J., Nunez, G. & Clarke, M.F. (1995). Bcl-XL protects cancer cells from p53-mediated apoptosis. Oncogene 11(7):1389:1394.

Schweichel, J.U. & Merker, H.J. (1973). The morphology of various types of cell death in prenatal tissues. Teratology 7(3):253-266

Scott, J., Selby, M., Urdea, M., Quiroga, M., Bell, G. I., & Rutter, W. J. (1983). Isolation and nucleotide sequence of a cDNA encoding the precursor of mouse nerve growth factor. Nature, 302(5908): 538-40.

Scott, S.A., & Davies, A.M. (1990). Inhibition of protein synthesis prevents cell death in sensory and parasympathetic neurons deprived of neurotrophic factor in vitro. J Neurobiol, 21(4): 630-8.

Sedlak, T.W., Oltvai, Z.N., Yang, E., Wang, K., Boise, L.H., Thompson, C.B. & Korsmeyer, S.J. (1995). Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. Proc. Natl. Acad. Sci USA 92(17):7834-7838.

Selvakumaran, M., Lin, H.K., Miyashita, T., Wang, H.G., Krajewski, S., Reed, J.C., Hoffman, B., & Liebermann, D. (1994). Immediate early up-regulation of bax expression by p53 but not TGF beta 1: a paradigm for distinct apoptotic pathways. Oncogene, 9(6): 1791-8.

Shelton, D.L., & Reichardt, L.F. (1984). Expression of the beta-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. Proc. Natl. Acad. Sci. USA, 81(24): 7951-7915.

Shimizu, S., Eguchi, Y., Kosaka, H., Kamiike, W., Matsuda, H., Tsujimoto, Y. (1995). Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-xL. Nature 1995 374(6525):811-813.

Shiraiwa, N., Inohara, N., Okada, S., Yuzaki, M., Shoji, S. & Ohta, S. (1996). An additional form of rat Bcl-x, Bcl-xbeta, generated by an unspliced RNA, promotes apoptosis in promyeloid cells. J. Biol. Chem. 271(22): 13258-13265.

Smeyne, R.J., Klein, R., Schnapp, A., Long, L.K., Bryant, S., Lewin, A., Lira, S.A., & Barbacid, M. (1994). Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature, 368(6468): 246-249.

Soppet, D., Escandon, E., Maragos, J., Middlemas, D. S., Reid, S. W., Blair, J., Burton, L. E., Stanton, B. R., Kaplan, D. R., Hunter, T., Nikolics, K., & Parada, L. F. (1991). The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. Cell, 65(5): 895-903.

Sorenson, C.M., Rogers, S.A., Korsmeyer, S.J., Hammerman, M.R. (1995). Fulminant metanephric apoptosis and abnormal kidney development in Bcl-2-deficient mice. Am.J.Physiol.268:F73-F81.

Spemann, H. (1938). Embryonic Development and Induction. Yale University Press, New Haven.

Squinto, S.P., Stitt, T.N., Aldrich, T.H., Davis, S., Bianco, S.M., Radziejewski, C., Glass, D.J., Masiakowski, P., Furth, M.E., Valenzuela, D.M., DiStefano, P.S., & Yancopoulos, G.D. (1991). trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell, 65(5): 885-893.

Steller, H. (1995). Mechanisms and genes of cellular suicide. Science, 267: 1445-1449.

Stockli, K.A., Lottspeich, F., Sendtner, M., Masiakowski, P., Carroll, P., Gotz, R., Lindholm, D., & Thoenen, H. (1989). Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor. Nature, 342(6252): 920-923.

Sulston, J.E., Schierenberg, E, White, J.G., & Thomson, J.N. (1983). The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev.Biol.100:64-119.

Sumantran, V.N., Ealovega, M.W., Nunez, G., Clarke, M.F.& Wicha, M.S (1995). Overexpression of Bcl-XS sensitizes MCF-7 cells to chemotherapy-induced apoptosis. Cancer Res. 55(12):2507-510.

Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millan, J. A., & Reed, J. C. (1995). Cloning and functional analysis of BAG-1: Anovel Bcl-2-binding protein with anti-cell death activity. Cell, 80: 279-284.

Tanaka, S., Saito, K., & Reed, J. C. (1993). Structure-function analysis of the Bcl-2 oncoprotein. Addition of a heterologous transmembrane domain to portions of the Bcl-2 beta protein restores function as a regulator of cell survival. J. Biol. Chem.268(15):10920-10926.

Tessarollo, L., Vogel, K.S., Palko, M.E., Reid, S.W., & Parada, L.F. (1994). Targeted mutation in the neurotrophin-3 gene results in loss of muscle sensory neurons. Proc. Natl. Acad. Sci. USA, 91:11844-11848.

Tewari, M., Quan, L.T., O'Rouke, K., Desnoyers, S., Zeng, Z., Beidler, D.R., Poirier, G.G, Salvesen, G.S. & Dixit, V.M. (1995). Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly (ADP-ribose) polymerase. Cell 81(5):801-809.

Theiler, K. (1972). The House Mouse (Development and normal stages from fertilisation to 4 weeks). Berlin: Springer-Verlag.

Thoenen, H. (1991). The changing scene of neurotrophic factors. Trends Neurosci14(5): 165-70.

Thompson, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. Science 267(5203):1456-1462.

Thompson, C.B. (1996). Transcription. A fate worse than death. Nature 382(6591):492-493.

Tomac, A., Lindqvist, E., Lin, L.H., Ogren, S.O., Young, D., Hoffer, B.J., & Olson, L. (1995). Protection and repair of the nigrostriatal dopaminergic system by GDNF *in vivo*. Nature, 373, 335-339.

Tsoulfas, P., Soppet, D., Escandon, E., Tessarollo, L., Mendoza, R.J., Rosenthal, A., Nikolics, K., & Parada, L.F. (1993). The rat trkC locus encodes multiple neurogenic receptors that exhibit differential response to neurotrophin-3 in PC12 cells. Neuron 10(5): 975-990.

Tsujimoto, Y., & Croce, C.M. (1986). Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proc. Natl. Acad. Sci. U S A, 83: 5214-5218.

Uhlmann, E.J., D'Sa-Eipper, C., Subramanian, T., Wagner, A.J., Hay, N. & Chinnadurai, G. (1996). Deletion of a nonconserved region of Bcl-2 confers a novel gain of function: suppression of apoptosis with concomitant cell proliferation. Cancer Res. 56(11): 2506-2509.

Ullrich, A., Gray, A., Berman, C., & Dull, T.J. (1983). Human beta-nerve growth factor gene sequence highly homologous to that of mouse. Nature, 303(5920): 821-5.

Unsicker, K., Reichert-Preibsch, H., Schmidt, R., Pettmann, B., Labourdette, G., & Sensenbrenner, M. (1987). Astroglial and fibroblast growth factor have neurotrophic functions for cultured peripheral and central nervous system neurons. Proc Natl Acad Sci USA, 84: 5459-5463.

Unsicker, K., & Westermann, R. (1992). Basic fibroblast growth factor promotes transmitter storage and synthesis in cultured chromaffin cells. Brain Res Dev Brain Res, 65(2): 211-6.

Valenzuela, D.M., Maisonpierre, P.C., Glass, D.J., Rojas, E., Nunez, L., Kong, Y., Gies, D.R., Stitt, T.N., Ip, N.Y., & Yancopoulos, G.D. (1993). Alternative forms of rat TrkC with different functional capabilities. Neuron, 10: 963-974.

Vaux, D.L. (1993). Toward an understanding of the molecular mechanisms of physiological cell death. Proc Natl Acad Sci U S A, 90(3): 786-9.

Vaux, D.L., Aguila, H.L., & Weissman, I.L. (1992). Bcl-2 prevents death of factor-deprived cells but fails to prevent apoptosis in targets of cell mediated killing. Int Immunol 4(7): 821-4.

Vaux, D.L., Cory, S., & Adams, J.M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature, 335(6189): 440-2.

Veis, D.J., Sorenson, C.M., Shutter, J.R., & Korsmeyer, S.J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell, 75(2): 229-240.

Vekrellis, K., McCarthy, M.J., Watson, A., Whitfield, J., Rubin, L.L., Ham, J. (1997). Bax promotes neuronal cell death and is downregulated during the development of the nervous system. Development 124(6):1239-1249.

Walicke, P.A. (1988). Basic and acidic fibroblast growth factors have trophic effects on neurons from multiple CNS regions. J Neurosci, 8: 2618-2627.

Walicke, P.A., Cowan, W.M., Ueno, N., Baird, A., & Guillemin, R. (1986). Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. Proc Natl Acad Sci USA, 83: 3012-3016.

Wang, H.G., Millan, J.A., Cox, A.D., Der, C.J., Rapp, U.R., Beck, T., Zha, H. & Reed, J.C. (1995). R-Ras promotes apoptosis caused by growth factor deprivation via a Bcl-2 suppressible mechanism. J. Cell Biol. 129(4):1103-1114.

Wang, L., Miura, M., Bergeron, L., Zhu, H., Yuan, J. (1994). Ich-1, an Ice/ced-3-related gene, encodes both positive and negative regulators of programmed cell death. Cell 78(5):739-750.

Weston, J.A. (1962). A radioautographic analysis of the migration and localization of trunk neural crest cells in the chick. Dev. Biol., 6: 279-310.

Wilkinson, G.A., Fariñas, I., Backus, C., Yoshida, C.K., & Reichardt, L.F. (1996). Neurotrophin-3 is a survival factor in vivo for early mouse trigeminal neurons. J. Neurosci.,16: 7661-7669.

Willingham, M.C., & Bhalla, K. (1994). Transient mitotic phase localization of bcl-2 oncoprotein in human carcinoma cells and its possible role in prevention of apoptosis. J. Histochem Cytochem 42(4):441-450.

Wong, G.H.W., Elwell, J.H., Oberley, L.W., & Goeddel, D.V. (1989). Manganous superoxide dismutase is essential for cellular resistance to cytotoxicity of tumor necrosis factor. Cell.58: 923-31.

Wright, E.M., Vogel, K.S., & Davies, A.M. (1992). Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. Neuron, 9(1): 139-150.

Wyatt, S., & Davies, A.M. (1993). Regulation of expression of mRNAs encoding the nerve growth factor receptors p75 and trkA in developing sensory neurons. Development, 119(3): 635-648.

Wyatt, S., Shooter, E.M., & Davies, A.M. (1990). Expression of the NGF receptor gene in sensory neurons and their cutaneous targets prior to and during innervation. Neuron, 4(3): 421-7.

Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E. & Dyson, N.J. (1996). Tumor induction and tissue atrophy in mice lacking E2F-1.

Cell 85(4):537-548.

Yan, Q., Matheson, C., & Lopez, O.T. (1995). *In vivo* neurotrophic effects of GDNF on neonatal and adult facial motor neurons. Nature, 373: 341-344.

Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B., & Korsmeyer, S.J. (1995). Bad, a heterodimeric partner for Bcl-xL and Bcl-2, displaces Bax and promotes cell death. Cell, 80: 285-291.

Yin, X.M., Oltval, Z.N., & Korsmeyer, S.J. (1994). BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature, 369(6478): 321-3.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., & Horvitz, H.R. (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell, 75(4): 641-652.

Zha, H., Aime-Sempe, C., Sato, T. &Reed, J.C. (1996). Proapoptotic protein Bax heterodimerizes with Bcl-2 and homodimerizes with Bax via a novel domain (BH3) distinct from BH1 and BH2. J. Biol. Chem. 271(13):7440-7444.

Zhan, Q., Fan, S., Bae, I., Guillouf, C., Liebermann, D.A., O'Connor, P.M. & Formace, A.J. Jr. (1994). Induction of bax by genotoxic stress in human cells correlates with normal p53 status and apoptosis. Oncogene 9(12):3743-3751.

Zhan, X, Bates, B, Mu, X.G. & Goldfarb, M. (1988). The human FGF Soncogene encodes a novel protein related to fibroblast growth factors. Mol. Cell Biol. 8: 3487-95.

Zhang, K.Z., Westberg, J.A., Holtta, E., & Andersson, L.C. (1996). BCL2 regulates neural differentiation. Proc. Natl. Acad. Sci. U S A, 93:4504-4508.

Zhong, L.T., Kane, D.J., & Bredesen, D.E. (1993a). BCL-2 blocks glutamate toxicity in neural cell lines. Brain Res Mol Brain Res, 19(4): 353-5.

Zhong, L.T., Sarafian, T., Kane, D.J., Charles, A.C., Mah, S.P., Edwards, R.H., & Bredesen, D.E. (1993b). bcl-2 inhibits death of central neural cells induced by multiple agents. Proc. Natl. Acad. Sci. USA, 90(10): 4533-7.