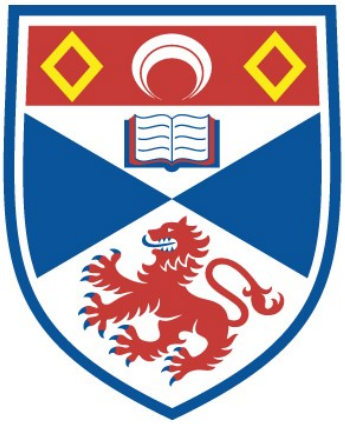
DEVELOPMENTAL CHANGES IN THE TROPHIC FACTOR RESPONSES OF PERIPHERAL NERVOUS SYSTEM NEURONS

Antony R. Horton

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



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DEVELOPMENTAL CHANGES IN THE TROPHIC FACTOR RESPONSES OF PERIPHERAL NERVOUS SYSTEM NEURONS

A Thesis Submitted for the Degree of Doctor of Philosophy

by

Antony R. Horton

September 1997.

School of Biomedical Sciences,
University of St. Andrews.

'Men ought to know that from the brain, and from the brain only , arise our pleasures joys, laughter, and jests, aswell as our sorrows, pains, griefs, and tears. Through it, in particular, we think, see, hear, and distinguish the ugly from the beautiful, the bad from the good, the pleasant from the unpleasant...'

-Hippocrates.

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Declarations

I, Antony Horton, hereby certify that this thesis, which is approximately 50 000 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..... signature of candidate.....

I was admitted as a candidate for the degree of Doctor of Philosophy in October 1993 at St. Andrews University. The higher study for which this is a record was carried out in the the University of St. Andrews between 1993 and 1997.

date..... signature of candidate.....

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy 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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Abstract

The aim of this project was to determine the neurotrophic factor survival requirements of PNS neurons during development and to clarify the role of certain receptors in mediating responsiveness. Members of the neurotrophin family of neurotrophic factors (NGF, BDNF, NT3 and NT4/5) and neurotrophic cytokines (CNTF, LIF, OSM, IL-6 and CT-1) were studied.

The activity of a recently identified neurotrophin, NT4/5, was investigated in vitro. In cultures of mouse neurons, mammalian NT4/5 promoted the survival of the same kinds of neurons as BDNF and was as potent as BDNF, which is consistent with the action of both neurotrophins on the same receptor, TrkB. However, both mammalian NT-4/5 and the *Xenopus* homologue were less potent than mammalian BDNF on chicken embryo neurons, which is consistent with the lower evolutionary conservation of NT4/5. Interestingly, mammalian NT4/5 exhibited differences in potency on certain populations of chicken neurons that responded equally well to BDNF, and this may reflect differences in TrkB receptors in these different populations of neurons.

To clarify the role of the common neurotrophin receptor in modulating the response of neurons to NGF, I then compared the actions of NGF with a mutated NGF protein that binds to TrkA, but does not bind to p75. At subsaturating concentrations, the NGF mutant was less effective than NGF in promoting the survival of embryonic sensory neurons and postnatal sympathetic neurons but was equally effective as NGF in promoting the survival of embryonic sympathetic neurons, indicating that binding of NGF to p75 enhances the sensitivity of NGF-dependent neurons to NGF at certain stages of development.

To investigate if neurotrophic cytokines act on developing sensory neurons, I studied their effects in vitro. Whereas trigeminal neurons were

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Abbreviations

BDNF Brain-derived neurotrophic factor

BSA Bovine serum albumin

cDNA Complementary deoxyribonucleic acid

ChAT Choline acetyl transferase

CMF-PBS Calcium and magnesium free phosphate-buffered saline

CNS Central Nervous System

CNTF Ciliary neurotrophic factor

CNTFRα Ciliary neurotrophic factor alpha

CT-1 Cardiotrophin-1

DRG Dorsal root ganglia

DMTG Dorso-medial trigeminal ganglion

E Embryonic day

EC50 Effective concentration at 50% survival

ERK1 Extracellular signal related kinase 1

ERK2 Extracellular signal related kinase 2

F12 Ham's nutrient mixture F-12

F14 Ham's nutrient mixture F-14

FGF Fibroblast growth factor

GDNF Glial cell line-derived neurotrophic factor

GDNFRα Glial cell line-derived neurotrophic factor recptor alpha

gp130 Glycoprotein-130

gp75 Glycoprotein-75

GPA Growth Promoting Activity

GPI Glycosyl phosphatidyl inositol

GM-CSF Granulocyte macrophage - colony stimulating factor

HBSS Hank's balanced salt solution

HIFCS Heat-inactivated foetal calf serum

HIHS Heat-inactivated horse serum

IC50 Inhibitory concentration at 50%

IL-6 Interleukin-6

IL-6Rα Interleukin-6 recptor alpha

Kb Kilobase

kD Kilodaltons

Kd Dissociation constant

L15 Leibovitz's L15 nutrient mixture

LIF Leukaemia inhibitory factor

LIFRβ Leukaemia inhibitory factor receptor beta

mRNA Messenger ribonucleic acid

MAPK Mitogen acivated protein kinase

NGF Nerve growth factor

NT-3 Neurotrophin-3

NT-4 Neurotrophin-4

NT-4/5 Neurotrophin-4/5

NT-5 Neurotrophin 5

NT-6 Neurotrophin-6

NTN Neurturin

NTNRα Neurturin receptor alpha

OSM Oncostatin-M

PI-3 Phosphatidyl inositol 3

PIPLC Phosphoinositol phospholipase C

P-ORN Poly-DL-ornithine

PBS Phosphate buffered saline

PLCγ-1 Phospholiapse C gamma-1

PNS Peripheral nervous system

RACE Rapid amplicication of cDNA ends

RNase Ribonuclease

RT-PCR Reverse transcription-polymerase chain reaction

SATO Sato's nutrient mixture

SC Sympathetic chain

SCG Superior cervical ganglion

SH2 Src-homology region 2

SOS Son-of-sevenless

TGFβ Transforming growth factor beta

TH Tyrosine hydroxlase

TK Tyrosine kinase

TRKA Tropomyosin kinase A

TRKB Tropomyosin kinase B

TRKC Tropomyosin kinase C

TMN Trigeminal mesencephalic nucleus

VLTG Ventrolateral trigeminal ganglion

CHAPTER 1

GENERAL INTRODUCTION

1.1 Early Development in the Vertebrate Nervous System.

Early development of the vertebrate nervous system commences with a process called neurulation. With the exception of the neurons of certain cranial sensory ganglia, all elements of the central and peripheral nervous systems are derived from a specialised region of ectoderm that lies along the dorsal midline of the developing embryo. This ectoderm thickens and under the influence of underlying structures, rolls up to form a hollow groove. The lateral edges of this groove then begin to fuse rostrally and caudally. When the process of fusion is complete, the original neural groove is converted into a structure called the neural tube. This neural tube then separates from the overlying ectoderm and forms the main elements of the central nervous system. At the point where the neural tube and ectoderm separate, certain cells detach from these structures to form a continous column called the neural crest. Cells of the neural crest give rise to most of the peripheral nervous system (PNS) incorporating most sensory and autonomic neurons, various support cells within the PNS and Schwann cells. A subset of cranial sensory neurons are not derived from the neural crest but from regions of epithelial thickening that appear in the cephalic ectoderm of the early vertebrate embryo (Von Kuppfer, 1894).

1.2 Development of Sensory Neurons.

The differentiation of sensory neurons proceeds after migration of their progenitor cells from the neural crest and neurogenic placodes to the site of the sensory ganglia, and is characterised by the appearance of axonal processes and the expression of certain neuron specific proteins, such as neurofilament protein (Le Douarin, 1986).

The neural crest gives rise to most of the neurons of the cranial and spinal sensory ganglia, and postganglionic neurons of the sympathetic and to the neurons of the autonomic and enteric nervous systems. The neurons of certain cranial ganglia, however, are derived either entirely from neurogenic placodes or from combinations of placodal and neural crest cells. In cranial sensory ganglia, the placode-derived neurons are always located distally with respect to neurons from the neural crest (Le Douarin, 1982). The origin of neurons in avian cranial sensory ganglia has been determined by a series of mapping experiments involving the transplantation of neural crest and placode derived cells (Weston, 1962; Le Douarin,1973). The result of these experiments are summarised in table 1.1. The cranial sensory ganglia, with the exception of a sub-population of neural-crest derived sensory neurons in the embryonic mid-brain (trigeminal mesencephalic nucleus or TMN), are situated on five of the twelve pairs of cranial nerves. The cell bodies of peripheral sensory neurons are located in the dorsal root ganglia, situated at the dorsal root of the spinal nerves, and in the cranial sensory ganglia.

Ganglion	Nerve	Embryonic origin
Trigeminal	V	
dorsomedial		Neural crest
ventrolateral		Placode
TMN		Neural Crest
Geniculate	VII	Placode
Vestibular	VIII	Otic placode
Acoustic	VIII	Otic placode
Jugular-superior	IX/X	Neural Crest
Petrosal	IX	Placode
Nodose	X	Placode

Table 1.1 Origins of the avian cranial sensory ganglia

1.3 Programmed Cell Death During Nervous System Development.

A striking feature in the development of the vertebrate nervous system is the occurrence of extensive neuronal death. After sensory neurons have differentiated within sensory ganglia axonal outgrowth to peripheral and central targets commences. The peripheral targets comprise tissues that are innervated by the sensory axons, whereas the central targets comprise second order neurons with which the sensory neurons synapse. During the period when neurons are innervating these targets, appropriate connections are established by means of selection. This selection is achieved by the production of limiting quantities of neurotrophic factors that are synthesised by the target tissues shortly after innervation commences. Inappropriately connected neurons do not receive an adequate supply of trophic factor and undergo a series of molecular events that ultimately results in their degeneration and death (for review see Oppenheim, 1991).

Two types of morphological change that accompany cell death have been distinguished. One is termed *necrosis,* and is characterised by cellular oedema that results in rupture of the plasma membrane and leakage of the cellular contents (Kerr *et al.*, 1972). The other degenerative process, termed *apoptosis*, involves a progressive contraction of cell volume accompanied by nuclear condensation and degradation of DNA into oligonulceosomal fragments (Wyllie *et al.*, 1980). During development, neurons die by apoptosis, whereas necrosis generally occurs in pathological situations. Four main features are now thought to typify the apoptotic cell death observed in animal cells. These are: constitutive expression of cell death proteins, degeneration involving a proteolytic cascade, control by intracellular regulatory proteins, and activation of the death program by gene transcription (Jacobson *et al.*, 1997).

Genetic studies of two invertebrates, the nematode *Caenorhabditis elegans* and *Drosophila melanogaster*, have provided most of the insight into

the genes regulating apoptosis. There is now a growing body of evidence to suggest that many of these proteins have been conserved throughout animal evolution (Hengartner *et al.*, 1996). The constitutive expression of cell death proteins was initially discovered in genetic studies of *C. elegans* that identified two genes, called *ced-3*, and *ced-4*, that are critical for the somatic cell death that occurs during nematode development (Chinnaiyan and Dixit, 1996; Jacobson *et al.*, 1996 for reviews). Mutation of either *ced-3* or *ced-4*, abolishes the apoptotic capability of the cells that are required to die during *C. elegans* development (Chinnaiyan and Dixit, 1996). Several mammalian homologues of the CED-3 protein have recently been identified. The first of these, interleukin-1β converting enzyme (ICE), is a cysteine protease necessary for the processing of pro-IL-1β into its active form (Yuan *et al.*, 1993). To date, around six homologues of CED-3 and ICE have been characterised and several more have been identified as expressed sequence tags (ESTs) of human genes comprising a new cysteine protease family termed 'caspases' (for cysteine aspase) (Chinnaiyan and Dixit, 1996; Jacobson *et al.*, 1996). All caspases are known to cleave their substrates after specific aspartic acids. In addition, they can activate themselves or other caspases *in vitro*, leading to the suggestion that they act in a proteolytic cascade (Nagata *et al.*, 1996). A further intracellular mechanism for the regulation of cell death has been conserved during evolution. The *ced-9* gene acts to inhibit cell death in *C. elegans*, and is homologous to the *bcl-2* gene which acts to inhibit cell death in mammals (Jacobson *et al.*, 1996). A number of proteins that have homology to Bcl-2 have now been identified in mammals. Some homologous proteins, such as Bcl-X_L inhibit apoptosis, whereas others, such as Bax and Bad, promote apoptosis (Chinnaiyan and Dixit, 1996; Jacobson *et al.*, 1996). Studies of *Drosophila melanogaster* have been useful in determining how cell death effectors may be activated (Chinnaiyan *et al.*, 1996). The *reaper* gene encodes a 65 amino acid residue polypeptide that is upregulated during cell death in *Drosophila* and is capable of integrating signals from several pathways to activate the apoptotic death program (White *et al.*,

1994; White *et al.*, 1996). The means by which *reaper* effects cell death is unclear, however, it is known to be transcriptionally upregulated to signal death and it possesses weak homology with the so-called 'death domain' of certain mammalian proteins, including Fas/APO-1, and TNFR-1 (Golstein *et al.*, 1995).

1.4.1 Control of Neuronal Survival by Neurotrophic Factors

Classic work, showing that sympathetic and certain sensory neurons require a supply of an exogenously derived agent during a critical stage of their development, led to the discovery of nerve growth factor (NGF) (reviewed by Levi-Montalcini, 1987). In addition to this work, studies demonstrating that NGF is synthesised in the target fields of NGF-dependent neurons in proportion to their innervation density during development (Korsching and Thoenen, 1983a; Heumann *et al.*, 1984; Shelton and Reichardt, 1984; Harper and Davies, 1990), and that NGF is delivered from these tissues to the cell bodies of the innervating axons by rapid axonal transport (Hendry *et al.*, 1974; Korsching and Thoenen, 1983b), provided a basis for the "neurotrophic theory".

The neurotrophic theory proposes that target fields directly influence the size of populations of neurons innervating them (reviewed by Purves *et al.*, 1988). Since a substantial number of neurons that innervate a particular target are lost by cell death shortly after target encounter (Oppenheim, 1991), limited availability of a trophic factor or factors in the target fields permits the survival of the required number of neurons (Thoenen and Barde, 1980). The striking changes in sensory and sympathetic innervation induced by addition of anti-NGF antibodies to neonatal mice (Levi-Montalcini, 1987), and more recently targeted mutation of the gene encoding NGF (Crowley *et al.*, 1994), has provided confirmation of the neurotrophic theory.

The neurotrophic theory is further reinforced by studies of the site and timing of NGF synthesis and NGF receptor expression during development. It has been demonstrated that the commencement of NGF synthesis in the target fields of NGF-dependent sensory and sympathetic neurons is concomitant with the onset of target field innervation (Davies *et al.*, 1987a;Korsching and Thoenen, 1988).

Additionally, it has also been shown that NGF receptors are not expressed until sensory neurons innervate their targets (Davies *et al.*, 1987a) suggesting that NGF cannot affect the development of these neurons at earlier developmental stages.

The discovery of a second neurotrophin, BDNF (Barde *et al.*, 1982), extended the neurotrophic theory still further. BDNF has been shown to promote the survival of subsets of embryonic neurons *in vitro* and can rescue these neurons if presented *in vivo* during the period of naturally occurring cell death (Hofer and Barde, 1988). Since the cloning of BDNF and several structurally related proteins, it has become apparent that NGF is part of a homologous family of proteins termed the "neurotrophins". To date, a total of six members of the neurotrophin family have been identified. Other known members of this family include, NT-3 (Ernfors *et al.*, 1990a; Hohn *et al.*, 1990; Jones and Reichardt, 1990 Maisonpierre *et al.*, 1990a; Rosenthal *et al.*, 1990), *Xenopus* NT-4 (Hallböök *et al.*, 1991), mammalian NT-4/5 (Berkemeier *et al.*, 1991; Ip *et al.*, 1992a) and NT-6 (Götz *et al.*, 1994).

Nerve Growth Factor

The initial discovery that a fraction of snake venom had nerve growth promoting activity (Cohen *et al.*, 1956) led to the isolation and purification of a non-dialyzable, heat-labile protein (Cohen, 1959). Subsequent findings demonstrated that the mouse submandibular salivary gland was an extremely rich source of this protein, termed nerve growth factor (Cohen, 1960).

NGF derived from the mouse submandibular gland is an acidic protein consisting of α, β, and γ subunits, with a molecular weight of 130kD (Varon *et al.*, 1968). Further work established that biological activity is conferred by the NGF β subunit, a 26kD dimer consisting of two identical peptides, each containing 118 amino acids (Angelletti and Bradshaw, 1971; Angelletti *et al.*, 1973; Green and Shooter, 1980). Recently, a detailed analysis of the tertiary structure of NGF using X-ray diffraction, has revealed an arrangement of three anti-parallel pairs of β strands that together form a flat surface where the two subunits associate (McDonald *et al.*, 1991). The NGF molecule possess four loop regions that contain many of the residues that vary between the neurotrophins, suggesting that these regions confer binding specificity to the neurotophin receptors (reviewed by Ebendal, 1992). Further functional analyses of modified and truncated NGF have demonstrated that certain amino acid residues contribute to the specificity of binding of NGF to its receptors, and to the structural stability of the molecule (Ibáñez *et al.*, 1990; Kahle *et al.*, 1992). Proteolytic cleavage of the first 9 amino acids at the amino terminus of recombinant human NGF, results in a 300-fold decrease in the affinity of NGF for TrkA (the NGF receptor tyrosine kinase), suggesting that these 9 residues are important for TrkA activation (Kahle *et al.* 1992). In addition, amino acid substitution of valine to alanine at position 21 considerably reduces the biological activity and receptor binding capacity of NGF (Ibáñez *et al.*, 1990).

Analysis of a mouse cDNA clone has shown that NGF is generated from a 305 amino acid precursor, termed pre-proNGF (Scott *et al.*, 1983). The NGF gene has also been isolated from several other species, and nucleotide sequence analysis has shown that the mature NGF protein has been highly conserved during evolution (Ullrich *et al.*, 1983; Meier *et al.*,1986; Ebendal *et al.*, 1986; Selby *et al.*, 1987; Whittemore *et al.*, 1988; Schwarz *et al.*, 1989). The mouse NGF gene spans 45 kb and consists of five exons separated by four introns. The mature NGF protein is encoded by a single 3' exon. Four transcripts encoding precursor proteins that differ in their amino termini are produced by alternative splicing of the 3' exon and the remaining 5' exons (Selby *et al.*, 1987). The two largest transcripts encode precursor proteins with molecular weights of 27kD and 34kD (Edwards *et al.*, 1988). The mRNA encoding the 27kD protein, possesses a hydrophobic signal peptide at the NH_2 terminus and is more abundant in most tissues. The 34kD precursor predominates in the mouse salivary gland and has a hydrophobic signal peptide 70 residues downstream from the initiation codon. Both proteins are further processed by glycosylation and proteolytic cleavage to give the biologically active mature form of NGF (Edwards *et al.*, 1988). The functional significance of the different transcripts is at present unknown.

The expression of NGF mRNA has been detected by sensitive hybridization techniques that have identified extremely low levels of NGF mRNA in RNA extracted from different tissues (Heumann *et al.*, 1984; Shelton and Riechardt, 1984), and in tissue sections (Bandtlow *et al.*, 1987). Careful examination of these NGF synthesising tissues has ascertained that many of them are innervated by NGF responsive neurons (Korsching and Thoenen, 1983a; Heumann *et al.*, 1984; Shelton and Reichardt, 1984; Korsching *et al.*, 1985). The exact identity of target cells that secrete NGF during normal development was first elucidated in a detailed study of the developing whisker pad in mouse embryos (Davies *et al.*, 1987a). Enzymatic dissociation of the

developing whisker pad into its main components, the epithelium (presumptive epidermis) and mesenchyme (presumptive dermis), and subsequent analysis of NGF expression demonstrated that the highest levels of NGF were expressed in the epithelium (Davies *et al.*, 1987a). Furthermore, subsequent work has demonstrated that the concentration of NGF mRNA in the epithelia of the opthalmic, maxillary and mandibular territories each of the trigeminal ganglion, is related to the innervation density of the territory (Harper and Davies, 1990). In conjunction with the spatial distribution of NGF mRNA, it has also been shown that NGF production in the target tissues coincides with the onset of target innervation, suggesting that NGF regulates the number of innervating neurons after the target cells have been contacted (Davies *et al.*, 1987a; Korsching and Thoenen, 1988; Harper and Davies, 1990). In addition to cells that normally receive innervation from NGF dependent neurons, several other cell types have been observed to express NGF both *in vivo* and *in vitro*. For example, NGF has been shown to be expressed *in vitro* by Schwann cells (Bandtlow *et al.*, 1987), astrocytes (Lindsay, 1979), and glial cell lines (Longo *et al.*, 1978). However, studies of the distribution of NGF mRNA by *in situ* hybridization in the adult rat brain, have demonstrated that regions where these types of cells occur *in vivo* are not clearly labelled, in contrast to the clear labelling of hippocampal pyramidal neurons (Ayer-LeLievre *et al.*, 1988).

The principal populations that are supported by NGF are nociceptive and thermoceptive neurons of both the spinal dorsal root ganglia and cranial sensory ganglia (reviewed by Davies 1994a). Mice that have undergone targeted disruption of the NGF gene display deficits in their responses to noxious mechanical stimuli. Furthermore, histological analysis of these mice has revealed exceptional cell loss in trigeminal and dorsal root sensory ganglia, and sympathetic ganglia (Crowley *et al.*, 1994). Significantly, the cell loss within dorsal root ganglia was restricted to subsets of neurons that were found to convey nociceptive and thermoceptive information (Crowley *et al.*, 1994). In

accordance with this study, overexpression of NGF in the epidermis of transgenic mice results in hypertrophy of sensory and sympathetic ganglia (Albers *et al.*, 1994). In contrast to previous studies suggesting an involvement of NGF in the development of basal forebrain cholinergic neurons, these neurons develop normally in mice with the NGF null mutation (Crowley *et al.*, 1994). Taken together, these findings confirm that sensory and sympathetic neurons are critically dependent on NGF.

Brain Derived Neurotrophic Factor

Brain derived neurotrophic factor isolated from pig brain was the second member of the neurotrophin family to be characterised. BDNF is a basic protein (pI ≥ 10.1), with a molecular weight of 12.3 kD (Barde *et al.*, 1982). The mature BDNF protein is 119 amino acids in length and, like NGF, exists as a stable homodimer linked by 3 disulphide bridges (Radziejewski *et al.*, 1992) BDNF displays approximately 50% sequence identity to NGF, including conservation of all six of the structurally important cysteine residues (Leibrock *et al.*, 1989). Further characterisation of BDNF has revealed that the BDNF gene spans 4.0 kb, encodes four 5' exons and possesses one 3' exon which encodes the mature protein (Timmusk *et al.*, 1993a; Metsis *et al.*, 1993). In mammals, BDNF is predominantly expressed in the CNS, where it is present at moderate levels in several different regions including the cingulate and piriform cortices, certain thalamic and hypothalamic nuclei, and in the postnatal hippocampus (Liebrock *et al.*, 1989; Ernfors *et al.*, 1990a; Ernfors *et al.*, 1990b; Maisonpierre *et al.*, 1990b; Phillips *et al.*, 1990; Friedman *et al.*, 1991; Huntley *et al.*, 1992; Metsis *et al.*, 1993). The expression of BDNF mRNA has also been demonstrated in the developing spinal cord and limb bud of the rat during the period when motor neurons are known to undergo programmed cell death (Henderson *et al.*, 1993). Similarly, BDNF expression has been observed at early developmental stages in the chick, within the developing otic vesicle, dorsal mesenchyme (adjacent to the developing wing bud), and in the optic tectum prior to the arrival of retinal ganglion cell axons (Hallböök *et al.*, 1993; Herzog *et al.*, 1994). Certain subpopulations of sensory and motor neurons express BDNF mRNA at early developmental stages, consistent with a proposed role in the maturation of these cells (Ernfors *et al.*, 1990b; Wetmore *et al.*, 1990; Schecterson and Bothwell 1992; Wright *et al.*, 1992). Interestingly, BDNF mRNA has been detected in the whisker pad and the mandible of the developing mouse, structures which are innervated by the mandibular branch of the trigeminal ganglion (Schecterson

and Bothwell, 1992). During development, the highest BDNF mRNA expressing regions are regions that are sensitive to touch, such as the digits, lips, tongue and mandible that contain specialised mechanoreceptors, called Merkel disks (Schecterson and Bothwell, 1992). While the expression of BDNF mRNA has generally been observed in the target tissues of innervating populations of BDNF responsive neurons, surprisingly, BDNF is also expressed in three types of sensory ganglia that originate from different embryonic sources in E8.5 mouse embryos (Schecterson and Bothwell, 1992). Neural crest derived dorsal root sensory ganglia, placode derived geniculate ganglia, and neurons of the trigeminal ganglia, that are derived from both sources, have all been observed to express BDNF mRNA, suggesting that an autocrine mechanism may operate within these ganglia, allowing early developing neurons to provide their own trophic support (Schecterson and Bothwell, 1992).

In agreement with its observed patterns of expression, BDNF has been shown to support different populations of peripheral and central neurons. Certain populations of placode and neural crest-derived sensory neurons that do not respond to NGF survive in the presence of BDNF *in vitro* (Lindsay *et al.*, 1985; Davies *et al.*, 1986a; Davies *et al.*, 1987b). Specific populations of central neurons have similarly been shown to respond to BDNF including embryonic motor neurons (Oppenheim *et al.*, 1992; Yan *et al.*, 1992; Sendtner *et al.*, 1992a; Koliatsos *et al.*, 1993), dopaminergic neurons from the substantia nigra (Hyman *et al.*, 1991; Knüsel *et al.*, 1991), cultured cerebellar granule neurons (Lindholm *et al.*, 1993a) and cells of the developing and adult retinal ganglion (Johnson *et al.*, 1986a; Thanos *et al.*, 1989). In addition to its survival promoting effects, BDNF is also known to influence the differentiation (Kalcheim and Gendreau 1988) and maturation of developing sensory neurons (Wright *et al.*, 1992) and certain central neurons (Marty *et al.*, 1996). Furthermore, it has been suggested that BDNF may act via an autocrine mechanism in sensory neurons (Wright *et al.*, 1992; Acheson *et al.*, 1995). In support of this proposal, the early

maturational change that is observed in E4.5 chick DRG neurons in response to BDNF, is retarded by antisense BDNF oligonucleotides (Wright *et al.*, 1992). Similarly, adult dorsal root sensory neurons which express BDNF mRNA, undergo a dose dependent decrease in survival, when treated with BDNF antisense oligonucleotides *in vitro* (Acheson *et al.*, 1995).

Homozygous mutant mice lacking the BDNF gene display substantially reduced numbers of cranial and spinal sensory neurons. In particular, significant numbers of sensory neurons are lost in the trigeminal, geniculate and vestibular ganglia and in the nodose-petrosal complex (Ernfors *et al.*, 1994a; Jones *et al.*, 1994). Surprisingly, in contrast to previous studies showing that motor neurons are supported by BDNF *in vivo* (Yan *et al.*, 1992; Oppenheim *et al.*, 1992; Sendtner *et al.*, 1992) and *in vitro* (Vejsada *et al.*, 1995), motor neuron development was largely unperturbed by targeted disruption of the BDNF gene (Ernfors *et al.*, 1994a; Jones *et al.*, 1994). Although the CNS of BDNF deficient mice shows no gross structural abnormalities, the expression of neuropeptide Y and calcium binding proteins is altered in many neurons, suggesting that these neurons may not function normally (Ernfors *et al.*, 1994a; Jones *et al.*, 1994).

Neurotrophin-3

Neurotrophin-3 was isolated and cloned as a result of a search for proteins homologous to NGF and BDNF. This protein, like NGF and BDNF, has been shown to have survival promoting effects on both central and peripheral neurons.

Polymerase chain reaction (PCR) using oligonucleotide primers based on amino acid sequence similarities in the NGF and BDNF coding sequences has been used to clone mouse (Hohn *et al.*, 1990), rat (Maisonpierre *et al.*, 1990a), and human (Jones and Reichardt, 1990; Rosenthal *et al.*, 1990) NT-3. The mature NT-3 polypeptide consists of 119 amino acid residues, has a pI of 9.3, and a relative molecular mass of 13.6kD (Hohn *et al.*,1990; Maisonpierre *et al.*, 1990a). Several regions of similarity are conserved between NGF, BDNF and NT-3, including all six of the cysteine residues (Hohn *et al.*, 1990). Sequence alignment of human NT-3 and BDNF predicts that NT-3 is synthesised in precursor form as a 257 amino acid protein (Rosenthal *et al.*, 1990; Jones and Reichardt, 1990). In comparison with BDNF and NGF, NT-3 is known to possess several regions of variability which lie in consensus with other neurotrophins (Hohn *et al.*, 1990; Ibáñez *et al.*, 1991). Whereas the variable regions of NGF and BDNF are thought to convey specificity of binding to their respective Trk tyrosine kinase receptors, the specificity of NT-3 binding to TrkC (the NT-3 high-affinity receptor) is conveyed by a central β strand and not by the variable loop regions of the molecule (Urfer *et al.*, 1994).

The NT-3 gene has been structurally analysed using genomic cloning, and rapid amplification of cDNA ends (RACE). These studies have shown that the gene consists of two small upstream exons and a larger downstream exon (Leingärtner and Lindholm, 1994). Two classes of NT-3 transcript are generated by alternative splicing of the small exons to the large exon (Leingärtner and Lindholm, 1994). Multiple mRNA transcripts of differing lengths can be

produced by combining the several transcription start sites in the small exons with three polyadenylation sites that are present in the large exon (Leingärtner and Lindholm, 1994). Regulation of NT-3 gene transcription by two promoters was demonstrated by transfection of cerebellar granule neurons with a DNA construct comprising the small exons and a reporter gene (Leingärtner and Lindholm, 1994). Additionally, it has been established that the NT-3 promoters contain silencer elements that control the differential expression of NT-3 transcripts in neurons and non-neuronal cells (Shintani *et al.*, 1993; Leingärtner and Lindholm, 1994).

During development, the expression of NT-3 mRNA is seen in several tissues that receive innervation from the PNS, and in several regions of the CNS. Northern blot analysis has demonstrated the expression of NT-3 mRNAs in the heart, kidney, liver, spleen, intestine and lung, and in several brain regions including the cerebellum, medulla oblongata and hippocampus in rodents. This expression pattern suggests that NT-3 could serve as a target derived factor for sensory and sympathetic neurons (Maisonpierre *et al.*, 1990a; Rosenthal *et al.*, 1990). In developing tissues, the expression of NT-3 appears to coincide with the onset of neuritogenesis, and a dramatic increase in overall expression is seen between E11 and E12 (Maisonpierre *et al.*, 1990b). The general expression of NT-3 (measured by Northern blotting) in the chicken embryo is strongest at E4.5 and subsequently decreases throughout development (Hallböök *et al.*, 1993). This is in broad agreement with other studies that have demonstrated a gradual developmental decrease in NT-3 expression in mammalian tissues, however, in mammalian tissues maximal expression usually occurs at slightly later ages (Rosenthal *et al.*, 1990; Maisonpierre *et al.*, 1990b; Buchman and Davies, 1993). The spatial distribution of NT-3 mRNA expression has been demonstrated by *in situ* hybridization in the developing chicken and rat, and is seen in the E4 chick epidermis, in the region of the branchial arches, and in the rat it is detectable in tissues of the inner ear, the iris and, significantly, in target fields of the

trigeminal ganglion, especially the epithelium of the whisker follicles (Hallböök *et al.*, 1993; Pirvola *et al.*, 1992; Ernfors *et al.*, 1992). Confirmation of the expression of NT-3 in the developing trigeminal target territory has been shown using quantitative Northern blotting. NT-3 mRNA is expressed at the highest levels in the epithelium of the most densely innervated target field, the maxillary target field, at E13, the age at which naturally occurring cell death commences in the trigeminal ganglion (Buchman and Davies, 1993). The widespread expression of NT-3 mRNA has also been demonstrated in muscle that is innervated by proprioceptive sensory neurons of the dorsal root ganglia (Ernfros *et al.*, 1990b; Hohn *et al.*, 1990; Maisonpierre *et al.*, 1990a; Rosenthal *et al.*, 1990; Schecterson and Bothwell 1992; Henderson *et al.*, 1993).

In the PNS, several studies have shown that NT-3 plays a role in the development of different populations of sensory and sympathetic neurons. Principally, NT-3 has been shown to promote the survival of developing muscle sensory neurons of the lumbar and cervical dorsal root ganglia, both *in vitro* (Wright *et al.*, 1992; Hory-Lee *et al.*, 1993; Gaese *et al.*, 1994) and *in vivo* (Ericksson *et al.*, 1994; Oakley *et al.*, 1995). Additionally, several other populations of sensory neurons are known to respond to NT-3, these include proprioceptive neurons isolated from the trigeminal mesencephalic nucleus of the E8 chick (Hohn *et al.*, 1990) and neurons of the spiral (Gao *et al.*, 1995; Ernfors *et al.*, 1995), nodose (Gaese *et al.*, 1994; Ockel *et al.*, 1996), and trigeminal ganglia (Buchman and Davies, 1993 Wilkinson *et al.*, 1996). Interestingly, trigeminal neurons only respond transiently to NT-3, undergoing a switch in trophic dependence from NT-3 to NGF, concomitant with observed changes in the level of NT-3 mRNA in the peripheral trigeminal target field (Buchman and Davies, 1993).

Other populations of PNS neurons respond to NT-3 in addition to sensory neurons. One study has shown that sympathetic neurons of the superior cervical ganglion undergo a switch in trophic dependence from NT-3 to NGF

shortly after formation of the ganglion (Birren *et al.*, 1993). Furthermore, the early development of sympathetic and sensory neurons may be more subtly influenced by NT-3. For example, sympathetic neuroblasts proliferate in response to NT-3 *in vitro* (DiCicco-Bloom *et al.*, 1993) and cultured DRG neurons undergo an enhanced maturational change in the presence of NT-3 (Wright *et al.*, 1992).

Certain populations of central neurons also respond to NT-3 during development. Dissociated hippocampal neurons show a moderate survival response in the presence of NT-3 *in vitro* (Ohsawa *et al.*, 1993) and sub-populations of hippocampal neurons express the immediate early gene cFos in response to NT-3 (Collazo *et al.*, 1992). Developing cerebellar granule neurons have also been shown respond to NT-3 by the induction of cFos *in vitro* (Segal *et al.*, 1992). In addition, these neurons synthesise NT-3 after addition of tri-iodo-thyronine, which in turn promotes hypertrophy and neuronal sprouting of Purkinje cells (Lindholm *et al.*, 1993).

In the adult CNS, NT-3 may play a role in regulating the response of different classes of neurons to injury. In noradrenergic neurons of the locus coeruleus, sustained application of different neurotrophins *in vivo* has shown that NT-3, but not NGF or NT-4/5, can prevent degeneration after excitotoxic lesion by 6-hydroxydopamine (Areñas and Persson, 1994). Additionally, NT-3 can enhance the collateral sprouting of corticospinal tract neurons after lesion (Schnell *et al.*, 1994), and has been shown to promote the survival of embryonic motor neurons *in vitro* (Henderson *et al.*, 1993). In contrast, experimental work conducted *in vivo* has provided some conflicting evidence as to the efficacy of NT-3 on motor neuron survival. For example, injection of NT-3 into neonatal rats does not effectively rescue axotomized motor neurons (Sendtner *et al.*, 1992a, Koliatsos *et al.*, 1993). Additionally, oligodendrocyte precursor cells from the optic nerve have been shown to survive and differentiate in response to

NT-3, suggesting that NT-3 may influence the development of different classes of non-neuronal cells within the CNS (Barres *et al.*, 1994),.

Recently, the function of NT-3 has been further clarified by several studies employing gene targeting in embryonic stem cells, to ablate the NT-3 coding sequence in mice (Ernfors *et al.*, 1994b; Fariñas *et al.*, 1994; Tessarollo *et al.*, 1994). Lack of NT-3 leads to severe deficits in the complement of sensory and sympathetic neurons. Neuronal loss in the PNS is principally observed in 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.*, 1994b; Fariñas *et al.*, 1994; Tessarollo *et al.*, 1994). The most severely affected neuronal population are proprioceptive sensory neurons of the dorsal root ganglia and their peripheral sense organs, the muscle spindles and Golgi tendon organs (Ernfors *et al.*, 1994b). The loss of this particular neuronal population was subsequently established by immunostaining for carbonic anhydrase (CA) and parvalbumin (PV), which are both markers for proprioceptive neurons. Both markers are absent in the dorsal root ganglia of NT-3$^{-/-}$ mutant animals (Ernfors *et al.*, 1994b)

In contrast with the severe effects that NT-3 gene deletion has on PNS neurons, neurons within the CNS do not appear to be seriously perturbed by the mutation (Ernfors *et al.*, 1994b). Although these findings may not agree with many previous *in vitro* studies on the effects of NT-3, they are consistent with *in vivo* work showing that NT-3 does not effectively promote the survival of motor neurons after transection (Sendtner *et al.*, 1992a; Koliatsos *et al.*, 1993).

More detailed analyses of mice lacking the NT-3 gene have identified deficiencies in specific subtypes of cutaneous mechanoreceptors, using electrophysiological techniques (Airaksinen *et al.*, 1996). In this study, heterozygous (+/-) animals were used, since the homozygous animals die shortly after birth. The data obtained demonstrate that two functionally specific subsets

of cutaneous afferents: low threshold slowly adapting mechanoreceptors (SA1 neurons) that respond tonically to skin indentation, and low threshold D-hair afferents that respond dynamically to skin stimulation, both require NT-3 during postnatal development (Airaksinen *et al.*, 1996). The number of receptor organs (Merkel cells) of the low threshold mechanoreceptors are decreased in NT-$3^{+/-}$ animals (Airaksinen *et al.*, 1996). Furthermore, the regulation of Merkel cell number by NT-3 has been verified by a study showing that elevated levels of NT-3 in the epidermis, substantially increase the total number of these cells (Albers *et al.*, 1996). Subsequent research has also shown that NT-3 is required for the early development of sensory precursor cells. In two separate studies sections of E11 DRGs from NT-$3^{-/-}$ mice were analysed by the TUNEL method which visualises apoptotic cells containing fragmented DNA. These studies show that some DRG neurons from NT-$3^{-/-}$ mice undergo excessive cell death prior to the developmental period of programmed cell death (ElShamy and Ernfors, 1996; Fariñas *et al.*, 1996). In addition, in contrast to earlier published work suggesting that sympathetic neuroblasts of the developing superior cervical ganglion (SCG) are transiently dependent on NT-3 for survival (Birren *et al.*, 1993), a recent detailed study of NT-$3^{-/-}$ animals has revealed that these ganglia develop normally, and that deficiencies in the SCG of these mice, occur late in development (Wyatt *et al.*, 1997).

Neurotrophin-4/5

A fourth neurotrophin with survival promoting activity was recently discovered in *Xenopus* and mammalian embryos, and has been designated neurotrophin-4/5.

The gene for neurotrophin-4/5 was first isolated in a phylogenetic analysis of DNA sequences from known members of the neurotrophin family (Hallböök *et al.*, 1991). An examination of NGF, BDNF and NT-3 genes from human, rat, chicken, viper, *Xenopus*, salmon and ray was carried out by PCR. This study showed that a fourth neurotrophin - designated; NT-4 - exists in both *Xenopus* and viper (Hallböök *et al.*, 1991). The *Xenopus* NT-4 gene encodes a precursor protein of 236 amino acids that is processed into a 123 amino acid mature form. *Xenopus* NT-4 has 50-60% amino acid identity with NGF, BDNF and NT-3, including several of the structurally important cysteine residues (Hallböök *et al.*, 1991). A mammalian NT-4 homologue was later identified and cloned, using sequences of the *Xenopus* and viper NT-4 (Ip *et al.*, 1992a). Analysis of human placental DNA by the polymerase chain reaction also revealed the existence of a neurotrophin, termed NT-5 (Berkemeier *et al.*, 1991). Since the sequences of mammalian NT-4 and NT-5 are identical, the mammalian NT-4 homologue is now referred to as NT-4/5.

Northern blot analysis and RNase protection assays have shown that mRNA for NT4/5 is present in several peripheral tissues, and brain regions during development (Berkemeier *et al.*, 1991; Ip *et al.*, 1992a; Timmusk *et al.*, 1993b). The levels of NT-4/5 are differentially regulated in the heart, liver and muscle, with a gradual decrease in expression from E13 to P1 (Timmusk *et al.*, 1993b). In adult rats, tissues which synthesise NT-4/5 include the thymus, muscle, lung, ovary, prostate, brain, heart, stomach and kidney (Berkemeier *et al.*, 1991; Ip *et al.*, 1992a). The expression of NT-4/5 mRNA has additionally been detected in several developing brain regions, including the cerebrum,

cerebellum, hippocampus, parts of the limbic system (Timmusk *et al.*, 1993b) and in the developing rat limb bud (Henderson *et al.*, 1993). Developmental variation of NT-4/5 expression has been demonstrated in structures that are innervated by neurons of the trigeminal ganglion, with the maximum levels of NT-4/5 expressed between E10 - E13 in the developing trigeminal target territory (Ibañez *et al.*, 1993; Arumäe *et al.*, 1993).

Several studies have recently demonstrated that different populations of neurons from the peripheral and central nervous system respond to NT-4/5 during development and in adulthood. In the peripheral nervous system, the survival promoting effects of NT-4/5 have been demonstrated on several populations of sensory neurons, including neurons of the early trigeminal, jugular and nodose ganglia (Ibáñez *et al.*, 1993; Davies *et al.*, 1993a), and neurons of the dorsal root and paravertebral sympathetic ganglia (Hallböök *et al.*, 1991; Berkemeier *et al.*, 1991). Additionally, neurons of the vestibular and spiral ganglia in the inner ear can be rescued from the detrimental effects of different neurotoxins, by administration of NT-4/5 both *in vitro* and *in vivo* (Zheng *et al.*, 1995a; Zheng *et al.*, 1995b).

In the central nervous system, it has been elucidated that NT-4/5 is an effective agent for preventing the death of developing and adult spinal motor neurons (Henderson *et al.*, 1993; Friedman *et al.*, 1995; Funakoshi *et al.*, 1995) and facial motor neurons (Koliatsos *et al.*, 1994). Furthermore, NT-4/5 can effect changes in the neurotransmitter phenotype of developing motor neurons (Wong *et al.*, 1993). In addition to its effects upon motor neurons, certain other populations of neurons within the CNS also show responses to NT-4/5. For example, rat retinal ganglion cells within the developing and adult visual system respond to NT-4/5 both *in vivo* and *in vitro* (Cui *et al.*, 1994; Cohen *et al.*, 1994; Sawai *et al.*, 1996). The survival promoting effects of NT-4/5 have been demonstrated in other areas of the visual system, for example, in the lateral geniculate nucleus of the ferret after monocular deprivation (Riddle *et al.*, 1995).

NT-4/5 has been shown to effect changes in the dendritic arborization of neurons in layers 4, 5 and 6 of the visual cortex (Cabelli *et al.*, 1995; McAllister *et al.*, 1995). Other populations of CNS neurons such as dopaminergic neurons of the rat substantia nigra (Studer *et al.*, 1995) and cerebellar granule cells (Gao *et al.*, 1995), are also known to undergo morphological changes in the presence of NT-4/5.

Ablation of the NT-4/5 gene results in phenotypic defects that typically affect sensory but not motor neurons (Conover *et al.*, 1995; Liu *et al.*, 1995; Erickson *et al.*, 1996). In NT-4/5 homozygous null mutant animals, the populations of neurons most severely affected are those of the nodose, petrosal, and geniculate ganglia (Conover *et al.*, 1995; Liu *et al.*, 1995). Since both NT-4/5 and BDNF are known to undergo signal transduction via the same receptor tyrosine kinase, $BDNF^{-/-}$ and $NT\text{-}4/5^{-/-}$ double homozygous knockout mice have been generated. In these mice, drastic reductions in the numbers of nodose-petrosal and geniculate neurons, more severe than those seen with either gene deletion alone, are evident (Conover *et al.*, 1995; Liu *et al.*, 1995). Evidence from these studies strongly suggests that motor neurons require additional factors for their survival during development since populations of facial motor neurons and lumbar spinal motor neurons are not significantly affected by either mutation alone, or a combination of the two (Conover *et al.*, 1995; Liu *et al.*, 1995). Furthermore, no obvious defects are observed in sympathetic neurons or dopaminergic neurons in the substantia nigra.

Neurotrophin-6

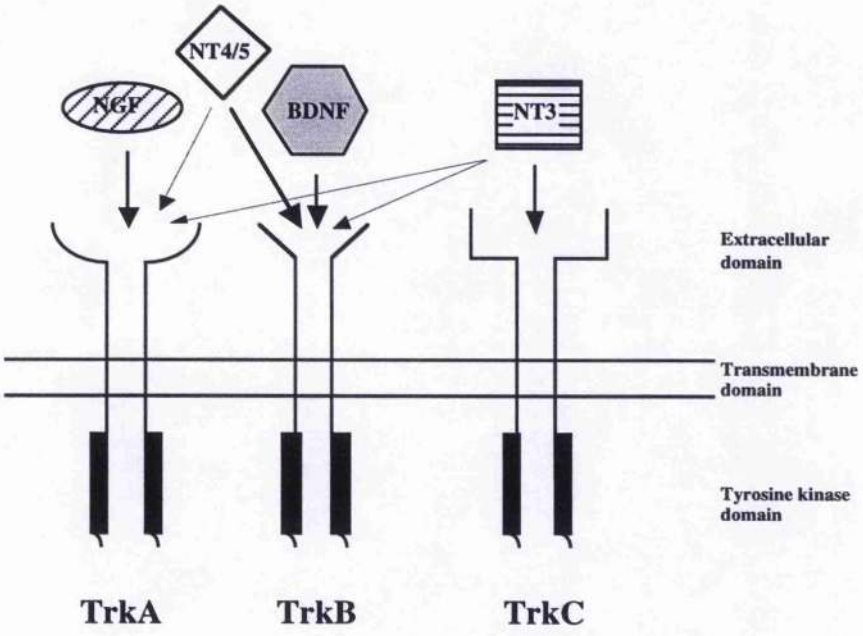
To date, neurotrophin-6 has only been identified in the teleost fish, *Xiphophorus maculatus* (Götz *et al.*, 1994). NT-6 was cloned from a genomic library of *Xiphophorus* DNA by low stringency hybridization to overlapping sequences of NGF (Götz *et al.*, 1994). Alignment of the NT-6 sequence with *Xiphophorus* NGF, BDNF or with salmon NT-3 revealed that it did not represent an homologue of the known neurotrophins (Götz *et al.*, 1994).

The predicted structure of NT-6 suggests that in precursor form, it is a protein of 286 amino acids, with a molecular weight of 31.4 kD (Götz *et al.*, 1994). In addition, six structurally important cysteine residues are conserved (Götz *et al.*, 1994). Other features of the molecule include; a putative signal sequence at the N terminus, a pro-region containing basic motifs necessary for the proteolytic cleavage of the mature protein, a 143 amino acid sequence encoding the mature form of the molecule (Götz *et al.*, 1994).

Northern blot analysis of the expression of NT-6 during teleost development has revealed a 1.4 kb transcript expressed from the onset of organogenesis (day 8) in several tissues. NT-6 mRNA is expressed in the developing and mature brain, and in the heart, skeletal muscle and other tissues of adult fish (Götz *et al.*, 1994). The highest expression of NT-6 according to *in situ* hybridization analysis, is in the valvula cerebelli, a rostral protrusion of the teleostean cerebellum under the midbrain tectum (Götz *et al.*, 1994).

Like NGF, NT-6 supports the survival of embryonic chick DRG and sympathetic neurons. However, lower levels of survival are seen with saturating concentrations of NT-6 compared to those observed with NGF (Götz *et al.*, 1994). Cultured nodose and ciliary neurons do not survive with NT-6, suggesting that the spectrum of NT-6 activity is similar to that of NGF (Götz *et al.*, 1994).

1.4.2 Neurotrophin Receptors



Adapted From Davies, 1994b

Figure 1.1 Schematic diagram summarising the interaction of neurotrophins with members of the Trk family of tyrosine kinase receptors.

NGF, BDNF, NT-3, and NT-4/5 recognise two types of transmembrane receptors: Trk receptors (TrkA, Trk B, or TrkC) and p75. The Trk family of receptor tyrosine kinases show a specificity in their preference for neurotrophin binding, whereas the p75 receptor interacts with each neurotrophin, albeit with slightly different binding properties. Although much has been learned about the signalling mechanism of the Trk receptors, the functions of p75 are complex. One of the major difficulties encountered in the study of p75 is that unlike the Trk receptor tyrosine kinases, it lacks an inherent enzymatic activity. However, accumulating evidence with regard to its structural homology to a family of receptors including TNF, CD 40 and Fas, suggests that the p75 receptor may be capable of fulfilling a number of different functions.

The Low Affinity NGF Receptor (p75)

The gene encoding the human low affinity NGF receptor was first identified by gene transfer assays, followed by immunological detection of the transfected receptor-expressing cells (Johnson *et al.*, 1986b). Genomic sequences containing the human NGF receptor locus were subsequently cloned from these cells (Chao *et al.*, 1986), whilst the rat low affinity NGF receptor gene was independently cloned from another NGF receptor-expressing cell line (Radeke *et al.*, 1987). The human gene encodes a 75 kD cell surface glycoprotein, designated gp75, or simply p75, consisting of a single transmembrane polypeptide of 427 amino acid residues, 399 of which correspond to the mature protein (Johnson *et al.*, 1986b; Radeke *et al.*, 1987; Large *et al.*, 1989). The extracellular domain consists of 222 amino acid residues, containing four cysteine repeats, that has approximately 30% sequence homology with a family of cell surface receptors which include the lymphocyte surface antigens; CD 27, CD 30, CD 40, OX 40, the Fas cell surface antigen, that mediates apoptosis, and the p55 tumour necrosis factor receptor (see Chao, 1994).

Experiments in which mutations were made in the extracellular domain of p75, have demonstrated that perturbation of the cysteine rich regions confers detrimental effects on the binding capacity of p75 for NGF, whereas mutating other regions of the molecule, including the cytoplasmic domain has, no such effects (Welcher *et al.*, 1991; Yan and Chao, 1991). This region has not been highly conserved during evolution, there being only 55% homology between the central domains of the human and chicken receptors (Johnson *et al.*, 1986b; Large *et al.*, 1989). However, comparison of the cytoplasmic domain of p75 with that of the tumour necrosis factor receptor (TNFR-1) and Fas (also known as CD95/APO-1), has revealed these proteins share homology within an 80 amino acid region known as the 'death domain' (Chapman, 1995). In addition, a

predicted structural similarity occurs between a region of the p75 cytoplasmic domain, the 14 residue wasp toxin mastoparan, and the hamster β_2-adrenergic receptor, all of which are presumed to form amphipathic α-helices that possibly interact with G-proteins (Feinstein and Larhammar, 1990).

The p75 receptor is widely expressed in both the central and peripheral nervous system (Ernfors *et al.*, 1988; Yan and Johnson, 1989). Since avian embryos are readily accessible for study during development, some of the early expression studies focused on the developing chicken embryo. In the chick CNS, p75 mRNA has been identified in a wide variety of cell populations at various stages of development, with expression seen in most CNS regions, from E4 onwards (Heuer *et al.*, 1990). In the PNS, *in situ* hybridization analysis has demonstrated that autonomic and primary sensory neurons express p75 from the earliest developmental stages, with levels of p75 mRNA increasing substantially from E5 in both sensory and sympathetic ganglia (Heuer *et al.*, 1990). Developmental studies of p75 expression by Northern blotting and *in situ* hybridization have shown that p75 mRNA is present in developing NGF dependent sensory neurons before they begin to innervate their targets, and the levels of mRNA substantially increase after target contact is made (Hallböök *et al.*, 1990; Wyatt *et al.*, 1990). Furthermore, in developing trigeminal ganglion neurons, the level of p75 mRNA is upregulated *in vitro* by BDNF and NGF during successive phases of development, when these neurons are responsive to BDNF and NGF (Wyatt and Davies 1993). Correspondingly, several other studies using postnatal and adult sensory, sympathetic, and CNS derived neurons, and the neuron-like PC12 phaeochromocytoma cell line, have suggested that NGF upregulates the level of p75 mRNA (Doherty *et al.*,1988; Miller *et al.*, 1991; Cavicchioli *et al.*, 1989; Higgins *et al.*, 1989; Lindsay *et al.*, 1990; Verge *et al.*, 1990; Miller *et al.*, 1994). However, the developmental expression of p75 mRNA in the trigeminal ganglia of $NGF^{-/-}$ mice does not differ from that seen in wild type animals (Davies *et al.*, 1995a).

Several populations of non-neuronal cells also express p75 receptors, and these including neuroepithelial cells such as melanocytes, meningeal cells, glial cells, and Schwann cells (DiStefano and Johnson, 1989). Schwann cells also secrete a proteolytically cleaved form of p75 with an unknown function (DiStefano and Johnson, 1989). Additionally, other non-neural populations of p75 expressing cells include, basal keratinocytes, epithelial cells lining the ducts of mammary and prostatic glands, and spleen cells (Thompson *et al.*, 1988; Chesa *et al.*, 1988).

The functional role of p75 in signal transduction is still controversial (see review by Chao 1994). Initial studies suggested that p75 alone is insufficient for the high-affinity binding of NGF and subsequent cellular responses (Chao *et al.*, 1986; Radeke *et al.*, 1987; Hempstead *et al.*, 1989). Further investigations which revealed that the TrkA, TrkB and TrkC tyrosine kinases are signal transducing receptors for NGF, BDNF, and NT-3 respectively, called into question whether p75 played any role in neurotrophin signalling (reviewed by Bothwell, 1991; Chao, 1992; Meakin and Shooter, 1992). Since p75 binds to all members of the neurotrophin family (reviewed by Chao, 1994), it has been suggested that it serves as a common subunit for different neurotrophins, with specificity conferred by the Trk tyrosine kinase subunit of the receptor complex (Bothwell *et al.*, 1991; Thoenen, 1991). In contrast, the dissociation rates of neurotrophin binding to p75 vary, with NGF having the fastest rate and NT-3 the slowest, suggesting that p75 may take part in ligand discrimination (Rodriguez-Tebar, 1992).

A number of studies have produced results suggesting that Trk receptors alone are sufficient to bind NGF with high-affinity (Klein *et al.*, 1991a; Lamballe *et al.*, 1991a; Jing *et al.*, 1992). Recently, however, contrasting studies have shown that the effects of neurotrophins can vary depending on the ratio between the p75 and Trk tyrosine kinase receptors (Hempstead *et al.*, 1992; Benedetti *et al.*, 1993). Additionally, tyrosine phosphorylation of TrkA

receptors is known to be influenced by the co-expression of p75 (Berg *et al.*, 1991; Verdi *et al.*, 1994; Barker and Shooter, 1994). The proposal that p75 does play a role in neurotrophin signalling comes from experiments showing that deletion of the cytoplasmic sequences of p75 leads to the loss of high-affinity binding in cultured PC12 cells (Hempstead *et al.*, 1990), and detailed *in vitro* survival experiments using NGF responsive neurons from p75 deficient mice (Davies *et al.*, 1993b; Lee *et al.*, 1994). In PC12 cells, that normally express both p75 and TrkA, high-affinity NGF binding sites form, whereas very few high-affinity sites are detected when truncated p75 receptors (lacking the extracellular region) are overexpressed in these cells (Benedetti *et al.*, 1993). Transfection of p75 expression constructs into TrkA expressing fibroblast cell lines produces high-affinity binding sites, whereas transfection of p75 constructs containing deletion mutations in the p75 cytoplasmic or extracellular domains cannot reproduce this effect (Battleman *et al.*, 1993). In addition, overexpression of p75 enhances NGF induced autophosphorylation of TrkA in a neuronal progenitor cell line (Verdi *et al.*, 1994). Evidence of a p75-TrkA complex has been provided by observations that physical interaction with TrkA immobilises p75 in PC12 cells (Wolf *et al.*, 1995). Furthermore, copatching experiments that utilise anti-p75 antibodies, have shown that both the extracellular and cytoplasmic domains of p75 are required for the p75-TrkA interaction (Ross *et al.*, 1996). More recently, p75 has been implicated in the internalisation of neurotrophins, since retrograde transport of BDNF and NT-3 in the chick isthmo-optic nucleus can be inhibited by anti-p75 antibodies (von Bartheld *et al.*, 1996).

Several studies have suggested that p75 may be involved in programmed cell death. For example, immortalised cerebellar neuronal cell lines expressing p75 display distinctly faster rates of apoptosis in the absence of NGF (Rabizadeh *et al.*, 1993). Lowering the levels of p75 expression in cultured NGF-dependent sensory neurons increases the survival of these neurons in the absence of NGF,

but only at late stages of development (Barrett and Bartlett, 1994). In contrast to its apparent cytotoxic action in the absence of bound NGF, recent work has suggested that p75 cytotoxicity is triggered by NGF binding (Frade *et al.*, 1996; Casaccia-Bonnefil *et al.*, 1996). Addition of anti-NGF antibodies or antibodies that inhibit NGF binding to p75 causes a significant reduction in the number of retinal neurons that undergo apoptosis in the chick embryo retina, prior to innervation of the tectum by retinal axons (Frade *et al.*, 1996). Furthermore, addition of NGF rapidly induces the death of mature oligodendrocytes *in vitro* (Casaccia-Bonnefil *et al.*, 1996). Interestingly, in both studies, the cells in question express p75 in the absence of TrkA, raising the possibility that NGF-bound p75, in the absence of TrkA conveys a death signal (Frade *et al.*, 1996; Casaccia-Bonnefil *et al.*, 1996).

Recent evidence suggests that the cytoplasmic domain of p75 takes part in intracellular signalling. Neurotrophin binding to p75 activates the sphingomyelinase pathway both in a glioma cell line and in transfected NIH 3T3 cells, producing the lipid second messenger ceramide (Dobrowsky *et al.*, 1994). The production of ceramide initiated by NGF via p75 is specific, and is dependent on the intracellular domain of the receptor (Dobrowsky *et al.*, 1994). This pathway is utilised by a number of cytokine receptors including the tumour necrosis factor receptor. Among the downstream targets activated by the TNF receptor is the transcription factor NF-κB, which, when activated by external stimuli, translocates to the nucleus where it binds to DNA and activates transcription (Baeuerle and Henkel, 1994). The p75 mediated activation of this transcription factor has been directly demonstrated in cultured Schwann cells (Carter *et al.*, 1996). Further studies have described interactions of p75 with the extracellular signal regulated kinases ERK1 and ERK2 (Volonte *et al.*, 1993), and have shown that peptide analogues of the mastoparan-like region within the cytoplasmic domain have biological function (Dostaler *et al.*, 1996). Furthermore, the activation of NF-κB following exposure to ceramide mimics

the effect of p75 mediated NGF signalling in cultured Schwann cells (Carter *et al.*, 1996). Sphingomyelin generated ceramide is a possible candidate for mediating the apoptotic effects of p75, since it has been previously established as a potent inducer of apoptosis via TNFR-1, which has homology to p75 (Rabizadeh *et al.*, 1993; Kolesnick and Golde, 1994; Hannun and Obeid, 1995). In support of this hypothesis, several recent studies have demonstrated that ceramide mediates apoptosis in cultured rat mesencephalic neurons (Brugg *et al.*, 1996), embryonic chick neurons (Weisner and Dawson, 1996), and PC12 cells (Hartfield *et al.*, 1996). In contrast, ceramide has been implicated in the prevention of cell death after NGF withdrawal in cultured sympathetic neurons, raising questions as to whether the actions of this second messenger depend on the cellular context or possibly on the maturity of the neuronal population studied (Ito and Horigome, 1995). Furthermore, the signal transduction mechanism which leads to apoptosis could be conveyed by a different signalling pathway. Since a region of the p75 cytoplasmic domain has homology with the Fas/TNFR-1 'death domain', it is feasible that the same apoptotic pathway is triggered by p75 (reviewed by Davies, 1997).

To date, the most definitive functional studies of p75 have resulted from the generation of mice that have null mutations in the coding region of the p75 gene (Lee *et al.*, 1992). These studies have shown that p75 deficient animals display deficits in populations of sensory and sympathetic neurons, with no major effects on neurons within the CNS (Lee *et al.*, 1992). Embryonic and post-natal neurons from trigeminal, dorsal root, and superior cervical ganglia of p75 deficient mice, have a decreased sensitivity to NGF, during certain stages of their development when compared with the equivalent populations of 'wild-type' neurons *in vitro* (Davies *et al.*, 1993b; Lee *et al.*, 1994). Mutant animals are particularly deficient in cutaneous nerve fibres immunoreactive for substance P and calcitonin gene-related peptide (CGRP), combined with a profound deficiency in thermoception (Lee *et al.*, 1992).

The Trk Family of Receptor Tyrosine Kinases.

Trk A

The *trk* (tropomysosin kinase) proto-oncogene was initially discovered by molecular analysis of a human colon carcinoma (Martin-Zanca *et al.*, 1986; Martin-Zanca *et al.*, 1989). Two receptor tyrosine kinase isoforms of 790 and 796 amino acid residues are encoded by this proto-oncogene (Martin-Zanca *et al.*, 1989; Meakin *et al.*, 1992; Barker *et al.*, 1993; Shelton *et al.*, 1995). The protein encoded by the *trk* proto-onocogene (alternatively referred to as Trk, TrkA, or gp140^{trk}) is a 140kD membrane spanning protein that possesses several characteristic features of cell surface receptors, including a 32 amino acid long putative signal peptide, an amino terminal moiety rich in potential consensus sites for *N*-glycosylation, a transmembrane domain, a kinase catalytic region highly homologous to that of other tyrosine kinases and a short 15 residue carboxy tail (Martin-Zanca *et al.*, 1989). The extracellular domain of TrkA exhibits two different cell-adhesion related motifs. The amino terminal moiety consists of an array of three leucine-rich motifs in tandem, flanked by two distinct cysteine-rich regions. Two immunoglobulin domains lie adjacent to the second cysteine cluster (Schneider and Schweiger, 1991). Affinity cross-linking experiments using ^{125}I-labelled NGF have demonstrated that NGF binds to this receptor with a dissociation constant of 10^{-9}M and binding stimulates receptor autophosphorylation (Klein *et al.*, 1991, Hempstead *et al.*, 1991;Kaplan *et al.*, 1991a). Furthermore, *in vitro* experiments have demonstrated that NGF and NT-3 confer a mitogenic response on fibroblast cells transfected with expression vectors encoding the TrkA receptor, although NT-3 elicits a more moderate response and is less efficient than NGF in competing for TrkA (Cordon-Cardo *et al.*, 1991). In addition, studies of Trk receptor kinases expressed in fibroblasts have shown that NT-3 can signal via TrkA (Klein *et al.*, 1991; Soppet *et al.*, 1991; Squinto *et al.*, 1991) and that NT-4/5 is also able to signal via TrkA

(Berkemeier *et al.*, 1991). Additionally there is some evidence that NT-3 can signal via TrkA in developing sensory and sympathetic neurons, since NT-3 promotes the *in vitro* survival of these neurons in mice that have homozygous null mutations in the *trkC* gene (Davies *et al.*, 1995a).

During development, the expression of *trkA* mRNA is confined to defined structures of the nervous system, and the onset of expression occurs during the early stages of neurogenesis (Martin-Zanca *et al.*, 1990; Schröpel *et al.*, 1995). Northern blot, and *in situ* hybridization analyses have revealed *trkA* mRNA expression in discrete regions of the peripheral nervous system, including the DRG and several cranial sensory and symapthetic ganglia (Martin-Zanca *et al.*, 1990; Tessarollo *et al.*, 1993; Schröpel *et al.*, 1995). Additionally, expression of the TrkA protein has been demonstrated using immunocytochemical localisation, by cells of the rat DRG and trigeminal ganglia, and by subpopulations of cells in the dorsal horn of the spinal cord (Averill *et al.*, 1995). In the CNS, TrkA mRNA expression has been observed in cholinergic neurons of the basal forebrain and striatum (Vazquez and Ebendal., 1991; Holtzman *et al.*, 1992; Merlio *et al.*, 1992; Steininger *et al.*, 1993), in non-cholinergic neurons of the thalamus (Venero and Hefti 1993), and in magnocellular neurons of several brainstem nuclei (Merlio *et al.*, 1992). Additionally it has been demonstrated that the expression of *trkA* mRNA in the cholinergic neurons of the adult rat forebrain is up-regulated by NGF (Holtzman *et al.*, 1992). Taken together, the results of these studies demonstrate that expression of the TrkA receptor is coincident with some of the known targets of NGF.

Outside of the nervous system, *trkA* mRNA expression has been observed in certain cells of the immune system, including activated $CD4^+$ T-lymphocytes and monocytes (Ehrhard *et al.*, 1993; Ehrhard *et al.*, 1994), and in cells of the spleen and thymus (Lomen-Hoerth *et al.*, 1995).

Signal transduction by TrkA occurs through ligand induced homodimerization of the two receptor subunits, resulting in autocatalytic tyrosine phosphorylation and, ultimately, a cascade of signalling events culminating with altered gene expression within the nucleus (Kaplan *et al.*, 1991b; Klein *et al.*, 1991a; Jing *et al.*, 1992). In all TrkA receptors, nine homologous tyrosine residues have been characterised, seven within and two flanking the kinase domain (Middlemas *et al.*, 1994; Loeb *et al.*, 1994; Stephens *et al.*, 1994). Experiments using cultured PC12 cells have identified phosphotyrosine binding sites for three intracellular *src*-homology region 2 (SH2) proteins in the cytoplasmic region of all Trk receptors (Stephens *et al.*, 1994). The adapter protein Shc, and PLCγ-1, both bind to tyrosine residues flanking the kinase domain. These are the juxtamembrane tyrosine residue Y490 and the carboxy terminal tyrosine residue Y785, respectively (Stephens *et al.*, 1994). The non-catalytic sub-unit of PI-3 kinase (p85) also binds to a tyrosine residue within the kinase domain namely, Y751 (Stephens *et al.*, 1994). Beyond these receptor binding proteins, a different adapter protein, known as GRB2, which possesses a phosphotyrosine binding SH2 domain and a proline binding SH3 domain, form a complex with the mammalian protein SOS (son-of-sevenless) (Suen *et al.*, 1993). Following TrkA receptor autophosphorylation, the SH2 binding region of the GRB2 protein is recruited to the membrane and associates with Shc (Ohmichi *et al.*, 1994; Obermeier *et al.*, 1994). The SOS protein is an exchange factor, that activates a small G-protein, p21Ras, by facilitating the exchange of GDP for GTP on the p21Ras protein (reviewed by McCormick 1994). A second Shc binding protein, called GAP, has also been established as an activator of p21Ras following binding of neurotrophins to the Trk receptors (D'Arcangelo *et al.*, 1993; Stephens *et al.*, 1994). The chain of signalling events downstream of p21Ras involves the serine/threonine kinase proto-oncogene *raf* which activates the mitogen-activated protein kinase kinase (MAPKK) by phosphorylation of two serine residues (Alessi *et al.*, 1994). The MAPKK protein is a dual specificity kinase, activating mitogen-activated

protein kinase (MAPK) by phosphorylation of tyrosine and threonine residues (Jaiswal *et al.*, 1993; Saito *et al.*, 1994). In PC12 cells, the MAPK consists of two related kinases, the extracellular signal-regulated kinases ERK1 and ERK2, that have a diverse array of cellular targets (reviewed by Davis, 1993). In non-neuronal cells, the MAP kinases have been demonstrated to activate transcription factors; ERK2 phosphorylates Myc and ERK1 phosphorylates Elk, which is involved in the induction of the immediate early gene *c-fos* (reviewed by Davis, 1993). The signal transduction pathway that occurs in primary sensory neurons may require additional elements to those that are common for other cell types, however, at this juncture this remains to be elucidated.

Mice that have targeted mutations in the *trk* gene develop severe sensory and sympathetic neuropathies, and most die within one month of birth (Smeyne *et al.*, 1994). These mice typically display extensive cell loss within trigeminal, dorsal root and sympathetic ganglia, and also have decreased cholinergic basal forebrain projections to the hippocampus and cortex (Smeyne *et al.*, 1994). The effects of *trk* gene deletion on these PNS ganglia are highly reminiscent of earlier studies that used immunological methods to inhibit the activity of NGF, and NGF gene deletion confirming that TrkA is the receptor for NGF *in vivo* (Levi-Montalcini, 1987; Crowley *et al.*, 1994). When comparing the effects of NGF and *trk* gene deletions, the striking difference between the two studies is the effect on basal forebrain cholinergic neurons. Whereas the CNS appears normal in $NGF^{-/-}$ mice (Crowley *et al.*, 1994), TrkA $^{-/-}$ animals display substantial loss of cholinergic fibres projecting from the medial septum to the hippocampus, and from the nucleus basalis to the cerebral cortex (Smeyne *et al.*, 1994). This may reflect a role for TrkA in fibre outgrowth as opposed to neuron loss possibly with NT-3 as the TrkA activating ligand (Smeyne *et al.*, 1994). Further detailed analyses of TrkA deficient mice has revealed that small myelinated and unmyelinated axons projecting from the dorsal root ganglia are massively depleted and is coupled with a loss of peptides associated with

nociceptive function (Silos-Santiago *et al.*, 1995). More recent analysis of TrkA deficient mice has revealed that the onset of neuronal depletion within the trigeminal ganglion is coincident with the switch to NGF dependence observed *in vitro* (Piñón *et al.*, 1996).

Trk B

The TrkB tyrosine kinase receptor was cloned as a result of an analysis of *trk* gene expression in the developing mouse (Klein *et al.*, 1989). The *trk*B gene locus demonstrates a complex pattern of transcription, describing at least eight different transcripts ranging in size from 0.7 to 9.0 kb, encoding at least two different classes of receptors (Klein *et al.*, 1989; Klein *et al.*, 1990; Middlemas *et al.*, 1991). The signal transducing isoform of TrkB designated gp145^{trkB} or TrkB^{TK+} , is an 821 amino acid molecule that is heavily glycosylated and contains several motifs that are common to tyrosine kinase receptors (Klein *et al.*, 1989; Klein *et al.*, 1990; Middlemas *et al.*, 1991). The overall homology between the extracellular domains of rat TrkA and TrkB^{TK+} receptors is 57%, with the kinase catalytic domains displaying the highest degree of homology at around 88% (Klein *et al.*, 1989). Two non-signal transducing isoforms of TrkB, designated TrkB^{TK-}, have thus far been identified. They possess the same extracellular domain as TrkB^{TK+}, however, they differ in their cytoplasmic domains, since they lack the kinase catalytic region (Klein *et al.*, 1990; Middlemas *et al.*, 1991; Allen *et al.*, 1994). The extracellular region of the TrkB receptor has been shown to possess three N-terminal tandemly linked leucine rich motifs, two cysteine clusters, and two immunoglobulin like domains (Schneider and Schweiger, 1991). Recently these leucine rich regions have been demonstrated to be capable of binding to BDNF, NT3 and NT-4 /5 (Windisch *et al.*, 1995a, Windisch *et al.*, 1995b; Ninkina *et al.*, 1997).

Expression of *trkB* mRNA has been detected in discrete regions of the developing nervous system of the mouse, both by Northern blot analysis, and *in situ* hybridization (Klein *et al.*, 1989). Northern blotting has revealed that *trkB* mRNA expression is highest in the developing brain, but it is also detected in a number of non-neural tissues, including the lung and muscle (Klein *et al.*, 1989). Non-neural tissues such as the lung and muscle display predominant expression of small RNA transcripts, encoding the non-catalytic $TrkB^{TK-}$ isoform of the receptor (Klein *et al.*, 1989). Within the developing nervous system, *in situ* hybridization analysis has revealed that expression is prominent in the brain, spinal cord, spinal sensory ganglia and paravertebral sympathetic chain ganglia (Klein *et al.*, 1989). The expression of *trkB* mRNA is also seen in structures resembling certain cranial ganglia, including the trigeminal ganglion (Klein *et al.*, 1989). *In situ* hybridization analysis of *trk*B in the chicken embryo has revealed a pattern of expression in developing peripheral ganglia that correlates well with the localisation of neurons known to respond to BDNF (Dechant *et al.*, 1993). Additionally, the onset of *trkB* mRNA expression commences early during embryogenesis, consistent with the view that BDNF plays a role in the early development of certain peripheral ganglia (Dechant *et al.*, 1993; Wright *et al.*, 1992). Developmental variations in the expression of the different TrkB isoforms have also been observed in certain cranial sensory ganglia (Ninkina *et al.*, 1996). High levels of mRNA encoding the $TrkB^{TK+}$ isoform are detected in trigeminal neurons during the period when these neurons respond to BDNF *in vitro*, whereas an increase in the non-catalytic $TrkB^{TK-}$ isoform is observed at later stages of development when these neurons are known to have undergone a switch in trophic dependence from BDNF to NGF (Buchman and Davies, 1993; Ninkina *et al.*, 1996). This study suggests that the loss of BDNF response shown by trigeminal neurons during development is imparted by increased levels of the non-catalytic receptor (Ninkina *et al.*, 1996). The $TrkB^{TK-}$ isoform has been proposed to play a role in limiting the spatial distribution of BDNF by sequestering the ligand, and subsequently creating distinct borders that separate

different BDNF responsive systems, such as the optic tectum, and the developing trigeminal system (Biffo *et al.*, 1995). The expression of full length and truncated TrkB has additionally been demonstrated in the developing visual system (Takahashi *et al.*, 1993; Biffo *et al.*, 1995; Garner *et al.*, 1996), where the truncated isoforms are found to be upregulated with increasing age (Allendorfer *et al.*, 1994). The $TrkB^{TK-}$ isoform is also highly expressed within adult sensory neurons (McMahon *et al.*, 1994), and its expression is increased in adult facial motor neurons in response to injury (Kobayshi *et al.*, 1996).

In addition to its role as the primary receptor for BDNF, TrkB has been demonstrated to bind NT-3 when ectopically expressed in non-neuronal cells, but with a lower affinity than BDNF (Klein *et al.*, 1991; Glass *et al.*, 1991; Squinto *et al.*, 1991; Soppet *et al.*, 1991). Recent studies have revealed that TrkB is not only the primary receptor for BDNF, but is also the cognate receptor for NT-4/5 (Klein *et al.*, 1992). Introduction of a point mutation in the extracellular domain of the TrkB receptor abolishes the binding of NT-4/5 whereas the binding of BDNF is unaffected (Klein *et al.*, 1992). Analysis of the extracellular region of the various Trk receptors has implicated the immunoglobulin domains in ligand discrimination between the neurotrophins (Urfer *et al.*, 1995), whereas the adjacent juxtamembrane region has also been suggested to participate in binding NT-3 and NT-4/5 (Clary *et al.*, 1994; Ströhmaier *et al.*, 1996). In contrast, recent studies have demonstrated that the leucine rich motifs of Trk receptors are the essential ligand binding regions (Windisch *et al.*, 1995a; Windisch *et al.*, 1995b). Recently, novel isoforms of TrkB have been identified in developing sensory neurons that have variations in the extracellular binding region, lacking two or all three of the leucine-rich motifs (Ninkina *et al.*, 1997). When expressed in fibroblast cell lines, these isoforms do not bind either BDNF, NT-3 or NT-4/5, and the transfected fibroblasts do not survive or show responses to neurotrophins (Ninkina *et al.*, 1997). This study suggests that the leucine-rich motifs in TrkB are essential for

ligand binding and signalling and that the immunoglobulin-like domains alone are insufficient to confer neurotrophin binding via TrkB

Targeted disruption of the *trk*B gene results in severe nervous system lesions and premature death of the affected animals (Klein *et al.*, 1993). The specific structures affected by this mutation include trigeminal and dorsal root ganglia within the peripheral nervous system, and the facial motor nucleus within the central nervous system (Klein *et al.*, 1993). Structures known to have a function in feeding, including the trigeminal ganglion, facial motor nucleus and vagal nucleus are particularly affected by the TrkB mutation, resulting in aberrant feeding behaviour in the neonatal animals (Klein *et al.*, 1993). All of these structures have previously been shown to express *trkB* transcripts during development (Klein *et al.*, 1993). Other neuronal deficiencies were observed in the CNS, in the spinal cord at lumbar levels L2-L5 (Klein *et al.*, 1993). Further detailed analysis of $TrkB^{-/-}$ mice has shown that the loss of neurons within the trigeminal ganglion occurs at an early developmental stage, consistent with the dependence of early trigeminal neurons on BDNF *in vitro* (Piñón *et al.*, 1996).

Trk C

The third member of the neurotrophin receptor tyrosine kinase family to be identified was TrkC (Lamballe *et al.*, 1991b). TrkC was initially identified as the product of a cDNA clone isolated from a porcine brain cDNA library, and displays the same structural features as the related TrkA and TrkB tyrosine kinase receptors (Lamballe *et al.*, 1991b). The mature TrkC protein has a molecular weight of 145kD, and the overall homology of porcine TrkC with the human Trk and mouse TrkB receptors is 67% and 68%, respectively. The extracellular domains exhibit 54% (TrkC and TrkA) and 53% (TrkC and TrkB) homology including conservation of several cysteine residues (Lamballe *et al.*, 1991b). The kinase domains of the Trk receptors exhibit a high degree of

homology, TrkC being 87% homologous to TrkA and 88% homologous to TrkB (Lamballe *et al.*, 1991b). The three receptors also share several characteristic features including a threonine residue at position 682, a tryptophan residue at position 757 and possesses a short, 15 residue, carboxy tail containing 8 identical amino acids (Lamballe *et al.*, 1991b). Several variants of the TrkC receptor are produced by differential RNA splicing. To date, four TK^+ and three TK^- receptor isoforms have been identified (Lamballe *et al.*, 1993; Tsoulfas *et al.*, 1993; Valenzuela *et al.*, 1993; Garner and Large, 1994). The TK^+ TrkC isoforms have the same overall structure, but differ in that the splice variants possess 14 (TrkC K14), 25 (TrkC K25), or 39 (TrkC K39) additional amino acid residues in the tyrosine kinase domain of the receptor (Lamballe *et al.*, 1993; Tsoulfas *et al.*, 1993; Valenzuela *et al.*, 1993). The 14 and 25 amino acid residue sequences are unique and are unrelated to any other known sequences, whereas the 39 amino acid-long insert consists of a combination of the 14 and 25 amino acid sequences (Tsoulfas *et al.*, 1993).

Most of the available information regarding the expression of *trkC* mRNA has been generated using probes that do not discriminate between the different isoforms of the receptor. Weak expression of *trkC* transcripts can first be detected at E7.5, in the early neuroectoderm of the mouse, by ribonuclease protection assay (Tessarollo *et al.*, 1993). By E9.5, *in situ* hybridization analysis reveals that the developing DRG, telencephalon, and spinal cord are all expressing *trkC* mRNA (Tessarollo *et al.*, 1993; Lamballe *et al.*, 1994). At E11.5, *trkC* expression can be seen in discrete regions of the developing PNS such as the dorsal root and trigeminal ganglia, and the ganglia of the VII, VIII, IX, and X cranial nerves (Tessarollo *et al.*, 1993; Lamballe *et al.*, 1994). At this stage of development, expression is also prevalent in many regions of the CNS, including the telencephalon, diencephalon, mesencephalon, rhombencephalon and neural tube (Tessarollo *et al.*, 1993; Lamballe *et al.*, 1994). In mid-gestation E13.5 embryos, *trkC* transcripts are present primarily in two structures within

the PNS, the trigeminal and the otic ganglion. In the CNS, *trkC* expression is seen at relatively high levels in the cerebellar Purkinje, and Golgi cells, the telencephalon, septal nuclei, spinal cord, and, at lower levels in the hypothalamus, mesencephalon, and brain stem (Tessarollo *et al.*, 1993; Lamballe *et al.*, 1994). At E15.5, the highest level of *trkC* transcripts in the PNS are in the otic and trigeminal ganglia, and the spinal nerves. At this age the DRG also display punctate labelling of peripheral cells (Tessarollo *et al.*, 1993; Lamballe *et al.*, 1994). Within the CNS, E15.5 embryos express *trkC* mRNA in the tectum, telencephalon, septal region, mesencephalon, brainstem, cerebellum. At this age *trkC* mRNA is expressed for the first time in the pontine nuclei and developing hippocampus (Tessarollo *et al.*, 1993; Lamballe *et al.*, 1994). In late stage embryos, at E17.5, the majority of *trkC* hybridization observed in the PNS is seen in the periphery of individual dorsal root ganglia. Within the CNS, *trkC* mRNA is observed in the caudatoputamen and associated fibres, located between the caudate and forebrain subventricular zone, in the medial septal nucleus and the hypothalamus, the cerebellum, tectum, medulla and spinal cord (Lamballe *et al.*, 1994).

During development, the expression of *trkC* transcripts has additionally been observed in various areas outside of the nervous system (Tessarollo *et al.*, 1993; Lamballe *et al.*, 1994). These areas include several facial structures such as the vibrissae of the snout, the dental papillae, the posterior tongue, sublingual glands and olfactory epithelium in the head (Tessarollo *et al.*, 1993; Lamballe *et al.*, 1994). In the body cavity, *trkC* mRNA is expressed in the submandibular gland, the subendothelial mesenchyme of arteries, the mesenchyme surrounding the urogenital ducts, the diaphragm, adrenal glands and within adipose tissue (Tessarollo *et al.*, 1993; Lamballe *et al.*, 1994).

In adult animals *trkC* mRNA expression continues to be seen in several regions within the CNS, including regions of the forebrain such as the cerebral cortex, hippocampus, thalamus, and hypothalamus and in large motor neurons

within the spinal cord (Merlio *et al.*, 1992; Tessarollo *et al.*, 1993; Lamballe *et al.*, 1994).

Neurotrophin-3 binds to the different isoforms of TrkC with similar affinity and kinetics, and induces the rapid phosphorylation of tyrosine residues within the cytoplasmic domain of the receptor (Lamballe *et al.*, 1993; Tsoulfas *et al.*, 1993; Valenzuela *et al.*, 1993). However, at this juncture, only the TK^+ isoform TrkC K1 is known to activate $PLC\gamma$ and phosphatidyl inositol-3 kinase (PI-3 kinase), although both the insert containing K14 and K25 isoforms possess recognition sites for $PLC\gamma$ and PI-3 kinase (Lamballe *et al.*, 1993). When ectopically expressed in cells of the NIH 3T3 fibroblast cell line, the TrkC K1, K14, and K25 isoforms have all been observed to induce DNA synthesis on exposure to NT-3 (Lamballe *et al.*, 1993). However, neither the K14 nor K25 isoform could induce the efficient transformation of these cells (compared with those expressing TrkC K1) upon exposure to NT-3 (Lamballe *et al.*, 1993). Further experiments have demonstrated that only TrkC K1 can induce the proliferation of transfected NIH 3T3 cells and the neuronal differentiation of PC12 cells, suggesting that the different isoforms of TrkC may act via different signalling pathways (Lamballe *et al.*, 1993; Tsoulfas *et al.*, 1993; Valenzuela *et al.*, 1993).

Inactivation of the gene encoding full length TrkC produces striking deficits within the PNS (Klein *et al.*, 1994). The homozygous mutant mice are reduced in size by postnatal day 4 (P4) and most die by P21 (Klein *et al.*, 1994). The behavioural phenotype of the mutant mice suggests a deficit in proprioception, and subsequent analysis of the DRG of these animals has demonstrated that the group Ia primary afferent sensory neurons subserving proprioception are substantially reduced in number (Klein *et al.*, 1994; Minichiello *et al.*, 1995). No gross defects are observed in CNS structures that express *trkC* transcripts, although $TrkC^{-/-}$ mice generally do not survive until adulthood (Klein *et al.*, 1994). Studies using antibodies that inactivate the TrkC

receptor during chick embryogenesis produce a phenotype that is remarkably similar to that resulting from *trkC* gene deletion. In particular there are severe reductions in the total complement of neurons within the DRG (Lefcort *et al.*, 1996). Detailed neuroanatomical analyses of the TrkC$^{-/-}$ mutant mice has recently 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$^{-/-}$ 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 an approximately 66% reduction in the complement of neurons within the lumbar DRG of neonatal NT-3$^{-/-}$ animals (Ernfors *et al.*, 1994a; Fariñas *et al.*, 1994) In contrast, in the lumbar DRG of *trk*C$^{-/-}$ animals, there is only an 18% reduction (Klein *et al.*, 1994 ; Minichiello *et al.*, 1995). Similarly, the number of neurons in the spiral ganglia of NT-3$^{-/-}$ mice is reduced by ~86%, whereas the decrease seen in TrkC$^{-/-}$ neonates is 51% (Schimmang *et al.*, 1996). 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.*, 1995a).

1.5 Other Neurotrophic Factors And Their Receptors.

Neurotrophic Cytokines.

Neurotrophic or 'neuropoietic' cytokines are a group of distantly related proteins that effect similar biological responses in a variety of different cell types. Members of this cytokine subfamily include; ciliary neurotrophic factor (CNTF), growth promoting activity (GPA), leukaemia inhibitory factor (LIF), oncostatin-M (OSM), interleukin-6 (IL-6), interleukin-11 (IL-11), granulocyte

macrophage colony stimulating factor (GM-CSF) and the newly isolated cardiotrophin-1 (CT-1). Although these proteins share less than 30% amino acid homology, they possess high a degree of structural homology, based on their predicted tertiary structures (Bazan *et al.*, 1991). Additionally, these cytokines are thought to be evolved from a common ancestral gene (Bruce *et al.*, 1992), and are known to transduce signals via shared multi-subunit receptors (Fig 1.2; Stahl and Yancopoulos 1994).

Ciliary Neurotrophic Factor

CNTF was initially identified as a result of its ability to promote the survival of embryonic chick ciliary neurons *in vitro* (Adler *et al.*, 1979). Ciliary neurons had been observed to survive in the presence of chick skeletal muscle cells, rat ocular tissues, and fragments of sciatic nerve (reviewed by Sendtner *et al.*, 1994). After a systematic analysis of different ocular tissues, Adler and colleagues demonstrated that the iris, ciliary body, and choroid layer were the richest sources of the putative survival factor that they termed 'ciliary neuronotrophic factor' (Adler *et al.*, 1979). Subsequent studies, utilising ion-exchange chromatography, revealed that the survival factor was an acidic protein with a molecular weight of 20-24kD (Barbin *et al.*, 1984; Manthorpe *et al.*, 1986). Based on partial amino acid sequence data, the cDNAs for rat and rabbit CNTF were cloned (Stöckli *et al.*, 1989; Lin *et al.*, 1989). Additionally, the mouse and human genomic clones have been identified and investigated (Kaupmann *et al.*, 1991; Lam *et al.*, 1991). Biochemical analysis of CNTF has revealed that it is a 200 amino acid long protein with a molecular weight of 22.7kD ($pI \geq 5.78$) (Stöckli *et al.*, 1989; Lin *et al.*, 1989). One unusual feature of CNTF is that it lacks a consensus sequence for glycosylation and a secretory signal peptide, suggesting that it is a cytosolic protein (Stöckli *et al.*, 1989; Lin *et al.*, 1989). The human CNTF homologue has subsequently been cloned and

was found to be 80% homologous to rat CNTF (Masiakowski *et al.*, 1991; Lam *et al.*, 1991; Negro *et al.*, 1991). Although no high degree of sequence homology exists between CNTF and other known proteins, structural similarities have been identified between CNTF, LIF, OSM, IL-6, and GM-CSF based on the tertiary sequences of these proteins, which distinguished four α-helices (Bazan, 1991). Experiments using site directed mutagenesis have determined that up to 14 N-terminal and 27 C-terminal amino acid residues can be removed from the CNTF molecule without drastically reducing its biological activity (Negro *et al.*, 1994). Longer deletions, or the elimination of internal stretches of amino acids, have been shown to abolish the biological activity of CNTF (Negro *et al.*, 1994).

Early experiments carried out *in vitro*, demonstrated that a partially purified form of CNTF had a broad spectrum of survival activity on PNS neurons (Manthorpe *et al.*, 1983). These results were later confirmed with purified recombinant CNTF, using several populations of chicken embryo neurons including E8 trigeminal, nodose, and dorsal root ganglion sensory neurons, parasympathetic ciliary neurons, and neurons of the paravertebral sympathetic chain ganglia (Manthorpe *et al.*, 1982; Manthorpe *et al.*, 1986). Similarly, neurons of developing rat nodose ganglion show increased survival in the presence of CNTF *in vitro* (Thaler *et al.*, 1994). Furthermore, within the PNS, CNTF has additionally been shown to exert its survival promoting effects upon postnatal neurons of the rat acoustic ganglion (Hartnick *et al.*, 1995). Interestingly, dorsal root ganglion sensory neurons have been observed to exhibit changes in their survival requirements. At E8, DRG neurons are unresponsive to CNTF, whereas around 40% of these neurons can be rescued by E10 (Manthorpe *et al.*, 1982). Likewise, E21 rat sympathetic neurons that are normally unresponsive to CNTF *in vitro* survive in the presence of CNTF after an initial period in culture with NGF (Kotzbauer *et al.*, 1994). The equivalent

age postnatal neurons demonstrate an immediate response to CNTF (Kotzbauer *et al.*, 1994).

Within different populations of CNS neurons, CNTF displays similar age-related effects to those seen with PNS neurons. For example, it does not promote the survival of chick spinal motor neurons at E4 (Longo *et al.*, 1982; Bloch-Gallego *et al.*, 1991), but does promote the survival of purified motor neurons at E6 (Arakawa *et al.*, 1990). In keeping with these findings, the administration of CNTF *in ovo* to E5-E9 chicks induces an increase in the number of spinal motor neurons (Oppenheim *et al.*, 1991). Likewise, a number of studies have concluded that CNTF has a trophic effects upon spinal cord motor neurons in developing and postnatal rodents, both *in vitro* and *in vivo* (Sendtner *et al..*, 1990; Sendtner *et al.*, 1992a; Henderson *et al.*, 1993; Forger *et al.*, 1993; Mitsumoto *et al.*, 1994; Li *et al.*, 1994; Vejsada *et al.*, 1995). Other populations of CNS neurons that respond to CNTF include hippocampal neurons and cerebellar Purkinje neurons (Ip *et al.*, 1991; Larkfors *et al.*, 1994).

In addition to the survival promoting effects of CNTF, several studies have revealed that CNTF has an effect on other properties of developing neurons. In the precursor neurons of the E7 sympathetic chain ganglia, CNTF can arrest cell division and concomitantly induces the expression of vasoactive intestinal peptide (VIP) immunoreactivity (Ernsberger *et al.*, 1989). Depolarization of cultured sympathetic neurons with elevated levels of K^+, has a similar effect to that of CNTF on the expression of VIP and neuropeptide Y (NPY), however , this effect cannot be repeated on sensory (DRG) neurons (Rao *et al.*, 1992). Similarly, CNTF has been demonstrated to influence the levels of choline acetyltransferase (ChAT) and tyrosine hydroxylase (TH) activity in neonatal rat sympathetic neurons (Saadat *et al.*, 1989). Additionally, CNTF has been shown to enhance the expression of TH immunoreactivity by cultured rat neurons from the locus coeruleus, and substantia nigra as well as upregulating

the expression of the endogenous neurotransmitters for these populations of neurons (Louis *et al.*, 1993; Magal *et al.*, 1993)

The expression of CNTF mRNA has chiefly been studied using Northern blot analysis. The highest level of expression detected by this method is in the PNS, within the sciatic nerve of adult rats (Williams *et al.*, 1984). CNTF mRNA cannot be detected in the new-born rat (Stöckli *et al.*, 1989), and the CNTF mRNA expression in the peripheral nerve does not occur until one month postnatally, suggesting that CNTF is not a physiological survival factor for embryonic neurons (Dobrea *et al.*, 1992). The expression of CNTF mRNA and protein has been suggested to coincide with the differentiation of Schwann cells of the peripheral nerve (Jessen and Mirsky, 1992). Northern blot analysis of total RNA from the skeletal muscle, liver, spleen and lung, has demonstrated the presence of very low levels of CNTF mRNA in the skeletal muscle of adult rats (Stöckli *et al.*, 1989; Ip *et al.*, 1993c, Giovannini *et al.*, 1993a). Taken together, the results of these experiments suggest that skeletal muscle is not a source of CNTF, rather it is more likely to originate in Schwann cells ensheathing the innervating nerve fibres within the muscle (Sendtner *et al.*, 1994). In contrast, cultured Schwann cells express only low levels of CNTF mRNA, however, CNTF-like biological activity can be detected in the conditioned medium of these cells (Carroll *et al.*, 1993; Meyer *et al.*, 1992).

CNTF is not expressed in the hindlimbs or brain in the critical period during the development of spinal cord motor neurons, nor is it expressed in significant levels within the cortex or hippocampus (Ip *et al.*, 1993c). This suggests that CNTF is not a target derived factor for CNS neurons. Interestingly, several studies have reported that CNTF mRNA is drastically reduced following peripheral nerve lesion, with the levels recovering slowly, until a significant increase was observed 4 weeks after lesion (Sendtner *et al.*, 1992b; Freidman *et al.*, 1992; Seniuk *et al.*, 1992). However, in marked contrast to the studies on PNS neurons, it has been shown that CNTF mRNA is rapidly

up-regulated in the CNS after lesion of the hippocampus and cortex, with the levels remaining elevated for up to 3 weeks after lesion, suggesting that CNTF may be released in response to the trauma (Ip *et al.*, 1993b). The cells responsible for the production of CNTF mRNA and protein are thought to be reactive astrocytes which invade the site of the lesion (Ip *et al.*, 1993b).

Disruption of the CNTF gene produces effects within the nervous system that are consistent with a role for CNTF in the maintenance of motor neurons in adult animals, but not during development (Masu *et al.*, 1993). Abolition of the gene by homologous recombination results in a progressive atrophy of adult mouse motor neurons, leading to a gradual decrease in muscle strength (Masu *et al.*, 1993). No evidence of perinatal disturbance in motor neuron development is apparent, since morphometric analysis of lumbar spinal cord, and facial motor neurons revealed no significant differences between postnatal $CNTF^{-/-}$ and wild-type mice (Masu *et al.*, 1993). However, 8-14 weeks after birth, morphological changes are evident in lumbar and facial motor neurons, and at 28 weeks the number of facial motor neurons are reduced by 22% (Masu *et al.*, 1993). No change in the number of ChAT, VIP or TH positive nerve fibres innervating the footpad and a second cholinergic target, the periosteum are apparent in the $CNTF^{-/-}$ animals (Masu *et al.*, 1993; Francis *et al.*, 1997).

Growth Promoting Activity

Growth promoting activity (GPA) was initially identified as a trophic factor for ciliary ganglion neurons after its extraction and partial purification from chick eye extracts (Nishi and Berg 1981). Following this, GPA was purified and characterised from a sciatic nerve preparation, revealing a 21.5 kD protein lacking a consensus signal sequence that had 57% amino acid identity with mammalian CNTF, suggesting that GPA may be the avian homologue of CNTF (Eckenstein *et al.*, 1990). Following its initial purification, the cloning of

a cDNA for GPA was carried out using an embryonic chick eye cDNA library, allowing the tissue specific expression of the molecule to be deduced (Leung *et al.*, 1992).

Northern blot analysis of GPA expression during development has demonstrated that the uvea of the eye, containing tissues innervated by the ciliary ganglion, contains the highest levels of mRNA for GPA. Expression was also seen in the E18 sciatic nerve (Leung *et al.*, 1992). The analysis of GPA expression in the chick eye at closely staged intervals from E7 to E19, has revealed that expression increases between E11 and E19, the period when ciliary ganglion neurons are undergoing programmed cell death (Leung *et al.*, 1992).

In experiments conducted *in vitro*, GPA was shown to promote the survival of several populations of neurons within the PNS (Eckenstein *et al.*, 1990; Heller *et al.*, 1993) GPA supports developing parasympathetic ciliary ganglion neurons and lumbar sympathetic chain neurons, and in contrast to previous studies carried out using CNTF, GPA promotes the survival of E8 DRG neurons (Eckenstein *et al.*, 1990; Manthorpe *et al.*, 1982). The effects of GPA have been also been compared with CNTF on the proliferation and functional differentiation of sympathetic neurons, revealing that these two factors produce strikingly similar effects but with different efficacy (Heller *et al.*, 1993).

Leukaemia Inhibitory Factor

Leukaemia inhibitory factor (LIF) was initially purified and characterised as a protein secreted from cultured heart cells that possessed the ability to induce sympathetic noradrenergic neurons to synthesise acetylcholine and form cholinergic synapses (Fukada, 1985). Purified conditioned medium from primary cultures of neonatal heart cells, revealed a protein with a molecular

weight of 45kD, which reduced to ~22kD after treatment with a deglycosylating enzyme (Fukada, 1985). Amino acid sequence data demonstrated that this cholinergic differentiation factor was identical to amino acid sequences of several known proteins, one of which, termed 'leukaemia inhibitory factor' induced the leukaemic myeloid M1 cell line to undergo differentiation into a macrophage-like phenotype (Gearing *et al.*, 1987). The amino acid sequence data was then used to obtain cDNA clones of murine and human LIF that display 75% homology, and give rise to a mRNA transcript of approximately 4.1 kb (Gearing *et al.*, 1987; Stahl *et al.*, 1990). The mature murine LIF protein comprises 179 residues, possesses 7 potential N-linked glycosylation sites, and has a pI of ~9.0 (Gearing *et al.*, 1987).

During development and embryogenesis, the expression of LIF mRNA has been observed at low levels in several tissues, by Northern blot, RNAse protection and *in situ* hybridization analyses (Bhatt *et al.*, 1991). Whereas LIF mRNA can be detected in most developing tissues throughout development, the highest levels of this cytokine are expressed in the uterine endometrial glands, 4 days post coitum, suggesting that LIF may play a role in the implantation of the developing blastocyst (Bhatt *et al.*, 1991). Within adult tissues, Northern blot analysis has revealed the expression of LIF transcripts in the brain, heart, thymus, liver and intestine, where the highest level of LIF signal are detected (Bhatt *et al.*, 1991). The developing skin of neonatal mice is also a site of high expression, where it is thought that embryonic stem cell populations may be responsible for LIF production (Bhatt *et al.*, 1991). RT-PCR analysis has specifically identified sites of LIF expression within peripheral tissues and in the CNS of postnatal rats (Yamamori, 1991), and in the spinal column, limbs, and DRG of the developing mouse (Murphy *et al.*, 1993). The expression of LIF mRNA within peripheral tissues is localised in the targets of cholinergic sympathetic neurons such as the developing sweat glands and within the skin of the rat footpad, presenting the possibility that LIF is a target derived neuronal

differentiation factor for these neurons *in vivo* (Rao and Landis, 1990; Yamamori, 1991). In the postnatal brain, RT-PCR technique has demonstrated expression of LIF transcripts within the superior colliculi and visual cortex, suggesting a possible role for LIF in the developmental plasticity of the visual system (Yamamori, 1991). Changes in the expression of LIF mRNA in response to injury of the adult peripheral nerve have also been detected, using RNAse protection assay and *in situ* hybridization (Banner and Patterson, 1994). In adult rats the expression of LIF mRNA was dramatically upregulated 24 hours after sciatic nerve transection, immediately proximal and distal to the lesion site, providing strong evidence that this cytokine may act in response to nervous system damage (Banner and Patterson 1994).

Within the nervous system, LIF has a wide range of effects on both central and peripheral neurons. In many cases the studies carried out thus far have indicated that the actions of LIF are similar, if not identical, to those of CNTF. Cultures of non-dividing mouse neural crest cells from E9 embryos, were observed to undergo differentiation into a sensory neuron-like phenotype and express the neuropeptide CGRP, in the presence of LIF (Murphy *et al.*, 1991). Furthermore, LIF supports the survival of cultured sensory neurons derived from E12 - E15 DRG, in a manner reminiscent of the actions of CNTF (Murphy *et al.*, 1991). Consistent with the role of LIF in development of the DRG, is the observation that mRNA for LIF is expressed in the central and peripheral targets of the ganglia, and also within the ganglia themselves, during the critical period when these neurons are innervating their targets and undergoing naturally occurring cell death, (Murphy *et al.*, 1993). Following application of ^{125}I-labelled LIF or CNTF to the lesioned sciatic nerve, both factors are retrogradely transported within the nerve *in vivo* , providing evidence that these factors may be involved in a response to injury (Hendry *et al.*, 1992; Curtis *et al.*, 1993; Curtis *et al.*, 1994). Similarly, ^{125}I-LIF binds to dissociated DRG neurons in *vitro* , and *in vivo* it is retrogradely transported by these neurons

after subcutaneous injection into the footpad (Hendry *et al.*, 1992). In addition to DRG neurons, other populations of developing sensory neurons survive in response to LIF and CNTF. In nodose ganglion cultures from E16.5 rats LIF and CNTF support the survival of 50% of the neurons (Thaler *et al.*, 1994). By birth less than 10% of these neurons survive with LIF or CNTF, suggesting a loss of responsiveness with age (Thaler *et al.*, 1994). Embryonic mouse neurons of the nodose and trigeminal ganglia have also been reported to survive in culture with both LIF and CNTF (Horton *et al.*, 1996). Furthermore, LIF and CNTF elicited the same survival response in dissociated postnatal sympathetic neurons *in vitro* (Kotzbauer *et al.*, 1994).

Evidence has accumulated that in addition to its survival and differentiation promoting effects, LIF effects a change in neurotransmitter and neuropeptide expression in cultured sympathetic and sensory neurons. In sympathetic neurons, for example, a switch from noradrenergic to cholinergic phenotype has been observed after addition of exogenous LIF *in vitro*. A similar switch has been observed *in vivo* in transgenic mice overexpressing LIF (Ure *et al.*, 1992; Bamber *et al.*, 1994). Similarly, LIF has been observed to induce mRNA for several neuropeptides in cultured sympathetic neurons (Fann and Patterson, 1994). There is also evidence that LIF may affect the expression of neurotransmitters and neuropeptides in developing sensory neurons, since non-neuronal cells from the DRG have been observed to secrete LIF, which has been demonstrated to inhibit catecholamine expression in cultured rat trigeminal neurons (Fan and Katz, 1993). Furthermore, neurons generated in neural crest cultures in the presence of LIF express both CGRP and substance P, suggesting that LIF may inhibit the synthesis of neurotransmitters not used by sensory neurons and promote the synthesis of neuropeptides (Murphy *et al.*, 1991; Murphy *et al.*, 1994).

In addition to the effects of LIF and CNTF within the PNS, both factors elicit similar responses upon CNS neurons. Both LIF and CNTF have been

observed to promote the survival of spinal motor neurons after deafferentation or axotomy *in vivo* (Qin-Wei *et al.*, 1994; Vejsada *et al.*, 1995), and in purified spinal cord motor neurons *in vitro* (Martinou *et al.*, 1992; Henderson *et al.*, 1993). Furthermore, LIF has been demonstrated to promote the differentiation of isolated spinal cord precursor cells into a neuronal phenotype (Richards *et al.*, 1996). In addition, both CNTF and LIF effect similar changes in ChAT activity in motor neuron cultures (Martinou *et al.*, 1992), and in cultures of neurons isolated from the ventral mesencephalon, which contains neurons of the cranial motor nuclei (Zurn and Werren, 1994).

Inactivation of the gene encoding LIF has no obvious deleterious effects on nervous system development, however, it has been observed to affect the implantation of blastocysts in the uteri of homozygous mutant mice, which is consistent with previous observations of LIF expression during embryonic development (Stewart *et al.*, 1992; Bhatt *et al.*, 1991). Further analysis of these mice has revealed that the noradrenergic to cholinergic switch that occurs *in vivo* in sympathetic neurons innervating the developing sweat gland occurs normally (Rao *et al.*, 1993). However, in the absence of LIF, neuropeptide induction in response to injury is suppressed both *in vivo* and *in vitro* in peripheral neurons (Rao *et al.*, 1993). Taken together, these results suggest that some functional redundancy may occur between cytokines. More recently, double gene targeting experiments have been performed by crossing $LIF^{-/-}$ and $CNTF^{-/-}$ mice in order to examine the extent of co-operative action of both factors *in vivo* (Sendtner *et al.*, 1996). Whereas $CNTF^{-/-}$ animals suffer relatively mild motor neuron degeneration the degenerative changes in the double mutant animals were more extensive, appeared earlier, and were functionally reflected by a marked reduction in grip strength (Sendtner *et al.*, 1996).

Oncostatin M

Oncostatin M (OSM) is a cytokine that acts as a growth regulator for a variety of mammalian cells *in vitro*. OSM was initially isolated and cloned from mRNA extracted from cells of the U937 cell line, after these cells had been induced to differentiate into macrophage-like cells by treatment with a phorbol ester (Malik *et al.*, 1989). Subsequently, a clone was also obtained from a human brain cDNA library (Malik *et al.*, 1989). Sequence analysis of these clones demonstrated that OSM is synthesised from a 2 kb mRNA encoding a 252 amino acid long precursor polypeptide, that undergoes further cleavage to yield a 196 residue mature protein with a molecular weight of ~28 kD (Malik *et al.*, 1989). The predicted OSM sequence has no homology with other known proteins, but the sequence of the 3' non-coding region contains an A+T-rich stretch with sequence motifs found in the 3' untranslated regions of many cytokine cDNAs (Malik *et al.*, 1989). Recently, OSM has been recognised as a member of the family of neurotrophic cytokines since they share an overall structural homology (Bazan, 1991). One further interesting finding is that the genes encoding OSM and LIF are tandemly linked on chromosome 22 in the human genome, suggesting they have a common evolutionary origin (Giovannini *et al.*, 1993b).

The expression of OSM is largely restricted to haematopoietic cells, including activated peripheral blood T-lymphocytes, and lipopolysaccharide induced human monocytes (Bruce *et al.*, 1992). The only known non-haematopoietic source of OSM mRNA has been detected in cells derived from the AIDS related Kaposi's sarcoma (Bruce *et al.*, 1992).

The effects of OSM *in vitro* have been determined largely using various cell lines, and overlap with those of other haematopoietic cytokines such as LIF (Bruce *et al.*, 1992). OSM induces the differentiation of murine M1 myeloid leukaemia cells into a macrophage-like phenotype in a similar way to LIF and

IL-6 (Rose and Bruce, 1991). Similarly, OSM has been demonstrated to inhibit the differentiation of cultured embryonic stem cells into a variety of phenotypes, an attribute shared by LIF (Piquet-Pellorce *et al.*, 1994). Additionally, both LIF and OSM stimulate the expression of acute-phase response proteins in a human hepatoma cell line (Piquet-Pellorce *et al.*, 1994). Within the developing nervous system, the only documented evidence of the action of OSM has been carried out using late stage murine DRG neurons. OSM displays a slightly lower survival promoting effect on these neurons than that elicited by CNTF or LIF (Ware *et al.*, 1995).

Transgenic mice have recently been generated that over-express the bovine form of OSM, which bears 58% sequence identity to the human OSM (Malik *et al.*, 1995). The OSM over-expressing fusion genes were injected into mouse embryos under the direction of various tissue specific promoters, resulting in gross abnormalities in various tissues (Malik *et al.*, 1995). In order to test the potential effects of bovine OSM on neurons *in vivo*, a neuron-specific promoter was used, which directs gene expression within the brain, spinal cord and peripheral ganglia (Malik *et al.*, 1995). Of the resulting transgenic mice, 50% died at birth and the remainder displayed restricted growth, accompanied by tremors, ataxia, and a progressive weakening of strength in the hindlimbs. However, no histological analyses of these transgenic animals has been carried out (Malik *et al.*, 1995).

Interleukin-6

The convergence of several lines of investigation on factors regulating B-lymphocyte growth and differentiation ultimately lead to the identification of a single growth regulatory protein termed 'interleukin-6' (reviewed by Lee, 1992). Molecular cloning of human and murine cDNAs encoding IL-6 revealed a 184 amino acid protein with two potential N-linked glycosylation sites , and a

relative molecular mass of ~26 kD (Hirano *et al.*, 1986; Chui *et al.*, 1988; Northemann *et al.*, 1989)

Northern blot analysis has identified IL-6 mRNA transcripts in several tissues of the adult rat, including several structures within the CNS (Schöbitz *et al.*, 1993; Kiefer *et al.*, 1993). The areas of IL-6 expression have been more specifically identified using *in situ* hybridization, and include; the large pyramidal neurons of the pyriform cortex, medial habenular nuclei, ventromedial and dorsomedial hypothalamus, hippocampal formation, optic tract, fimbria, corpus callosum, the internal and external capsule and the granular cell layer of the cerebellum (Schöbitz *et al.*, 1993; Gadient *et al.*, 1994). No expression is observed in brainstem structures or in the spinal cord, and none has been reported in either cranial or peripheral sensory or autonomic ganglia (Schöbitz *et al.*, 1993). IL-6 expression has also been demonstrated in cultured microglia and astrocytes (Cadman *et al.*, 1994) and is up-regulated in the peripheral nerve and facial motor nucleus after nerve injury (Reichert *et al.*, 1996; Kiefer *et al.*, 1993).

The pleiotropic functions of IL-6 are numerous and have been well documented, with the principal role of this cytokine related to immune functions, such as the production of antibodies by B-cells (Hirano *et al.*, 1986), induction of acute phase response proteins by hepatocytes (Gauldie *et al.*, 1987) and the proliferation of B-lymphoma cells (Piquet-Pellorce *et al.*, 1994). An increasing body of evidence now suggests that IL-6 also plays a role in the physiological and pathological events that take place in the developing and mature nervous system. Within the CNS, IL-6 is a survival factor for cultured embryonic and postnatal midbrain catecholaminergic neurons (Kushima *et al.*, 1992), developing cholinergic neurons of the spinal cord (Kushima and Hatanaka *et al.*, 1992), and basal forebrain cholinergic neurons (Hama *et al.*, 1989). IL-6 also promotes the differentiation of PC12 cells into a neuron like phenotype (Satoh *et al.*, 1988) and induces ChAT synthesis and substance P

expression in sympathetic neurons (Fann and Patterson, 1994). Furthermore, IL-6 in conjunction with the soluble IL-6 receptor (see below) were demonstrated to promote neurite outgrowth when added to explant cultures of embryonic DRG (Hirota *et al.*, 1996).

IL-6 deficient mice generated by gene targeting suffer defects in their capacity for liver regeneration and are protected against bone loss induced by ovariectomy (Kopf *et al.*, 1994; Poli *et al.*, 1994; Cressman *et al.*, 1996). IL-6$^{-/-}$ animals do not suffer any obvious phenotypic defects within the central or peripheral nervous system either during development or in adulthood (Kopf *et al.*, 1994; Poli *et al.*, 1994; Cressman *et al.*, 1996).

Cardiotrophin-1

Cardiotrophin-1 (CT-1) was initially cloned as a result of a search for factors that induce cardiac myocyte hypertrophy *in vitro* (Pennica *et al.*, 1995a). Sequencing of a 1.7kb cDNA encoding CT-1 revealed that this cytokine has a relative molecular mass of 21.5 kD, and possesses a potential N-linked glycosylation site, and, like CNTF, does not possess a hydrophobic signal sequence (Pennica *et al.*, 1995a; Pennica *et al.*, 1996a). Furthermore, the amino acid sequence of CT-1 displays some similarity with that of LIF and CNTF, with 24% and 19% identity, respectively (Pennica *et al.*, 1995a). The predicted tertiary structure of CT-1 additionally bears some similarity to CNTF and LIF, indicating that this cytokine is also a member of the neurotrophic cytokine family (Pennica *et al.*, 1995a).

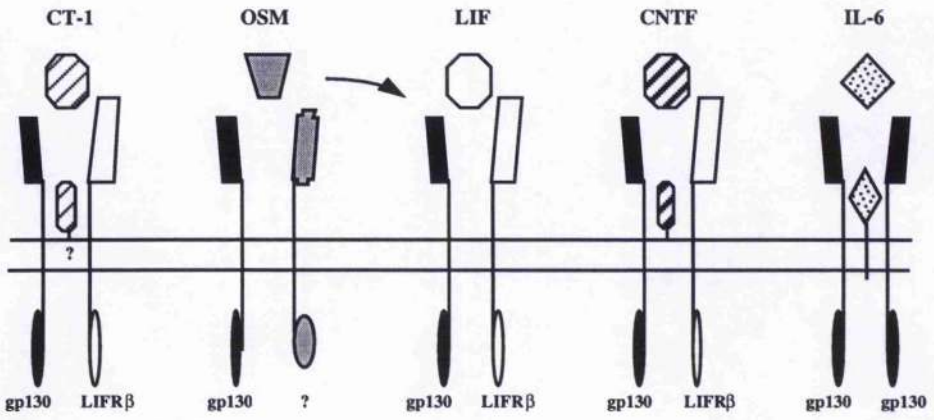
CT-1 protein has been detected in the murine heart tube between E8.5-E10.5 and in myocardial cells later in development (Sheng *et al.*, 1996). After E12.5, CT-1 protein expression is present in a number of tissues, including skeletal muscle, bone, liver, kidney, and epithelia (Sheng *et al.*, 1996). In the

PNS, CT-1 is weakly expressed in the DRG of E11.5 embryos, with expression increasing at later developmental stages (Sheng *et al.*, 1996). Similarly, of CT-1 is also expressed in the spinal cord and brain at later stages of development, however the levels are not as high as in those observed in the PNS (Sheng *et al.*, 1996). More detailed analysis of CT-1 mRNA expression using *in situ* hybridization, and RT-PCR, has revealed that CT-1 is also present in the developing limb-bud at E13.5-E14.5, the stage at which motor neurons innervating this structure commence programmed cell death (Pennica *et al.*, 1996).

The effects of CT-1 that have been thus far documented are similar to those of CNTF and LIF. For example, CT-1 induces; acute phase proteins in cultured hepatocytes (Peters *et al.*, 1995), inhibits the proliferation of the M1 myeloid leukaemic cell line, and inhibits the differentiation of embryonic stem cells (Pennica *et al.*, 1995b). CT-1 has also been demonstrated to have effects on cells of the central and peripheral nervous system. Within the CNS, unlike CNTF, CT-1 can promote the survival of cultured midbrain dopaminergic neurons, however, it is not as potent as glial cell line-derived neurotrophic factor (Pennica *et al.*, 1995b). CT-1 can also induce a neurotransmitter switch in cultured rat sympathetic neurons, increasing ChAT activity whilst inhibiting tyrosine hydroxylase activity (Pennica *et al.*, 1995b). CT-1 promotes the survival of embryonic ciliary neurons *in vitro*, however, at low concentrations, CT-1 is not as potent as CNTF (Pennica *et al.*, 1995b). In addition, CT-1 promotes the long term survival of purified E14 spinal cord motor neurons in culture , and is more potent over time than either CNTF or LIF (Pennica *et al.*, 1996b).

At this juncture, targeted gene disruption experiments have not been conducted, and it will be of interest to see whether CT-1 gene inactivation has an effect on the *in vivo* survival of central or peripheral neurons.

1.5.2 Cytokine Receptors.



Adapted from Stahl and Yancopoulos, 1994

Figure 1.2 Schematic diagram showing the interaction of neurotrophic cytokines their receptors.

The neurotrophic cytokines; CNTF, LIF, OSM, IL-6, and CT-1 are now known to utilise subunits of a multicomponent receptor signalling system (see Fig. 1.2 above). This receptor sharing between cytokines largely accounts for the degree of pleiotropy and functional redundancy displayed by these proteins, and may have evolved to allow signalling by many members of the cytokine family, such that each member can elicit actions upon distinct targets (reviewed by Stahl and Yancopoulos, 1994). The distribution of the receptor components also helps to explain the known target cell specificity of the individual cytokines. The receptor subunits have been designated the suffix α or β, correspondent with their capacity to transduce a signal across the cell membrane. Thus, the α components are non-signalling receptors capable of binding to their cognate ligands, that play a role in recruitment of the β receptor subunits, whereas the β components themselves are the signal transducing elements. So far, α subunits have been described for CNTF (CNTFRα) and IL-6 (IL-6Rα), however, there remains the possibility that CT-1 also requires an additional α subunit, whereas LIF or OSM do not. However in the case of OSM an additional OSM specific signalling component may replace LIFRβ.

The β receptor subunits form homo or hetero-dimers upon binding of the appropriate ligand. The LIF receptor constitutes a hetero-dimer comprised of LIFRβ and glycoprotein-130 (gp130) subunits. The other cytokines, with the exception of IL-6, similarly form these heterodimers, but in addition may also require an α component (Bruce *et al.*, 1992). The IL-6 receptor is comprised of a homodimer of two gp130 subunits and the accessory IL-6Rα component (Bruce *et al.*, 1992). The receptor components are brought together at the cell surface in a stepwise manner, upon binding of the appropriate ligand (reviewed by Stahl and Yancopoulos, 1994). Thus, in the case of CNTF, the ligand first binds to the CNTFRα component, which recruits gp130, and finally, forms a complex with LIFRβ. Constitutively associated with the β subunits are non-receptor tyrosine kinase proteins of the Jak/Tyk family, that become activated upon β-subunit dimerization. These proteins in turn activate a variety of intracellular signalling molecules, such as the STAT family of DNA binding transcriptional activators (Stahl and Yancopoulos, 1994).

CNTFRα

The CNTF alpha receptor (CNTFRα) was initially cloned using a strategy that employed an 'epitope tagged' form of CNTF, which when bound to neuronal cell lines, served as the basis for extraction of the receptor (Squinto *et al.*, 1990; Davis *et al.*, 1991). CNTFRα has a structure unrelated to the receptors for the neurotrophins, but is similar to the receptor for IL-6, having an approximate 30% sequence homology (Davis *et al.*, 1991). Sequence analysis of cDNAs encoding the isolated receptor have revealed a protein of approximately 41kD, containing four potential N-linked glycosylation sites. A high degree of sequence homology is apparent between the human and rat forms of CNTFRα, with approximately 94% of the nucleotides conserved between species (Ip *et al.*, 1993c). One interesting feature of the CNTFRα receptor is the absence of a

cytoplasmic domain (Davis *et al.*, 1991). The receptor is anchored to the cell membrane via a glycosyl-phosphatidylinositol (GPI) linkage that can be cleaved on treatment with phosphatidylinositol-specific phospholipase C (PIPLC), an enzyme that cleaves the anchors of GPI linked proteins (Davis *et al.*, 1991). Furthermore, a soluble form of CNTFRα has been identified that can form functional receptor complexes upon cells expressing gp130 and LIFRβ. This soluble form of CNTFRα has been detected in cerebrospinal fluid and in skeletal muscle after nerve injury, suggesting a physiological role for the released form of the receptor after injury (Davis *et al.*, 1993a).

Northern blot analysis, and *in situ* hybridization have detected the expression of CNTFRα transcripts in many tissues of the adult rat, especially within tissues of neuronal origin (Davis *et al.*, 1991; Ip *et al.*, 1993c). Widespread expression is seen within the CNS in several brain regions including cortical layer V, and motor neurons of the ventral spinal cord. Prominent expression is observed in the facial and trigeminal motor nuclei, consistent with the view that CNTF acts on different motor neuron populations (Davis *et al.*, 1991; Ip *et al.*, 1993c). During development, CNTFRα mRNA is also detected within the PNS in the sciatic nerve, and the dorsal root, ciliary and superior cervical ganglia, consistent with previous *in vitro* work demonstrating different effects of CNTF on neurons from these ganglia (Davis *et al.*, 1991; Ip *et al.*, 1993c). Additionally, prominent expression of CNTFRα mRNA occurs during development within the neuroepithelial linings of brain vesicles, within developing DRGs and in the spinal cord of E11-E15 rats, which contains many dividing neuronal precursor cells (Fredriksen and McKay, 1988; Murphy *et al.*, 1991; Ip *et al.*, 1993c). The expression of CNTFRα mRNA has also been demonstrated in non-neural cells within the developing embryo, and the adult rat, with marked expression in adult skeletal muscle and in developing structures associated with the heart and intestinal tract (Ip *et al.*, 1993c).

Reconstitution experiments using cell lines transfected with combinations of the various receptor components have revealed that the CNTFRα is not sufficient for signalling, and forms a functional receptor only when it is co-expressed with LIFRβ and gp130 subunits (Baumann *et al.*, 1993; Davis *et al.*, 1993b; Gearing *et al.*, 1994). Similarly, introduction of the LIFRβ and gp130 subunits, which are alone sufficient for LIF signalling, does not constitute a fully functional receptor for CNTF (Baumann *et al.*, 1993; Davis *et al.*, 1993b; Gearing *et al.*, 1994). CNTF binds to the CNTFRα-LIFRβ complex or CNTFRα-LIFRβ-gp130 with high affinity (KD ~10^{-12} M) whereas CNTF binds to CNTFRα alone or CNTFRα-gp130 with low affinity (KD ~10^{-9} M). However, whether the high affinity CNTFRα-LIFRβ complex has a physiological role is not clear (Gearing *et al.*, 1994; Wong *et al.*, 1995).

Targeted mutation of the gene encoding CNTFRα has a profound effect on motor neuron development and results in perinatal lethality (DeChiara *et al.*, 1995). The phenotype of mice with this mutation is strikingly different to that of mice that have undergone CNTF gene inactivation, which display much milder changes within the CNS (DeChiara *et al.*, 1995; Masu *et al.*, 1993). Typically, CNTFRα$^{-/-}$ mice have a reduction in the total complement of motor neurons in the brainstem trigeminal, facial and hypoglossal motor nuclei (27%, 41% and 51% respectively), and a 33% reduction was noted in the lumbar motor neuron pool (DeChiara *et al.*, 1995). Since comparison of CNTF$^{-/-}$ and CNTFRα$^{-/-}$ mice has demonstrated that inactivation of the CNTF gene leads to a less severe phenotype than inactivation of the CNTFRα gene, it has been proposed that CNTFRα may be a receptor for an as yet unidentified ligand, as none of the other identified cytokines forms a receptor complex with CNTFRα (DeChiara *et al.*, 1995).

IL-6Rα

The interleukin-6 receptor (IL-6Rα) was initially isolated and cloned from a human natural killer-like cell line (Yamasaki *et al.*, 1988). Subsequently, a homologous IL-6Rα cDNA has been isolated from rat liver that has 71% identity with the human leukocyte IL-6Rα in the coding region (Baumann *et al.*, 1990). These studies have demonstrated that IL-6Rα is an 80kD protein, with ~468 amino acid residues, 6 potential N-linked glycosylation sites, and a short hydrophobic sequence that is a putative intracytoplasmic domain (Yamasaki *et al.*, 1988; Baumann *et al.*, 1990).

Northern blot and *in situ* hybridization analysis have shown that the expression of IL-6Rα within the nervous system is co-localised with its ligand, IL-6 (Schöbitz *et al.*, 1992; Schöbitz *et al.*, 1993). The mRNAs for both IL-6 and IL-6Rα are expressed in the dentate gyrus and regions CA1 and CA4 of the hippocampus, the habenulae, the dorsomedial and ventromedial hypothalamic nuclei, the optic tract and the pyriform cortex of the adult rat (Schöbitz *et al.*, 1992; Schöbitz *et al.*, 1993). This widespread tissue distribution has been suggested to correlate with a role for IL-6 in the co-ordination of metabolic, behavioural and neuroendocrine changes in the brain, and since the cytokine and receptor are co-localised, may imply an autocrine or paracrine mode of action (Schöbitz *et al.*, 1992; Schöbitz *et al.*, 1993). Further experiments have demonstrated an increased immunoreactivity to IL-6Rα and IL-6 in Schwann cells after lesion of the hypoglossal nerve, suggesting a role for IL-6 and its receptor in injury responses (Hirota *et al.*, 1996). Within the PNS, IL-6Rα mRNA is expressed by cultured primary sympathetic neurons, and its expression is upregulated after treatment with IL-6 (März *et al.*, 1996). The expression of IL-6Rα by sympathetic neurons suggests a potential role for IL-6 in modulating sympathetic function.

The mode of signalling that IL-6 employs is similar to that of CNTF. Initially, IL-6 binds to IL-6Rα and subsequently recruits the gp130 signalling subunit (Taga *et al.*, 1989). Deletion of the intracytoplasmic region of IL-6Rα does not reduce the capacity of IL-6 to induce growth inhibition on a murine cell line, suggesting that the intracytoplasmic domain is not necessary for effective signalling (Taga *et al.*, 1989). Similarly, a soluble form of the receptor (sIL-6Rα) lacking the transmembrane and cytoplasmic domains forms complexes with gp130 and has functional capabilities (Taga *et al.*, 1989; Lust *et al.*, 1995).

Transgenic mice that constitutively express IL-6Rα in conjunction with IL-6 have been reported to display accelerated regeneration of the axotomized hypoglossal nerve, further suggesting a role for IL-6 and IL-6Rα in nerve regeneration after trauma *in vivo* (Hirota *et al.*, 1996).

LIFRβ

The LIF receptor-β was isolated and cloned by expression screening of a human placental cDNA library using iodinated LIF (Gearing *et al.*, 1991). The LIF receptor is homologous to the IL-6 β receptor, gp130 (discussed below), which suggests that the two receptors might share a common signal transduction pathway (Gearing *et al.*, 1991). A murine cDNA encoding the LIF receptor has subsequently been isolated by cross hybridization, and shares ~70% amino acid identity to the human sequence (Gearing *et al.*, 1991). The cDNA for human LIF encodes a 190kD protein, with 1097 residues comprising a pre-pro LIFRβ, which consists of a signal sequence, a large 789 residue extracellular domain, a transmembrane domain and a 238 residue cytoplasmic domain (Gearing *et al.*, 1991). The receptor possesses 20 potential N-linked glycosylation sites, 19 of which reside in the extracellular domain, and sequence comparison between the LIFRβ and gp130 receptors revealed ~65% amino acid identity in the transmembrane region (Gearing *et al.*, 1991).

Expression of LIFRβ mRNA during development has been demonstrated by *in situ* hybridization, and a novel technique using heterozygous embryos raised for the purpose of gene deletion experiments (see below), where the *lacZ* reporter gene was incorporated into the targeting vector and used to examine the developmental distribution of the receptor (Li *et al.*, 1995). Expression in the developing nervous system is apparent in the hindbrain and neural crest at E9.5, and from E14.5 through to adulthood, expression is prominent in the brainstem motor nuclei, including the hypoglossal and facial nuclei, and also the nucleus ambiguus (Li *et al.*, 1995). LIFRβ is also expressed in spinal motor neurons, and within the PNS, in dorsal root ganglia (Li *et al.*, 1995).

Reconstitution and affinity cross-linking experiments have revealed that coexpression of LIFRβ and gp130 are required for the formation of functional receptors for LIF, OSM, CNTF, and CT-1 (Baumann *et al.*, 1993; Davis *et al.*, 1993b; Gearing *et al.*, 1994; Pennica *et al.*, 1995b). Further experiments have demonstrated that the signalling mechanism of LIFRβ involves activation of the MAP kinases ERK1 and ERK2 (Boulton *et al.*, 1994; Thoma *et al.*, 1994), in addition to recruitment of adapter proteins known such as SHC, GRB2, and activation of PLCγ, PI-3 kinase, and members of the STAT family of transcription activating factors (Boulton *et al.*, 1994; Stahl *et al.*, 1994). Activation of STAT signalling proteins by tyrosine phosphorylation has been shown to be conferred by members of the Jak/Tyk family of non-receptor protein tyrosine kinases that are pre-associated with LIFRβ and gp130 (Boulton *et al.*, 1995).

Targeted mutation of the gene encoding LIFRβ causes perinatal death, and results in a reduction in the total number of spinal cord and brainstem astrocytes, concomitant with a reduction in MAP kinase activation, and loss of the *in vitro* survival response of DRG neurons to LIF, OSM and CNTF (Ware *et al.*, 1995). Detailed histopathological analysis of LIFRβ$^{-/-}$ mice has revealed

losses of > 35% in the complement of facial motor neurons, in addition to the loss of 40% of motor neurons in the lumbar spinal cord and 50% of neurons within the nucleus ambiguus (Li *et al.*, 1995). The effect of gene deletion on motor neuron populations is more severe in LIFRβ$^{-/-}$ mice than that seen in mice deficient for LIF or CNTF or both (Sendtner *et al.*, 1996), and has striking parallels with the loss of many brainstem motor neuron populations observed in CNTFRα$^{-/-}$ mice (DeChiara *et al.*, 1995).

gp 130

Molecular cloning of a human gp130 cDNA has revealed that it is a 130kD glycoprotein, of 918 amino acid residues, with a single transmembrane domain, 14 potential N-linked glycosylation sites, and an extracellular domain that consists of six fibronectin type III modules (Hibi *et al.*, 1990). Functional analysis of gp130 using amino acid substitution, has demonstrated that the receptor possesses a region of ~61 residues in the cytoplasmic domain that are critical for the generation of a signal (Murakami *et al.*, 1991). A murine homologue of the gp130 receptor has been cloned, and revealed its nucleotide sequence is 76% homologous to the human cDNA (Saito *et al.*, 1992). Both murine and human gp130 display significant homology to the receptors for prolactin, growth hormone, erythropoietin, GCSF, LIFRβ and several of the receptors for other interleukins (Hibi *et al.*, 1990; Gearing *et al.*, 1991; Murakami *et al.*, 1991; Saito *et al.*, 1992).

Ubiquitous expression of gp130 has been observed by Northern blot analysis. Gp130 mRNA is present in the brain, heart, thymus, spleen, kidney, lung, and liver of the adult mouse (Saito *et al.*, 1992). The levels of gp130 mRNA are significantly increased in several of these tissues, when examined after several hours *in vivo* treatment with IL-6 (Saito *et al.*, 1992). During

development, the expression of gp130 in whole embryos is evident as early as E6, with levels reaching a peak by E8, after which they gradually decline (Saito *et al.*, 1992). Several haematopoietic cell lines have been demonstrated to express gp130 mRNA (Hibi *et al.*, 1990; Saito *et al.*, 1992). Additionally, gp130 expression has been demonstrated in the neuron-like PC12 phaeochromocytoma cell line and also in cultured sympathetic neurons, using RT-PCR and *in situ* hybridization (März *et al.*, 1996). Both gp130 and IL-6Rα mRNAs are up-regulated in sympathetic neurons after co-treatment with IL-6 and TNFα (März *et al.*, 1996). Furthermore, gp130 protein is up-regulated in the axotomised peripheral nerve, supporting the view that IL-6 signalling plays a role in the response to injury (Yao *et al.*, 1997).

The association of gp130 and the IL-6Rα to form a functional receptor for IL-6 was initially demonstrated by co-precipitation experiments utilising IL-6 specific antibodies (Taga *et al.*, 1989). These experiments suggested that the two polypeptide chains in the IL-6Rα extracellular domain interact with gp130, since the intracytoplasmic domain could be abolished without any functional loss occurring (Taga *et al.*, 1989). The IL-6 receptor complex was originally thought to consist of a heterodimer of IL-6Rα and gp130 components (Kishimoto *et al.*, 1992). Subsequently, it was observed that this model was dissimilar from the proposed receptors for LIF and CNTF which were demonstrated to consist of LIFRβ - gp130 heterodimers (Ip *et al.*, 1992b). Therefore, the receptor for IL-6 was proposed to be a complex of IL-6-bound IL-6Rα, together with a homodimer of two gp130 subunits (Ip *et al.*, 1992b). This proposal has been verified by studies using transfected cells that overexpress LIFRβ and gp130 subunits. In these studies, disulphide linked homodimers of gp130 were retrieved after stimulation with IL-6, and only these homodimers were associated with tyrosine kinase activity (Davis *et al.*, 1993b; Murakami *et al.*, 1993). The heterodimeric LIFRβ - gp130 complex has now been demonstrated as a signal transducing receptor for LIF, CNTF, OSM, IL-6 and

CT-1 (Davis *et al.*, 1993b; Murakami *et al.*, 1993; Sporeno *et al.*, 1994; Pennica *et al.*, 1995b). Intracellular signal transduction has been demonstrated upon ligand binding, and is conveyed by pre-associated members of the Jak/Tyk non-receptor tyrosine kinases, as with LIFRβ (Stahl *et al.*, 1994)

Targeted inactivation of the gene encoding gp130 results in a reduction in the total complement of haematopoietic cells and striking defects within the developing heart, leading to embryonic lethality (Yoshida *et al.*, 1996). The specific developmental defects result in a loss of cardiac myocytes, cells whose development is known to be regulated by the cytokine CT-1 (Pennica *et al.*, 1995b; Zheng *et al.*, 1996). No obvious developmental defects are apparent within the nervous system of $gp130^{-/-}$ mice, however, since the $gp130^{-/-}$ mutation results in embryonic lethality, it is possible that any nervous system defects due to the mutation may not be evident until late stages of development or adulthood. A more detailed analysis of these embryos is currently being undertaken (Yoshida *et al.*, 1996).

1.6 The TGFβ Family of Neurotrophic Factors and Their Receptors.

The transforming growth factor β (TGFβ) family of proteins comprises a number of structurally related growth factors that regulate cell growth, differentiation, motility, organisation and cell death, during development (for review, see Massagué 1994). To date, around 30 members of the TGFβ superfamily have been identified, that have numerous functions in different tissues (Massagué *et al.*, 1994). The effects of members of the TGFβ family on neuronal survival are limited, however, TGFβ1 and TGFβ2 have been demonstrated to promote the survival of midbrain dopaminergic neurons and protect against the neurotoxin MPTP (Poulsen *et al.*, 1994; Kreiglstein *et al.*, 1995). Recently two proteins of the TGFβ family, glial cell line-derived

neurotrophic factor (GDNF) (Lin *et al.*, 1993), and neurturin (NTN) (Kotzbauer *et al.*, 1996), have been identified that have extensive survival promoting effects on developing PNS and CNS neurons (Lindsay and Yancopoulos, 1996; Kotzbauer *et al.*, 1996; Buj- Bello *et al.*, 1995).

Signalling by the majority of TGFβ family members utilises a receptor complex consisting of two transmembrane serine/threonine kinases, whereas GDNF and NTN are now known to signal via the Ret receptor tyrosine kinase, coupled with unique GPI-linked α components similar to the CNTFRα (Massagué *et al.*, 1996; Durbec *et al.*, 1996) The receptors for GDNF and NTN have been designated GDNFRα (Jing *et al.*, 1996; Treanor *et al.*, 1996) and NTNRα (Baloh *et al.*, 1997; Buj-Bello *et al.*, 1997; Klein *et al.*, 1997) respectively.

Objectives of This Study.

Since the discovery of NGF, the survival of different populations of developing PNS neurons has been found to be regulated by an increasing number of neurotrophic factors. These neurotrophic factors regulate survival via cell surface receptors that transduce signals across the cell membrane and in some cases, the same cell surface receptors are utilised by different neurotrophic factors. In this study I attempt to clarify the role of certain neurotrophic factors and their signalling mechanisms during nervous system development.

In the first part of this study, I have focused on a newly identified member of the neurotrophin family, NT-4/5, that like BDNF, binds to the TrkB tyrosine kinase receptor. Initially, I compared the survival promoting activity of mammalian NT-4/5 with BDNF on different populations of neurons from mammalian and chicken embryos, at different stages of their development. I then extended this study to investigate the survival responses of different populations of chicken BDNF responsive neurons to a homologue of NT-4/5, *Xenopus* NT-4.

Since all of the neurotrophins bind to the low affinity p75 receptor, in the second part of this study I have examined the responses of different populations of mammalian and chicken neurons at different developmental stages, using a mutated NGF protein that does not bind to the p75 receptor. Thus I have used an alternative approach to the analysis of neurotrophin function in the p75 'knockout' mouse, in order to further clarify the role of the p75 receptor in neurotrophin signalling during nervous system development.

In the final part of this study, I have studied the ability of developing cranial sensory neurons to survive in the presence of a group of neurotrophic factors that do not belong to the neurotrophin family. The structurally related neurotrophic cytokines, CNTF, LIF, OSM, IL-6 and the newly identified CT-1,

are known to signal via different cell surface receptors and intracellular pathways to the neurotrophins. I have examined the survival promoting effects of this group of proteins in comparison with members of the neurotrophin family at closely staged intervals during the development of populations of sensory neurons that respond to different neurotrophins.

CHAPTER 2

GENERAL METHODS

2.1 Introduction.

Cranial sensory neurons have many advantages for studying the different phases of neuronal development. These neurons and their progenitor cell populations are well-defined, may be easily obtained for *in vitro* experimental studies, and are accessible to experimental manipulation *in ovo*, in the case of avian embryos, from the earliest stages of their development (Davies, 1989; Vogel and Davies 1993). The peripheral target fields of several cranial sensory ganglia, such as the trigeminal ganglion, are clearly delineated, and this has facilitated detailed studies of the influence of the target field on various aspects of neuronal development (Lumsden and Davies, 1983; Lumsden and Davies, 1986; Davies, 1987; Buchman and Davies, 1993). In contrast to the functional heterogeneity of dorsal root ganglia, cranial sensory neurons are for the most part segregated into functionally distinct populations. Perhaps as a consequence of their functional segregation, populations of cranial sensory neurons have quite distinct neurotrophic factor requirements. This has previously facilitated studies of the function of different neurotrophic factors in regulating neuronal survival (Davies *et al.*, 1986a; Allsopp *et al.*, 1993a; Allsopp *et al.*, 1993b Paul and Davies, 1995).

In this chapter, techniques for culturing different populations of embryonic chicken cranial sensory and autonomic neurons, and selected populations of embryonic mouse cranial sensory and autonomic neurons will be described. The description will be restricted to the establishment of dissociated cultures. Because non-neuronal cells can be effectively removed from these cultures, analysis of the effects of a particular factor on neurons is not complicated by any indirect effects mediated by non-neuronal cells.

2.2 Dissection Techniques.

Instruments and Equipment

All dissections and subsequent preparation of neuronal cultures were carried out in a laminar flow hood using standard sterile technique. For the final stages of dissection, a stereomicroscope with zoom lens was employed, using a fibre optic light source for illumination.

In the initial stages of most dissections, toothed forceps, straight and curved watchmaker's forceps, and a scalpel were required. Tungsten needles were required to complete dissections and to remove adherent connective tissue from the dissected neural tissue. These were made from 0.5 mm diameter tungsten wire as follows (Figure 2.1). The wire was cut into 3 to 5 cm lengths using wire cutters. The wire was then bent 1 cm from the end, to an angle of around 60^{o}. This end was immersed with the bent portion near horizontal in 5M KOH. A current of 3 to 12 volt DC was passed through the wire and a second copper electrode was immersed in this solution. The tungsten was then etched away over several minutes so as to form a taper from the bend to the tip of the needle. To form a sharp point at the tip, the bent portion was placed vertically in the solution for several seconds. The needles were washed in water to remove the alkali. For dissection, the needles were held in chuck-grip platinum wire holders. Forceps and scalpels were sterilised by flaming in alcohol. Tungsten needles were sterilised in a Bunsen burner flame, which additionally tempered the wire thus increasing its rigidity.

Plasticware: Sterile plastic dishes (60 and 100 mm diameter, supplied by Corning).

Solutions: All dissections were carried out using Liebowitz's L15 medium without sodium bicarbonate (supplied by GIBCO, see appendix).

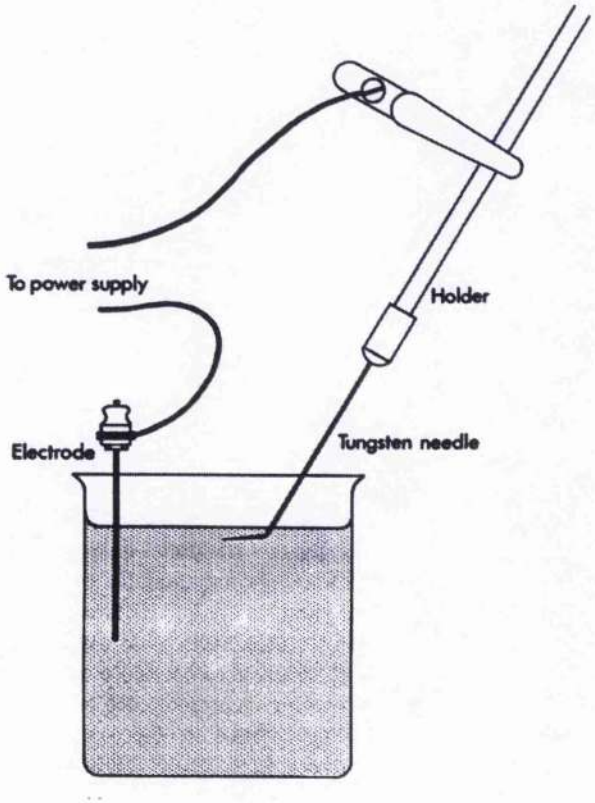


Figure 2.1 Diagram detailing the method for making electrolytically sharpened tungsten needles. (From Davies, 1988)

2.2 Dissection of chicken embryo cranial sensory neurons.

Naturally occurring cell death occurs in cranial sensory ganglia during the mid-embryonic stages. Because each population of cranial sensory neurons is dependent on a particular neurotrophic factor or combination of factors, studying these neurons at this stage of development has previously been used for analysing the function and co-operation of neurotrophic factors (Davies, *et al.*, 1986a; Davies *et al.*, 1986b; Davies and Lindsay 1985; Allsopp *et al.*, 1993a). The location of the cranial sensory ganglia in the E10 chick embryo is shown in Figure 2.2. The precise embryonic stage was determined using the criteria of Hamburger and Hamilton (1951).

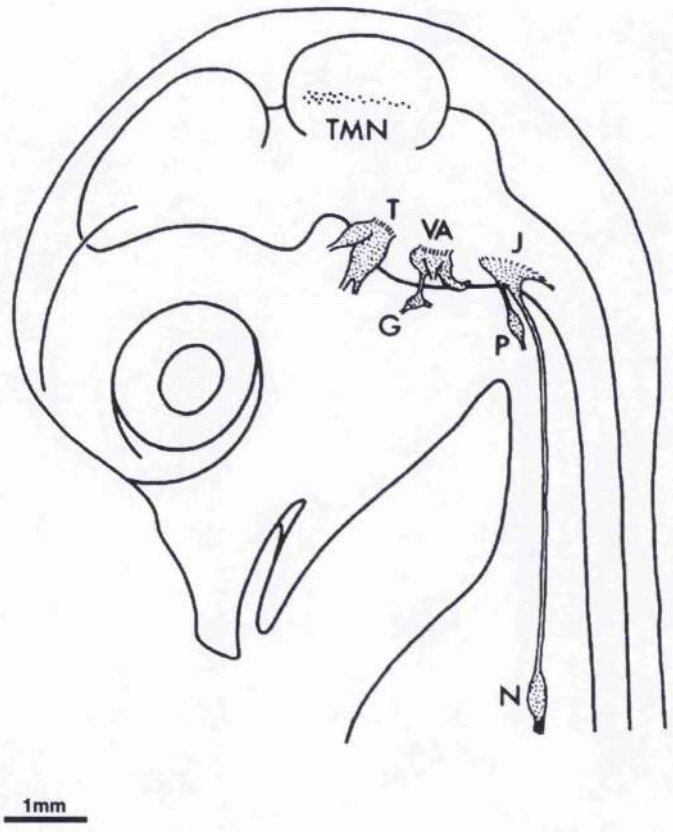


Figure 2.2 Lateral aspect of the head and neck of an E10 chicken embryo showing the locations of the trigeminal mesencephalic nucleus (TMN) and the trigeminal (T), geniculate (G), vestibulo-acoustic (VA), petrosal (P), jugular (J) and nodose (N) ganglia. (From Davies and Lindsay, 1985)

Dissection of mid-embryonic trigeminal sensory ganglia.

1. Fertile chicken eggs were incubated in a forced-draft, humidifed incubator at 38°C for the required time. To remove embryos, the eggs were held with their blunt end uppermost (where the airspace is located) and each egg was swabbed with 70% alcohol and allowed to dry. The shells were cracked in a line around the airspace by tapping with forceps and this portion of the shell was removed. The membrane lining the airspace was then removed. The embryo, together with its adherent membranes, was then carefully removed with a pair of curved forceps.

2. Embryos were washed in a 60 mm dish containing L15 medium and all membranes were removed with a pair of watchmaker's forceps.

3. Tungsten needles were used to isolate parts of the developing head containing the trigeminal ganglia (Figure 2.3a).

4. The part of the head containing the trigeminal ganglia was bisected along the saggital plane, in the following way. A tungsten needle was inserted into the cavity of the developing fourth ventricle and the tissue was turned so that its ventral aspect lay next to the bottom of the dish. The tungsten needle was then pressed against the bottom of the culture dish from where it was inserted. To cut through the roof of the fourth ventricle, the bisection was completed by pushing a tungsten needle down through the tissue to the bottom of the dish.

5. The hindbrain was removed from the medial aspect of each half of the bisected tissue using tungsten needles

6. Ganglia were dissected from the surrounding tissues by bringing two tungsten needles down on either side of each ganglion and removing any adherent connective tissue with these needles. Ganglia were further sub dissected into their dorsomedial and ventrolateral aspects (Figure 2.3b)

7. The ganglia were collected and removed to a separate 35 mm dishes using a siliconised Pasteur pipette.

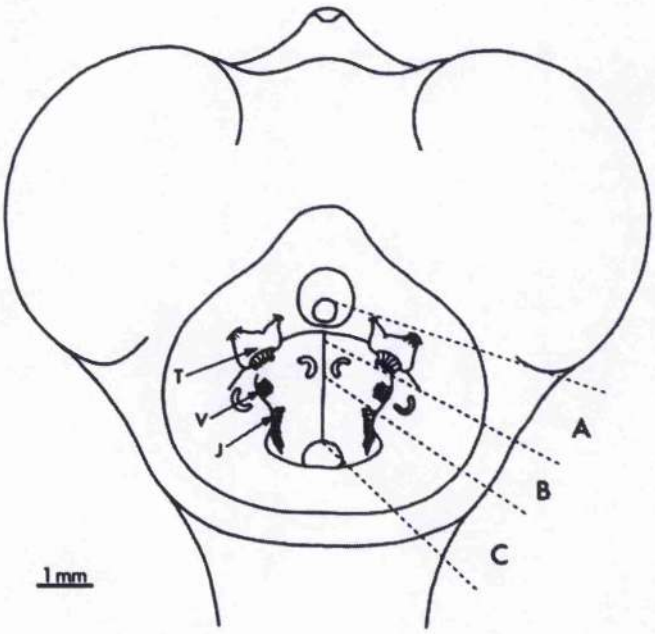


Figure 2.3a Dorsal aspect of the cranial base after removal of the brain, showing the lines for sub dissecting this tissue into blocks that contain the trigeminal ganglion (block A), vestibulo-acoustic, and geniculate ganglia (block B) and the jugular and petrosal ganglia (block C). The trigeminal ganglion (T) and the roots of the vestibulo-acoustic (V) and jugular (J) ganglia are shown. (Modified from Davies, 1988).

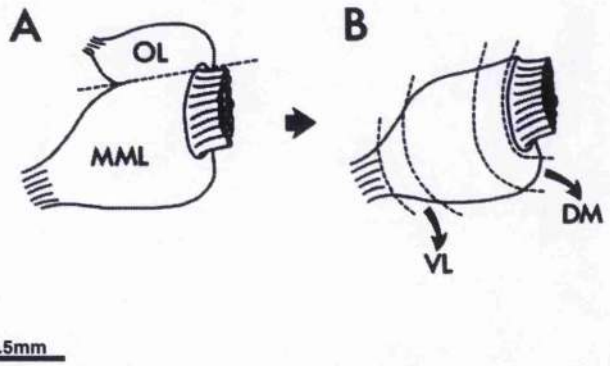


Figure 2.3b Sub dissection of the trigeminal ganglion into dorsomedial (DM) and ventrolateral (VL) poles. The ophthalmic lobe (OL) is separated from the maxillo-mandibular lobe (MML) along the interrupted line shown in **A**. The MML is sub dissected along the lines shown in **B** to obtain the DM and VL poles. (Modified From Davies, 1988).

Dissection of the mid-embryonic trigeminal mesencephalic nucleus (TMN).

This population of neural crest-derived, BDNF-dependent, primary sensory neurons was dissected from the midbrain (Davies, 1986).

1. The brains were collected in L15 medium (20 to 40 in most preparations).

2. These were transferred to a fresh plastic dish containing L15, and the dissection was completed with tungsten needles as shown in Figure 2.4.

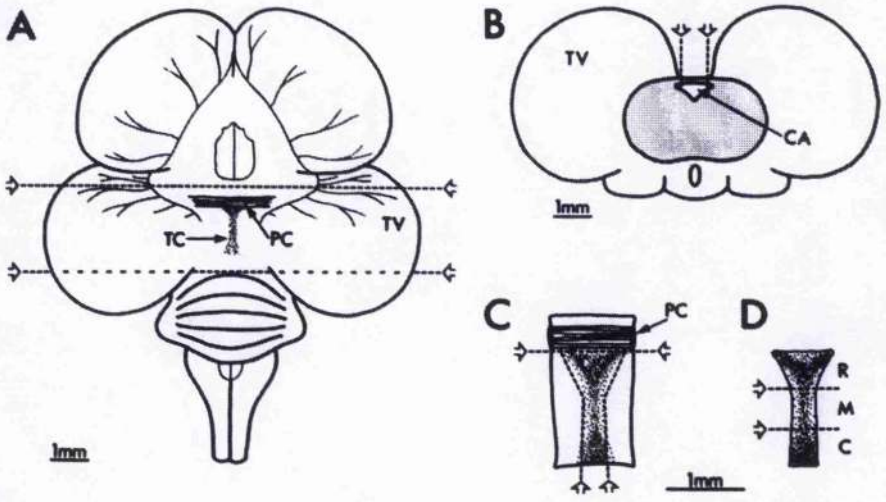


Figure 2.4 Successive stages in the dissection of the median part of the TMN from an E12 chick embryo. **A**, Dorsal aspect of the brain showing the location of the two coronal incisions (interrupted lines) for isolating the midbrain. **B**, Caudal aspect of the isolated midbrain showing the location of the two parasagittal incisions for removing the roof of the cerebral aqueduct after carefully stripping off the overlying pia mater. **C**, Dorsal aspect of the cerebral aqueduct showing the location of the incisions for cutting out the median part of the TMN. **D**, Sub dissection of the median part of the TMN. Abbreviations; tectal vesicle (TV), tectal commissure (TC), posterior commissure (PC), cerebral aqueduct (CA) rostral (R), middle (M), caudal (C). (Adapted from Davies, 1986)

Dissection of the mid-embryonic nodose ganglia.

The ganglia are located either side of the midline in tissues lying in front of the vertebral column at the base of the neck.

1. Embryos were extracted from eggs using a pair of curved forceps placed beneath the neck and the embryos were decapitated close to the base of the skull.

2. The skin was removed from the front of the neck and upper thorax using a pair of watchmakers forceps and the great vessels emerging from the heart (the aorta, superior and inferior vena cava, and pulmonary artery) were exposed by separating the overlying muscular and skeletal tissues at the upper part of the thorax.

3. Using one blade of a pair of watchmakers forceps, which was inserted deep to the root of the great vessel (i.e., between these and the underlying vertebral column), the great vessels were held and their attachment to the heart severed using a second pair of watchmakers forceps. The great vessels and the attached tissues lying in front of the neck were gently pulled away from the underlying vertebral column, using the second pair of forceps to loosen the tissues so that they peeled away from the vertebral column without tearing it (Figure 2.5).

4. This tissue, which contains both nodose ganglia, was gathered in a fresh 65 mm dish containing L15 medium. The tissue was then sub-dissected using tungsten needles. The nodose ganglia are recognised by their opaque white exterior, spindle shape and attached vagus nerve. The vagus nerves were removed and the ganglia were cleaned of all adherent tissue using tungsten needles.

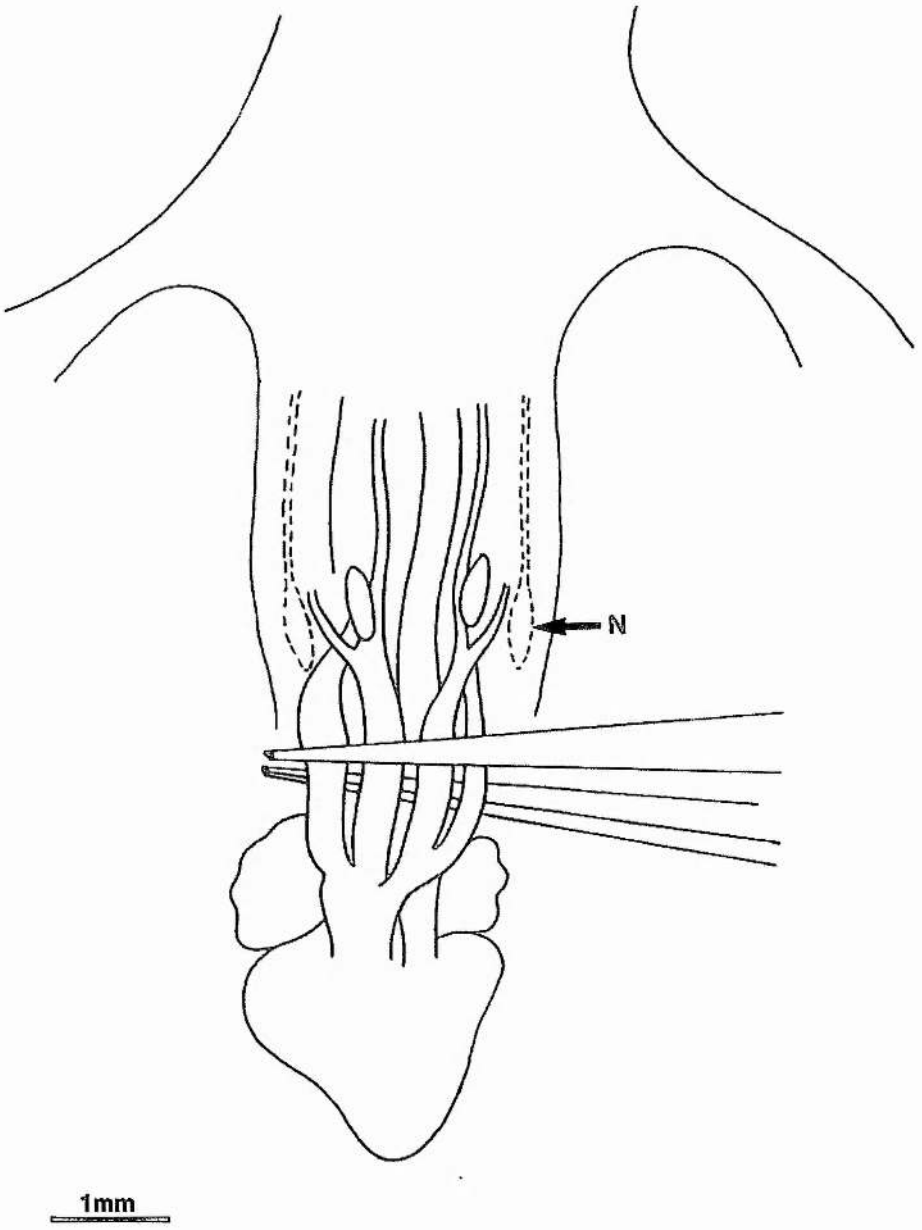


Figure 2.5 Ventral aspect of the thoracic region of an E10 chicken embryo after removal of the skin and reflection of the ventral thoracic wall to either side of the midline, exposing the heart and great vessels. The great vessels are clasped with a pair of forceps prior to removing the attached tissues. The connective tissue lying either side of the great vessels contains the two nodose ganglia (N) attached to the vagus nerves (shown by the interrupted lines).

Dissection of paravertebral sympathetic chain ganglia.

These ganglia are located either side of the midline, lying proximal to the vertebral column and ventral to the dorsal root ganglia.

1. Embryos were removed from eggs using a pair of curved forceps placed beneath the neck and the embryos were decapitated close to the base of the skull.

2. Skin was removed from the front of the neck and upper thorax using a pair of watchmakers forceps. A pair of curved forceps was used to remove the thoracic and abdominal viscera, exposing the spinal column and lumbosacral dorsal root ganglia.

3. The posterior thoracic and abdominal walls were gently washed with L15 from a Pasteur pipette.

4. Using the blade of a pair of watchmakers forceps, the ganglia were gently peeled away from the vertebral column (Fig. 2.6) Care was taken to ensure the ganglia were kept intact. Ganglia were then removed using a siliconised Pasteur pipette and pooled in a fresh 65mm dish containing L15 medium.

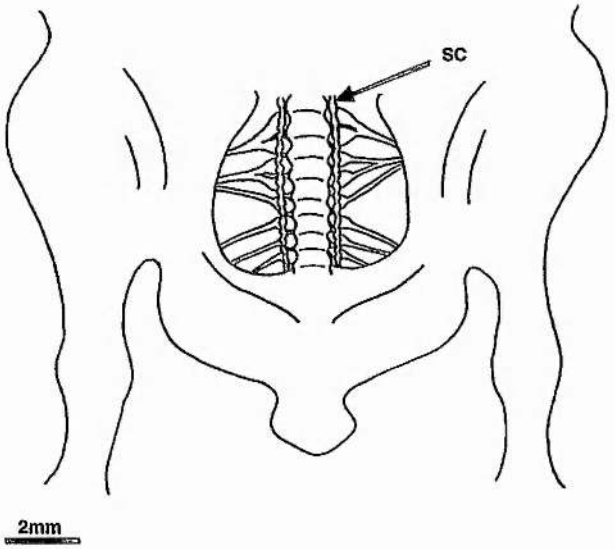


Figure 2.6 Ventral aspect of the lumbosacral region of an E10 chick embryo after evisceration showing the location of the paravertebral sympathetic chain ganglia (SC) (Adapted from Davies, 1988)

2.3 Dissection of cranial sensory ganglia from mouse embryos.

Mouse trigeminal and nodose ganglia, like those of the developing chick are readily accessible for study (see Fig. 2.7). The dissection technique for obtaining these ganglia differs with embryonic age. In all cases, the embryos were removed from pregnant females under sterile conditions .

1. The pregnant females were killed at the required stage of gestation (Theiler, 1972) by cervical dislocation.

2. 70% alcohol (from a wash bottle) was then sprayed onto the abdomen.

3. A small incision in the skin on the front of the abdomen was made, and the skin just above and below the incision was taken between the index finger and thumb of each hand. The skin was pulled away from the incision, immediately tearing it and exposing the abdominal muscles beneath.

4. Holding the anterior abdominal muscle with a pair of toothed forceps, a small incision was made with a pair of fine scissors, taking care not to cut into the intestines. Once air has entered the peritoneal cavity through this hole, an incision can be easily extended without cutting the intestines and contaminating the dissection with bacteria.

5. Each uterine horn was removed by holding with a pair of toothed forceps and cutting it free with a pair of scissors.

6. E9 to E12 embryos were removed from the uterine horns by using a pair of fine toothed forceps to pinch a small part of the musculature on the anti-mesometrial border of the uterine horn next to each embryo. The uterine muscle was then cut open with a pair of fine scissors. After this procedure, the embryo, contained within the intact chorion and amnion, was extruded by contraction of the remaining uterine muscle. Care was taken not to tear these membranes as naked embryos are forcibly extruded by muscular contraction of the uterine wall.

Removing embryos from the uterus within their membranes can minimise this damage. Embryos from E13 onwards are more easily removed from the uterine horns within their membranes. This entailed making one continuous incision along the anti-mesometrial border of each uterine horn thus exposing the embryos enclosed within their membranes.

7. The embryos were detached from the uterine horns whilst still within their membranes. This was done by detaching them from the placenta with a pair of watchmaker's forceps. The embryos were transferred to a fresh 65mm dish containing L15 medium, and the chorion and amnion were removed using watchmaker's forceps.

8. For very young embryos this procedure was carried out by aspirating the embryos into the blunt end of a Pasteur pipette after the elongated, narrow part of the pipette had been carefully broken off allowing the wide part of the pipette to be inserted into a pipette bulb.

Dissection of trigeminal ganglia from E10 to E12 embryos.

1. Using tungsten needles, two coronal incisions through the head were made, one just above each eye, the other between the maxillary and mandibular processes of the first branchial arch (Figure 2.8 A). The trigeminal ganglia can be seen as two opaque structures in the tissue slice obtained (Figure 2.8 B).

2. The ganglia can be easily removed from the tissue slice and freed of any adherent mesenchymal tissue using tungsten needles. To free the ganglion of this tissue, one needle was used to steady the ganglion and the other to pinch off the adherent tissue against the bottom of the dish.

3. The dissected ganglia were then transferred to a 35 mm dish containing L15 medium using a siliconised Pasteur pipette

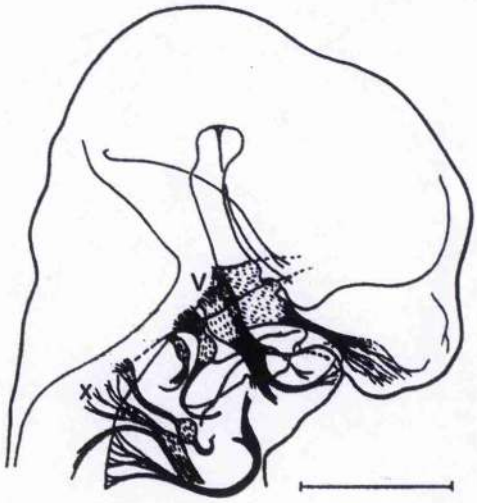


Figure 2.7 Drawing showing the medial aspect of the head of an E11 mouse embryo. The location of the trigeminal (V) and nodose (X) ganglia are marked. Scale bar = 1 mm.
(Adapted from Davies and Lumsden, 1986)

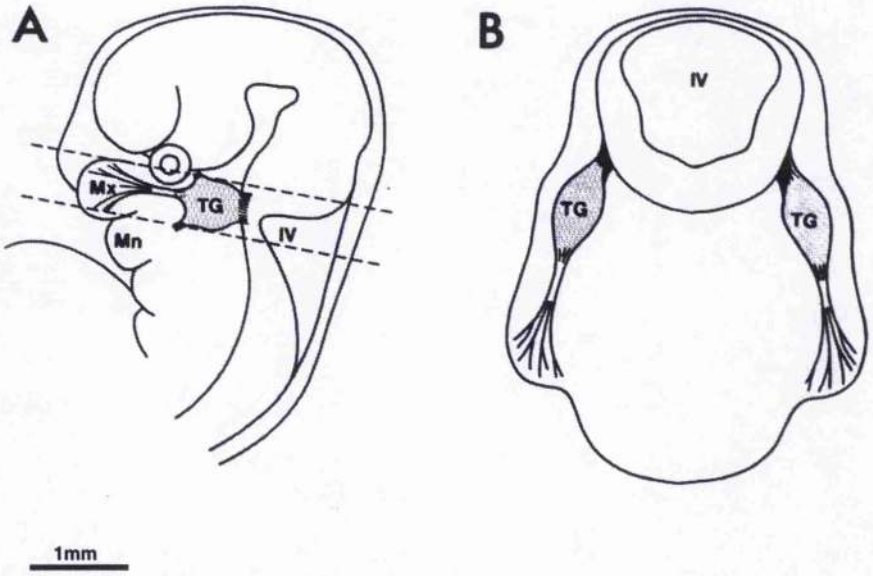


Figure 2.8 Drawings showing the dissection of the trigeminal ganglia from an E11 mouse embryo. **A**, lateral aspect of the E11 head showing the location of the transverse incision (interrupted lines) for obtaining a slice of tissue that contains both trigeminal ganglia. **B**, rostral aspect of this slice showing the location of the trigeminal ganglia. Trigeminal ganglion (TG), maxillary process (Mx), mandibular process (Mn), fourth ventricle (IV).
(Adapted from Davies and Lumsden, 1984).

Dissection of trigeminal ganglia from E13 and older embryos.

This dissection is similar to that of earlier ganglia except that a pair of fine scissors were used in the early stages of the dissection to cut through the cartilage (or bone in late foetal stages) of the developing head. The scissors used for this dissection have very fine serrations along the blades which help to grip the tissue to stop it from sliding out of the blades.

1. The top of the skull was removed, in a plane just above the eyes and whisker pads, using a pair of forceps to steady the embryo.

2. A second cut parallel to the first, passing through the mouth was then made.

3. Tissue slices were transferred to a fresh 65 mm dish containing L15 medium and two further cuts were made with the pair of scissors in front and behind the trigeminal ganglia.

4. These pieces of tissue were transferred to a fresh 65 mm dish containing L15 medium and tungsten needles were used to free the ganglia from the surrounding tissues and remove adherent connective tissue.

Dissection of nodose and superior cervical ganglia from mouse embryos.

In contrast to avian embryos, the nodose ganglion (otherwise known as the inferior vagal ganglion) remains situated close to the base of the skull in mouse embryos. The dissection is similar at all ages except that a pair of fine scissors is required during the initial stages of the dissection in E13 and older embryos.

1. The top of the skull and underlying forebrain were removed using the same plane of section described for the first incision of the trigeminal dissection.

2. The embryos were decapitated and the head cut in half along the saggital plane (using tungsten needles up to E12 and a pair of scissors or a number 15 scalpel in E13 and older embryos).

3. Using tungsten needles, the hindbrain was removed from each bisected head.

4. The cleft-like jugular foramen was then opened to the midline (Figure 2.9). For E12 and E13 embryos this was done by inserting one tungsten needle into the jugular foramen so that it lay beneath the base of the skull, medial to the foramen, and bringing a second needle into apposition with the first, so as to cut through the intervening tissues. For older embryos a pair of iridectomy scissors was used to achieve this.

5. A pair of tungsten needles was used to open up the jugular foramen which revealed the nodose ganglion lying at the base of this foramen. The nodose ganglion is spherical in structure with a prominent vagus nerve attached to its distal aspect. The ganglion is clearly distinguished from the superior cervical sympathetic ganglion (first visible at E13) which is an elongated structure that is attached caudally to the sympathetic chain, which is much thinner than the vagus nerve.

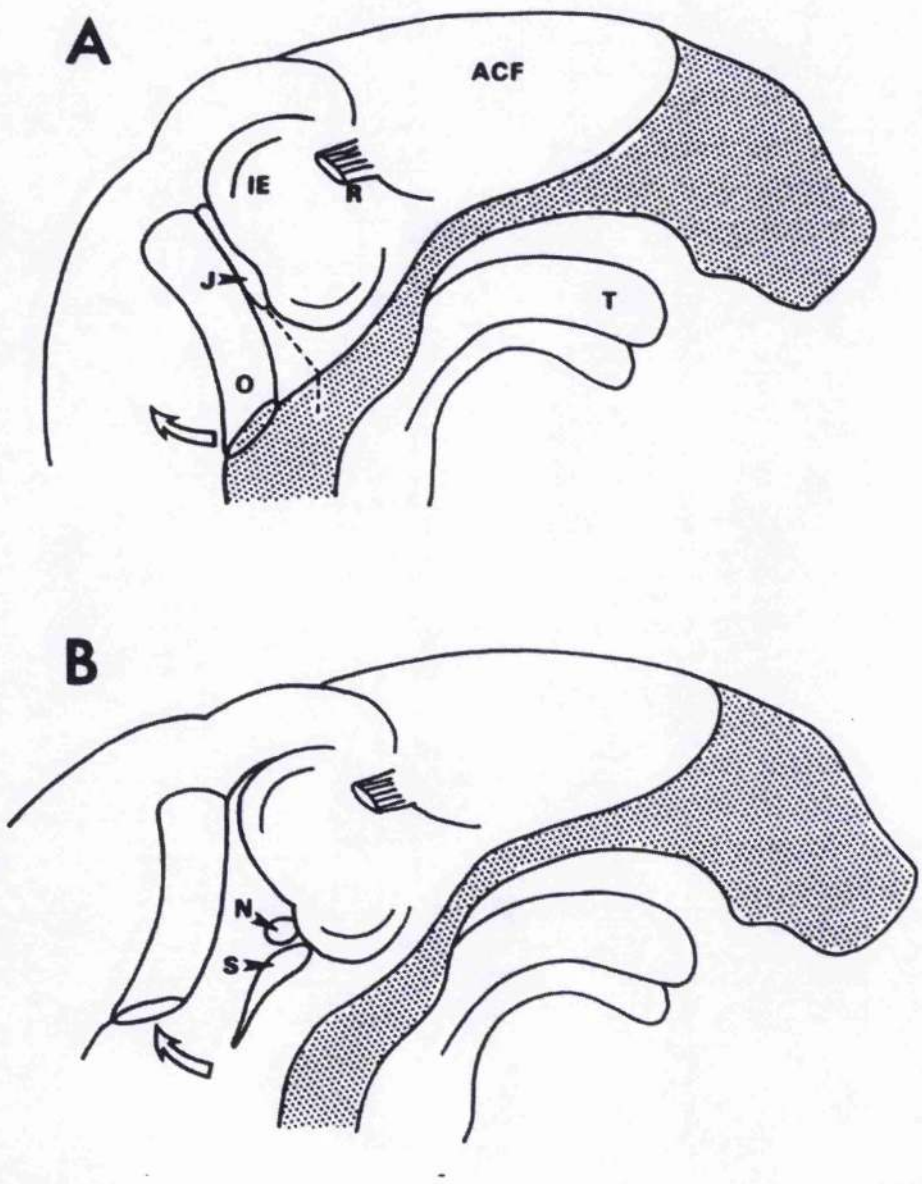


Figure 2.9 Drawings of the medial aspect of the left half of an E14 mouse embryo head showing successive stages in the dissection of the nodose ganglion. The bisected midline structures lying along and in front of the cranial base are stippled. **A.** The incision passing from the jugular foramen (J) to the midline is shown by the 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 the large curved arrow. T, tongue; ACF, anterior cranial fossa; R, root of the trigeminal nerve; IE, inner ear. **B.** The nodose ganglion (N) and superior cervical sympathetic ganglion (S) are revealed after extending the jugular foramen to the midline and reflecting the large ossified part of the occipital bone backwards.

2.4 Tissue dissociation techniques.

Dissected neural tissue was incubated with trypsin, washed and triturated to give a single cell suspension. The procedure is similar for mammalian and avian tissue of all embryonic stages, but the strength of the trypsin and incubation times were adjusted for the particular tissue.

1. Using a Pasteur pipette, the dissected tissue was transferred to a 10 ml conical tube containing 1 to 2 ml of calcium/magnesium-free, Hanks Balanced Salt Solution (CMF-HBSS). The tissue was washed, by agitation and the CMF-HBSS was then removed. For chicken ganglia, 0.9 ml of fresh CMF-HBSS was added, plus 0.1 ml of 1% trypsin (Worthington) in CMF-HBSS (stored as a stock solution in 0.1 ml aliquots at -20°C). For early mouse ganglia, 0.95 ml of fresh CMF-HBSS and 0.05 ml of 1% trypsin were added .

2. The lower end of the tube was immersed in a water bath, at 37°C, for 10 min for early sensory ganglia from chicken embryos, 15 to 20 min for mid-embryonic ganglia from chicken embryos, 5 min for early sensory ganglia from mouse embryos and up to 10 min for older embryonic mouse sensory ganglia. These times were adjusted for different batches of trypsin. The optimum time was discovered to be a compromise between neuronal damage due to over-trypsinization, and neuronal damage due to the vigorous trituration required to dissociate under-trypsinized tissue. If the tissue started to disaggregate before trituration, the time was too long. If the tissue dissociated with difficulty and incompletely with trituration, the time was not long enough.

3. After trypsinization, most of the trypsin solution was removed using a Pasteur pipette. The tissue was washed with 2 x 10 ml of Hams F12 or F14 medium containing 10% heat-inactivated horse serum to remove and inactivate the residual trypsin. Removal of the medium was facilitated by pelleting the tissue between washings in a bench top centrifuge, at 2000 x g, for 1 to 2 min

(this was essential for small ganglia). If the neurons were to be separated from the non-neuronal cells by differential sedimentation, it was necessary to wash the tissue with about 5 ml of HBSS to remove traces of serum.

4. After washing, the tissue was dissociated into a single-cell suspension by trituration. This step was critical . Trituration was carried out using a siliconized Pasteur pipette with the tip whose tip had previously been heated in a Bunsen burner flame to form a fine bore. The tissue was triturated in 1.5 ml of culture medium or, if the neurons were to be separated from non-neuronal cells by differential sedimentation (see below), HBSS without serum. The tissue and media were taken up into the pipette and the contents were slowly expelled with firm pressure. If done correctly, the early ganglionic tissue were completely dissociated after three to five passages. When using older tissue, some connective tissue fragments were left after all of the neurons had dissociated. These larger tissue fragments were allowed to settle after the first two or three passages. The supernatant suspension was then taken into a fresh tube and gentle trituration was continued (over-trituration causes substantial neuronal damage, especially in the case of large, older neurons). The trituration was monitored using an inverted phase contrast microscope, by examining a drop of the dissociated cell suspension on a glass slide. One characteristic of over-trituration is the loss of neuronal processes. Neurons that have been triturated carefully should have long processes attached to their cell bodies.

Because of the increasing amounts of collagenous connective tissue in late embryonic and postanatal ganglia, this tissue sometimes requires treatment with collagenase prior to trypsinization. Therefore in instances where late stage embryonic or postnatal tissues were used, the tissue was incubated with 500 μg/ml collagenase (Worthington) in F12, for 10 to 15 min, at 37°C. The tissue was then washed in HBSS prior to trypsinization.

2.5 Separation of neurons from non-neuronal cells.

A variety of different non-neuronal cells synthesise and release neurotrophic factors in culture. Therefore, it is essential to remove these cells prior to culture when studying the effects of neurotrophic factors on any population of neurons. If they are not removed, the percentage neuronal survival in control cultures may be unacceptably high. Furthermore, if non-neuronal cells are present, it cannot be concluded whether the effect of a factor or reagent on neuronal survival is due to a direct action of the factor on the neurons, or whether it is mediated via the non-neuronal cells.

Several different methods may be used to remove satellite cells and Schwann cells from mid to late embryonic dissociated ganglionic tissue. The method of differential sedimentation described here was found to be the most effective, reliable and rapid for removing non-neuronal cells from cranial sensory ganglia.

Differential adhesion was found to be advantageous when the size difference between neurons and non-neuronal cells is very small (as in the case of the vestibular ganglion), and may be desirable in these instances. However, neither method works very well for very early ganglia which contain progenitor cells in addition to satellite and Schwann cells. In these cases the potential effects of factors produced by non-neuronal cells can be reduced by setting up very low density cultures in a relatively large volume of culture medium (which was also routinely done for older embryonic cultures) or by setting up single cell cultures (Wright, *et al.*, 1992).

A further complication encountered when setting up early ganglionic cell cultures is that progenitor cells may differentiate into neurons in culture. Therefore, when studying the effects of factors on the survival of early neurons, it must be borne in mind that an increase in the number of neurons in early

ganglionic cell cultures could result not only from the enhanced survival of differentiated neurons, but also from an enhanced rate of neuronal differentiation. An increase in the number of neurons in early ganglionic cell cultures due to growth factor treatment, may also reflect an increase in the number of progenitor cells. An increase in progenitor cells may occur as a result of proliferation or enhanced survival. Such an increase will create a larger pool of cells that can subsequently differentiate into neurons. To distinguish between these alternatives it is necessary to follow the fates of individual neurons in these cultures (Buchman and Davies, 1993; Paul and Davies, 1995).

Differential sedimentation of embryonic chicken neurons.

This technique depends on differences in sedimentation rates of cells in a liquid medium. Generally, the larger the cell, the faster its rate of sedimentation, therefore neurons sediment more quickly than non-neuronal cells because of their greater size. Several variations of the method described by Miller and Phillips (Miller and Phillips, 1969) have been applied to embryonic neural tissue (Lam, 1972; Barkley, *et al.*, 1973; Cohen, *et al.*, 1973; Cohen *et al*; 1978; Berg and Fischbach, 1978). This method is applicable to all populations of neurons in the peripheral nervous system and is very efficient (at least 60% of the neurons in the starting tissue are recovered uncontaminated by other cells). Furthermore, since the cells sediment through culture medium, neuronal viability is very high.

1. Sedimentation was carried out in a 100 ml cylindrical, siliconised glass dropping funnel with a ground glass outlet tap (Figure 2.10). Funnels were sterilised before use by autoclaving. Each funnel was filled to a height of 8 to 10 cm with F14 medium plus 10% heat-inactivated horse serum (thoroughly

mixed and filtered through a 0.2 μm Millipore filter beforehand). Funnels were clamped vertically in a stand, and placed on a vibration-free surface at 2 ± 0.5°C, overnight.

2. The dissociated cell suspension was usually made up to a volume of about 2 ml in HBSS. The cell suspension was carefully layered on the medium, by running it down the inside of the funnel, and the foil on top of the dropping funnel was then replaced.

3. After an hour, the foil was removed from the spout, and 4 to 5 ml aliquots were run off into sterile tubes.

4. Samples of 0.5 ml were taken from each fraction, and placed in a 24 multi-well plate (with 16 mm diameter wells). These were examined with a phase contrast microscope to determine which fractions contained only neurons. Mid-embryonic and older neurons were clearly distinguished from other cells by their characteristic rounded phase bright cell bodies and elongated processes.

5. All of the neuronal fractions were pooled and were plated in Nunc tissue culture dishes, as described below.

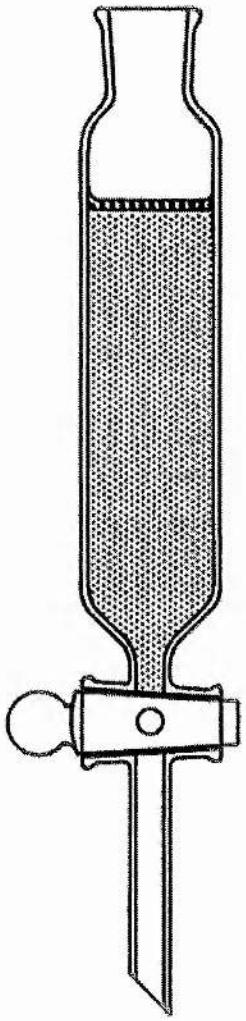


Figure 2.10 Drawing of a dropping funnel showing a cell suspension (heavy stipple) layered on culture medium (light stipple).
(Adapted from Davies, 1988)

2.6 Cell Culture Techniques.

Preparation of the culture substratum.

Neurons were grown on a laminin/polyornithine substratum. For most experiments, 35 mm diameter plastic tissue culture dishes were used (Nunc dishes available from provided the best results). The following substratum was used :

1. 1 ml of 0.5 mg/ml poly-DL-ornithine in 0.15M borate buffer (pH 8.6 and filter-sterilised using a 0.2 μm filter prior to use) was placed in each dish and left to stand overnight at room temperature.

2. The polyornithine solution was then aspirated, and the dishes were washed three times with sterile distilled water. Dishes were then left to air-dry in a laminar flow hood.

3. A 150 μl aliquot of a 20 μg/ml solution of laminin in F14 medium was placed in the centre of each dish, and a pipette tip was used to spread this over about two-thirds of the dish surface. Dishes were then placed in a tissue culture incubator for at least 4 h.

4. Dishes were removed from the incubator and washed twice with F14 culture medium (it is important not to allow the dishes to dry between washes as medium will form crystalline deposits on the base of the dish). 1 ml of medium was placed in each dish after washing.

Preparation of the culture medium.

Powdered F14 (a special formulation from Imperial labs see appendix) was made up with highly purified water that was sequentially passed through a charcoal filter, reverse osmosis system and a Milli-Q system before being double distilled. The F14 medium was made up from a frozen X10 concentrate (stored in 50ml aliquots kept at -40°C). The X1 F14 was made up by adding 1g of sodium bicarbonate to 450ml of water, then a 50ml aliquot of X10 F14 was added and CO_2 was bubbled through the medium until the pH reached between pH6.5 and pH7. 100 mg of streptomycin and 60 mg of penicillin were also added. The medium was then filter sterilised through a 0.2 μm filter, and stored at 4°C.

For culturing embryonic chicken neurons, the medium was supplemented with 10% heat-inactivated horse serum that was filtered through a 0.2 μm filter before use. Serum obtained from GIBCO generally gave the best results. For embryonic mouse neurons, the use of serum was avoided. Instead, a defined medium was used, containing the following supplements added to X1 F14 medium: 2 mM glutamine, 0.35% bovine serum albumin (Pathocyte-4, ICN), 60 ng/ml progesterone, 16 μg/ml putrescine, 400 ng/ml L-thyroxine, 38 ng/ml sodium selenite and 340 ng/ml tri-iodo-thyronine. The use of this medium was employed as it was not conducive for the growth of non-neuronal cells. Generally, mouse neurons were not separated from their non-neuronal counterparts using differential sedimentation, owing to their similarity in size.

Seeding the neurons.

To carry out a dose response to a neurotrophic factor, a suitable range of dilutions was first set up in culture medium at double the required final concentration.

The culture medium was removed from the washed dishes and 1 ml of each dilution was placed in triplicate dishes. Control dishes (usually at least four) each received 1 ml of medium. To avoid possible observer bias, dishes were often coded .

The most reliable results were obtained when the neurons were plated at a density of between 400 and 800 per dish. The suspension of neurons was placed in a suitable volume of medium in a large screw-top tube (e.g. 50 ml centrifuge tube) and the neurons were evenly distributed in the medium by gently rocking the tube end-over-end several times. The neurons were dispersed within each dish using a microlitre pipette, in two lots of 0.5 ml (to ensure even distribution). It was important to avoid touching the medium in the dishes with the pipette tip, otherwise small quantities of neurotrophic factor could be transferred to other dishes. Dishes were returned to a humidified CO_2 incubator at 37° C (4% CO_2 was used for F14 medium). Because of the very low neuron density required, the most reliable method of estimating the correct neuron density at the time of plating neurons was observation by eye. For this purpose, a 1ml aliquot of the cell suspension was placed in a test culture dish and the resulting neuronal suspension was examined with a phase contrast microscope.

Quantification of neuronal survival.

To quantify neuronal survival, a standard graticule for examining the same area of each culture dish was used. These were made from the base of a 90 mm plastic dish using a scalpel blade to inscribe a 12 x12 mm square divided into 2 mm squares, using graph paper as a template.

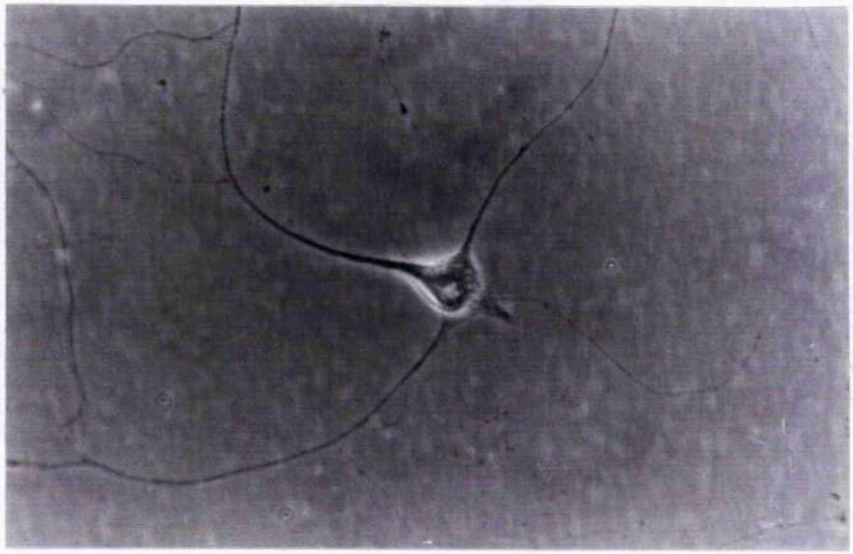
1. The graticule was mounted on the stage of an inverted phase contrast microscope with the inscribed surface uppermost.

2. To determine the number of neurons seeded per dish, several dishes were examined after 6 hours in culture. Each dish was centred over the graticule, and the number of neurons within the inscribed area was counted. A small percentage of neurons (usually less than 10%) are damaged during dissociation, and do not attach to the substratum. These neurons were ignored and only those that had attached were counted.

3. The number of surviving neurons in all dishes was counted after at least 48 hours in culture. In almost all cases, long neurites had grown from these neurons. Viable neurons were those neurons with rounded, phase bright cell bodies with a neurite length $\geq$ 5 times the diameter of the cell body (Fig 2.11)

4. The survival responses of neurons to a particular neurotrophic factor are expressed as percentage survival. This is, the quotient of the number of attached neurons in the graticule area at six hours and the number surviving in this same area at 48 hours.

A.



B.

Figure 2.11 **A.** Phase contrast photomicrograph of an E10 DMTG neuron after 48 hrs in culture in the presence of NGF. **B.** Phase contrast micrograph of an E10 DMTG neuron after 48 hrs in culture in the absence of trophic factors. Scale bar = 25.0μm.

Neurotrophic Factors.

The neurotrophins used in this study were obtained from the following sources.

Recombinant human NGF, was bacterially expressed in *Eschericia coli*, a gift of Dr. Arnon Rosenthal, Genentech Inc. Recombinant human NGF-mutant, was bacterially expressed in *Eschericia coli*, a gift of Dr. Arnon Rosenthal, Genentech Inc., USA. Recombinant human BDNF, was bacterially expressed in *Eschericia coli*, a gift of Dr. Arnon Rosenthal, Genentech Inc., USA. Recombinant human NT-4/5, was bacterially expressed in *Eschericia coli*, a gift of Dr. Arnon Rosenthal, Genentech Inc., USA. Recombinant *Xenopus* NT-4, was bacterially expressed in *Eschericia coli*, a kind gift of Prof. Yves Barde, Max-Planck Institute, Munich, Gerrmany

The neurotrophic cytokines used in this study were obtained from the following sources. Recombinant human CNTF, was bacterially expressed in *Eschericia coli*, a gift of Dr. Arnon Rosenthal, Genentech Inc., USA. Recombinant human LIF, was bacterially expressed in *Eschericia coli*, a kind gift of Dr. Perry Bartlett, Walter and Eliza Hall Institute, Melbourne, Western Australia. Recombinant human OSM, was bacterially expressed in *Eschericia coli*, a kind gift of Dr. Perry Bartlett, Walter and Eliza Hall Institute, Melbourne, Western Australia. Recombinant human IL-6, was bacterially expressed in *Eschericia coli*, a kind gift of Dr. Perry Bartlett, Walter and Eliza Hall Institute, Melbourne, Western Australia. Recombinant human CT-1, was bacterially expressed in *Eschericia coli*, a gift of Dr. Dianne Pennica, Genentech Inc., USA.

CHAPTER 3

THE ROLE OF NEUROTROPHIN-4/5 DURING CRANIAL SENSORY NEURON DEVELOPMENT.

3.1 Introduction

The neurotrophins NGF, BDNF and NT-3 have been structurally and functionally conserved during evolution. NGF, BDNF, and NT-3 from frog and mammals share 90-95% of their primary structure (Hallbook *et al.*, 1991) and neurons from aves, reptiles amphibians and rodents respond equally well to the mammalian factors (Thoenen and Barde, 1980; Levi-Montalcini and Aloe, 1985; Barde *et al.*, 1987; Hohn *et al.*, 1990; Maisonpierre *et al.*, 1990b; Rosenthal *et al.*, 1990; Buchman *et al.*, 1993). NT-4/5 is a recently identified member of the neurotrophin family that has been isolated from both *Xenopus* and mammalian tissues (Hallböök *et al.*, 1991; Berkemeier *et al.*, 1991; Ip *et al.*, 1992a). The biological activity of NT-4/5 had been examined in several previous studies (Henderson *et al.*, 1993; Ibáñez *et al.*, 1993; Zheng *et al.*, 1995a; Zheng *et al.*, 1995b), however, a detailed comparison of the species specificity and biological activity of *Xenopus* NT-4 and mammalian NT-4/5 had not been undertaken.

Cranial sensory neurons have been useful in studying the specificity of neurotrophins (Davies, 1987). In contrast to the functional heterogeneity of neurons in dorsal root ganglia (DRG), cranial sensory neurons are segregated into populations that deal with different kinds of sensation. As a possible consequence of this functional segregation, discrete populations of cranial sensory neurons require different neurotrophins when cultured during the period of naturally occurring cell death. In the chick embryo, the small diameter cutaneous sensory neurons of the jugular ganglion and the dorsomedial part of the trigeminal ganglion (DMTG) are supported by NGF (Davies and Lindsay, 1985), but show little response to BDNF (Davies *et al.*, 1986b) or NT-3 (Hohn

et al., 1990). The large diameter cutaneous sensory neurons of the ventrolateral part of the trigeminal ganglion (VLTG) are supported by BDNF (Davies *et al.*, 1986b), but show a negligible response to NGF (Davies and Lumsden, 1983) or NT-3 (Hohn et al., 1990). The proprioceptive neurons of the trigeminal mesencephalic nucleus (TMN) are supported by BDNF or NT-3 (Davies *et al.*, 1986a; Hohn *et al.*, 1990), but are unresponsive to NGF (Davies *et al.*, 1987). The enteroceptive neurons of the nodose ganglion contain subpopulations of neurons that are supported by either BDNF or NT-3 (Lindsay *et al.*, 1985; Davies *et al.*, 1986b; Hohn *et al.*, 1990), but are unresponsive to NGF (Lindsay and Rohrer, 1985). Cranial sympathetic and parasympathetic neurons also have distinct neurotrophin requirements. Superior cervical sympathetic ganglion (SCG) neurons are supported by NGF and NT-3 (Chun and Patterson, 1977; Greene, 1977; Rosenthal *et al.*, 1990), and the parasympathetic neurons of the ciliary ganglion are supported by ciliary neurotrophic factor (CNTF), but not by NGF, BDNF, NT-3 or NT-4/5 (Barbin *et al.*, 1984; Davies *et al.*, 1986b; Hohn *et al.*, 1990). Additionally, recent studies have shown that the survival of NGF-dependent cranial sensory neurons is transiently supported by BDNF and NT-3 during the early stages of target field innervation before the onset of naturally occurring neuronal death (Buchman *et al.*, 1993; Buj-Bello *et al.*, 1993).

Comparing NT-4/5 to other neurotrophins, I show that NT-4/5 and BDNF are survival factors for the same populations of mouse cranial neurons and that they are equipotent. In addition, I demonstrate that *Xenopus* NT-4 (hereafter referred to as xNT-4) and mammalian NT-4/5 are much less effective than mammalian BDNF as survival factors for chick neurons, and that some populations of neurons respond to both xNT-4 and NT-4/5, while others respond solely to xNT-4.

3.2 Results.

3.2.1 NT-4/5 is a Survival Factor for Specific Mouse Embryo Neurons

Low-density, dissociated cultures of trigeminal, nodose, and superior cervical ganglion (SCG) neurons were established from mouse embryos that were between 10 and 18 days gestation (E10 to E18). These neurons were grown either in medium alone (control cultures) or in medium supplemented with NGF, BDNF or NT-4/5. Except for nodose neurons at a very early stage of their development, virtually all neurons died in control cultures by 48 hours. Thus, the effects of neurotrophins on neuronal survival could be clearly observed at this time. Furthermore, because the serum-free medium used in these cultures prevented the growth of fibroblasts and glial cells, neuronal survival was not influenced by the release of growth factors from non-neuronal cells. For each experiment the number of neurons that initially attached to the plates was determined 6-9 hours after plating, and the percentage of neurons surviving after 48 hours was calculated. As depicted in Figure 3.1, similar numbers of trigeminal, nodose, and SCG neurons survived in the presence of BDNF and NT-4/5, whereas the survival response to NGF was markedly different. BDNF and NT-4/5 were both potent survival factors for E11 trigeminal neurons, but were unable to support the survival of this neuronal population at E15. In contrast, NGF did not support the survival of E11 trigeminal neurons but was a potent survival factor for these neurons from E13 onward. The majority of nodose neurons were supported by BDNF and NT-4/5 throughout their development from E11 to E18. Less than 5% of the SCG neurons were supported by either BDNF or NT-4/5 at E18, whereas the majority of these neurons were supported by NGF.

To determine whether BDNF and NT-4/5 act on the same subsets of trigeminal ganglion neurons, cultures of these neurons were grown in the

presence of saturating concentrations (2ng/ml) of BDNF or (50ng/ml) NT-4/5 alone and with both neurotrophins. Cultures of E11 neurons containing individual factors or a combination of BDNF and NT-4/5 sustained the survival of a similar number of neurons for 48 hours (Fig. 3.1 and data not shown). The lack of an additive effect indicates that BDNF and NT-4/5 promote the survival of the same neuronal populations.

To determine if the early survival-promoting effects of BDNF and NT-4/5 on NGF-dependent cutaneous sensory neurons are exerted over the same period of development, I compared the ability of BDNF and NT-4/5 to prevent the death of cultured trigeminal neurons at ages ranging from E10 to E15. Figure 3.2 shows that age-related changes in the response of trigeminal neurons to NT-4/5 and BDNF are very similar. E10 and E11 neurons were the most responsive. There was a four-fold decrease in the number of responding neurons between E11 and E12, and there was a further smaller decrease in responsiveness at later ages. This indicates that BDNF and NT-4/5 are active on trigeminal neurons over the same period of development.

To investigate the relative potencies of BDNF and NT-4/5, I conducted a series of dose response experiments. Trigeminal and nodose neurons were cultured in the presence of different concentrations of BDNF or NT-4/5 and the number of surviving cells was determined 48 hours later. BDNF and NT-4/5 supported the survival of E10 and E11 trigeminal neurons to the same extent over a broad range of factor concentrations (Figs 3.3 and 3.4). Likewise, there was no significant difference in the dose responses of nodose neurons to BDNF and NT-4/5 in cultures set up at E12 (Fig. 3.5).

Cultures of sensory ganglia from early developmental stages often contain progenitor cells that proliferate and differentiate *in vitro* (Rohrer *et al.*, 1985; Wright *et al.*, 1992). It is therefore possible that the increase in the number of neurons in E11 cultures following neurotrophin treatment was due to

enhanced proliferation or differentiation of progenitor cells and not due to the survival of post-mitotic neurons. To exclude this possibility, cohorts of neurons were identified by their bipolar morphology 6 hours after plating, BDNF or NT-4/5 was then added and the fate of the individual neurons was monitored for the next 42 hours. In these cohort experiments, 85 ± 3.8 % (mean ± SEM, n = 3) of neurons grown with BDNF and 89.7 ± 5.2 % (mean ± SEM, n = 3) of neurons grown with NT-4/5 survived from 6 to 48 hours in culture. In control cultures, none of the neurons identified at 6 hours survived to 48 hours. This indicates that BDNF and NT-4/5 have a direct survival-promoting effect on early trigeminal neurons. The fact that early trigeminal and nodose neurons cultured with BDNF or NT-4/5 contained more neurons at 48 hours than at 6 hours after plating (Figs. 3.1 and 3.2) suggests that proliferation or differentiation of progenitor cells occur in these cultures. It is therefore feasible that, in addition to their survival-promoting effects, BDNF or NT-4/5 act as mitogens or differentiation accelerating factors for neuronal progenitors in these cultures. Finally, although I did not undertake a quantitative study of neuronal morphology, there were no obvious differences in cell body size, or in neurite length or branching, between neurons surviving with BDNF and neurons surviving in parallel cultures with NT-4/5.

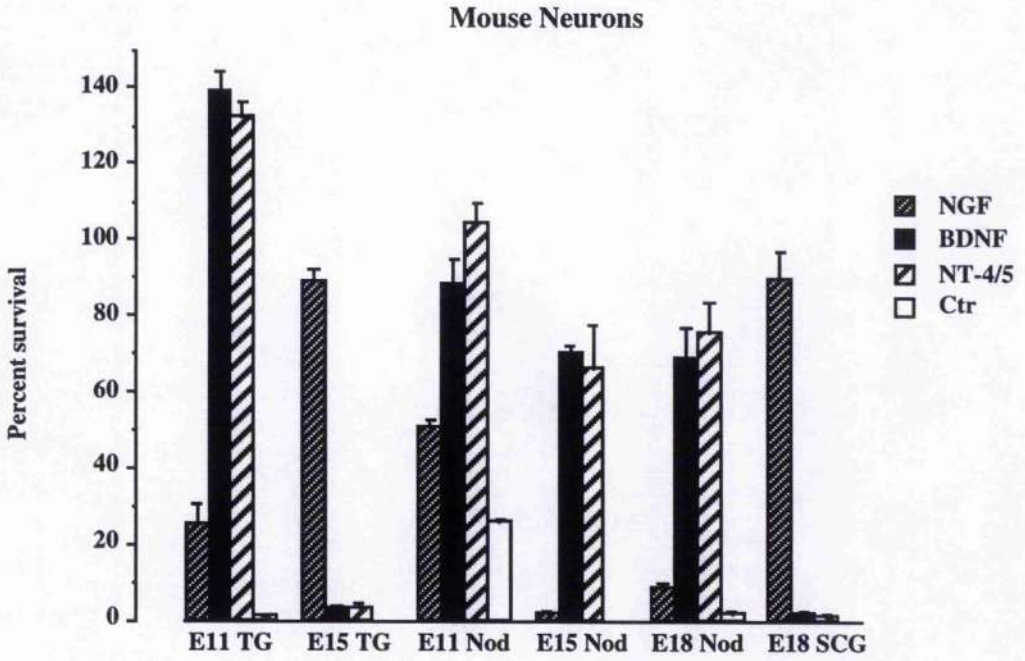


Figure 3.1 Survival of mouse embryo neurons in response to neurotrophins.

The bar graph shows the percentage of neurons surviving after 48 hours in low-density, dissociated cultures containing 2 ng/ml of NGF, BDNF or NT-4/5. The mean ± SEM of triplicate cultures are shown.

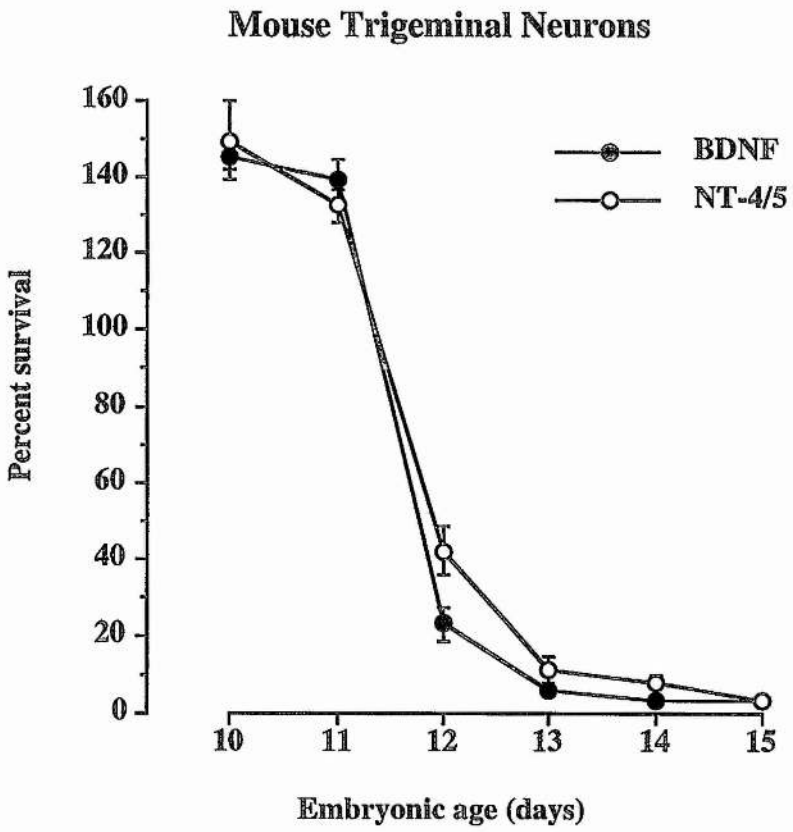


Figure 3.2 The survival of E10 to E15 mouse trigeminal neurons in cultures containing BDNF or NT-4/5.

The graph shows the number of neurons surviving after 48 hrs incubation with 2 ng/ml BDNF (filled circles) or 2 ng/ml NT-4/5 (open circles) expressed as a percentage of the number of neurons identified 6 to 9 hours after plating. The mean ± SEM of triplicate cultures from three separate experiments at each age are shown in both graphs.

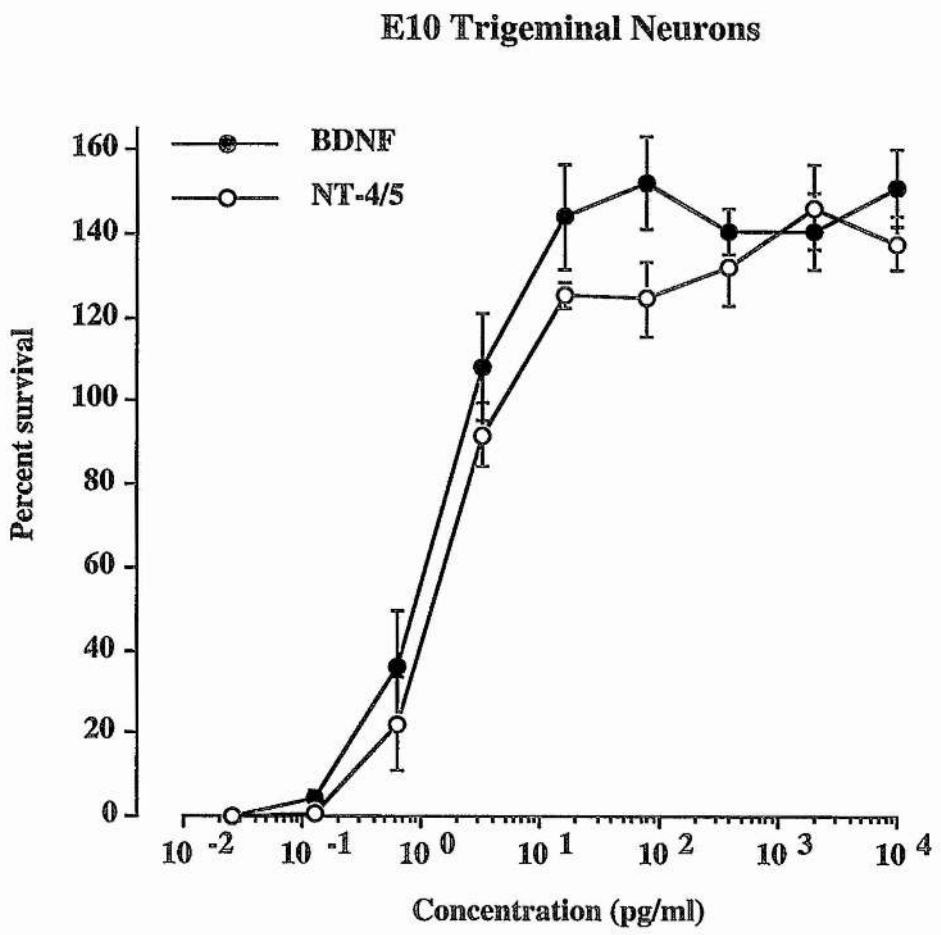


Figure 3.3 Dose response of embryonic mouse trigeminal neurons to BDNF and NT-4/5.

The graph shows the percentage of E10 trigeminal neurons surviving after 48hrs incubation with different concentrations of BDNF or NT-4/5. The number of neurons identified 6 to 9 hours and 48 after plating were used to calculate the percentage. The mean ± SEM of triplicate cultures from a representative experiment is shown.

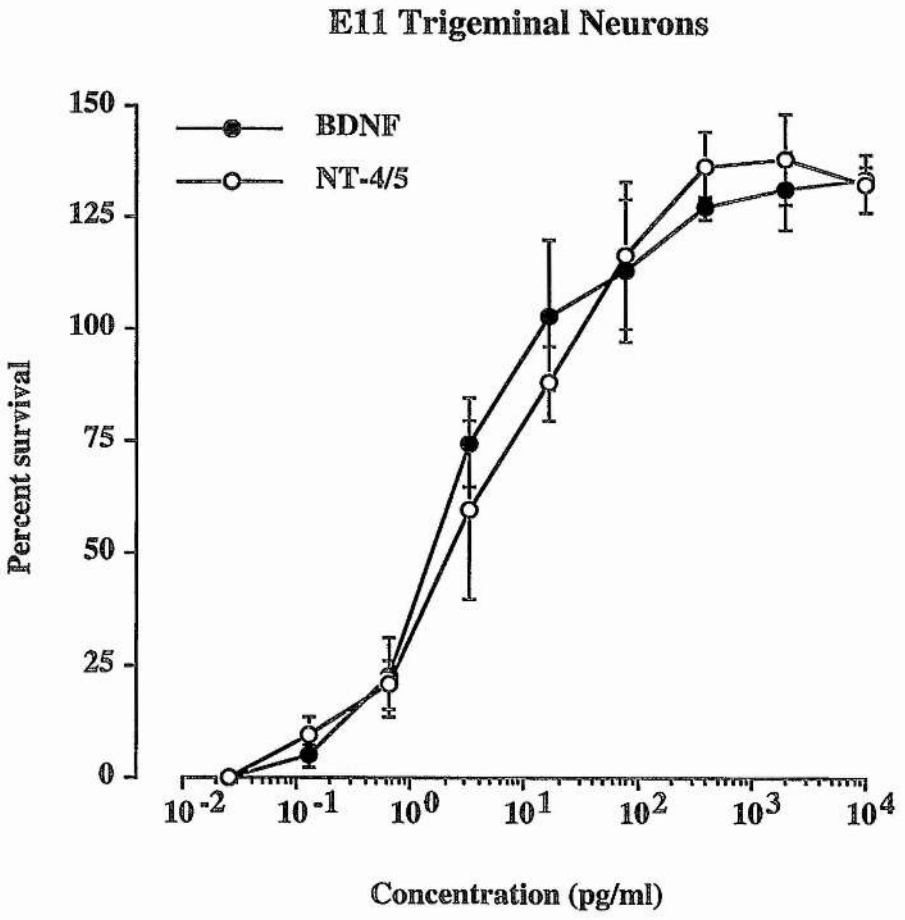


Figure 3.4 Dose response of embryonic mouse trigeminal neurons to BDNF and NT-4/5.

Graph depicting the percentage of E11 trigeminal neurons surviving after 48hrs incubation with different concentrations of BDNF or NT-4/5. The mean ± SEM of triplicate cultures from a representative experiment is shown.

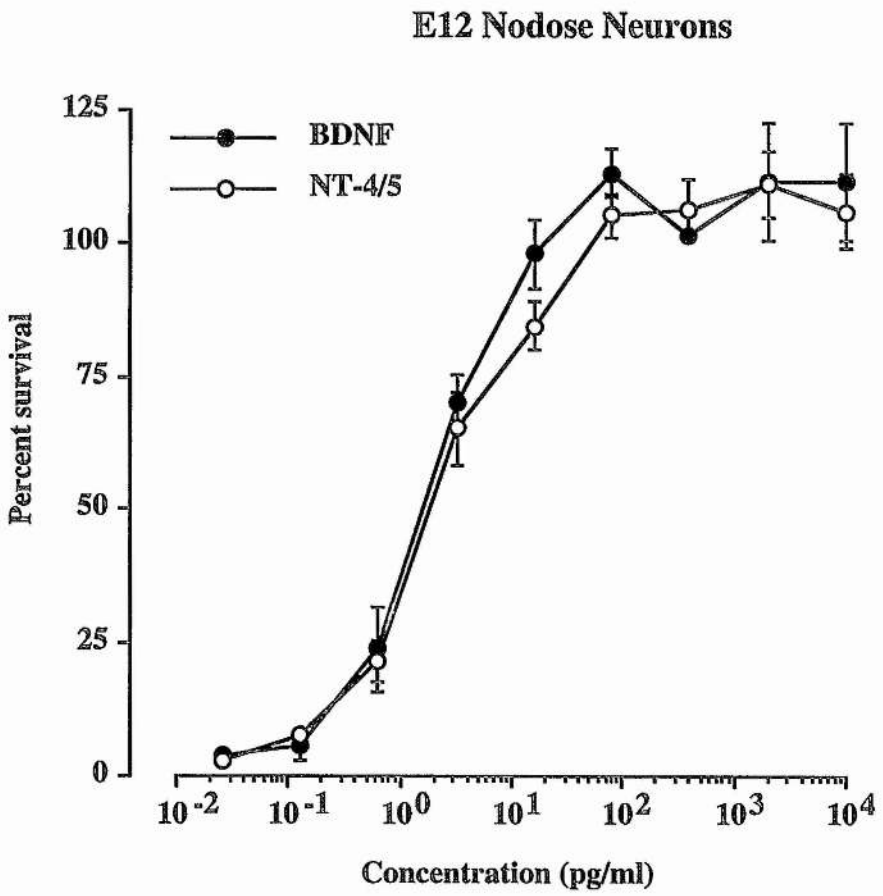


Figure 3.5 Dose response of embryonic mouse nodose neurons to BDNF and NT-4/5.

The graph shows the percentage of E12 nodose neurons surviving after 48hrs incubation with different concentrations of BDNF or NT-4/5. The mean ± SEM of triplicate cultures from a representative experiment is depicted.

3.3.2 Effect of NT-4/5 on Chick Embryo Neurons, Comparison with xNT-4

The specificity and the potency of NT-4/5 was also tested on cranial sensory and autonomic neurons from E10 chick embryos and was compared with that of xNT-4. At this developmental age, cranial chick embryo neurons can be dissected as homogeneous populations and can be separated from non-neuronal cells by differential sedimentation (see previous chapter). Furthermore, these neurons have an absolute dependence on neurotrophins for survival, and without exogenously added factors will all die by 48 hours. Surprisingly, NT-4/5 had little effect on any embryonic chick cranial neurons at a concentration which is above saturation for responsive embryonic mouse neurons (2 ng/ml) . I therefore examined the efficacy of NT-4/5 at 50 ng/ml. As shown in figure 3.6, addition of NT-4/5 at this concentration had a negligible effect on the survival of NGF-dependent DMTG neurons. In cultures of E10 BDNF-dependent VLTG, nodose, and TMN neurons, approximately 10% were supported by 50ng/ml of NT-4/5 (Figs. 3.7-3.9). The percentage of surviving neurons in these cultures was however, lower than that seen with the equivalent concentration of xNT-4 (consistently ~ 30%). When BDNF and xNT-4 were added in combination, no additional survival was observed beyond that seen with saturating concentrations of BDNF alone (Figs. 3.7 and 3.8), indicating that BDNF and xNT-4 act on the same subsets of trigeminal and nodose neurons. In the same experiments, the majority of neurons in cultures containing the most effective neurotrophic factor alone or in combination with NT-4/5 (at 50 ng/ml) sustained the survival of similar numbers of neurons (Figs. 3.6 - 3.8).

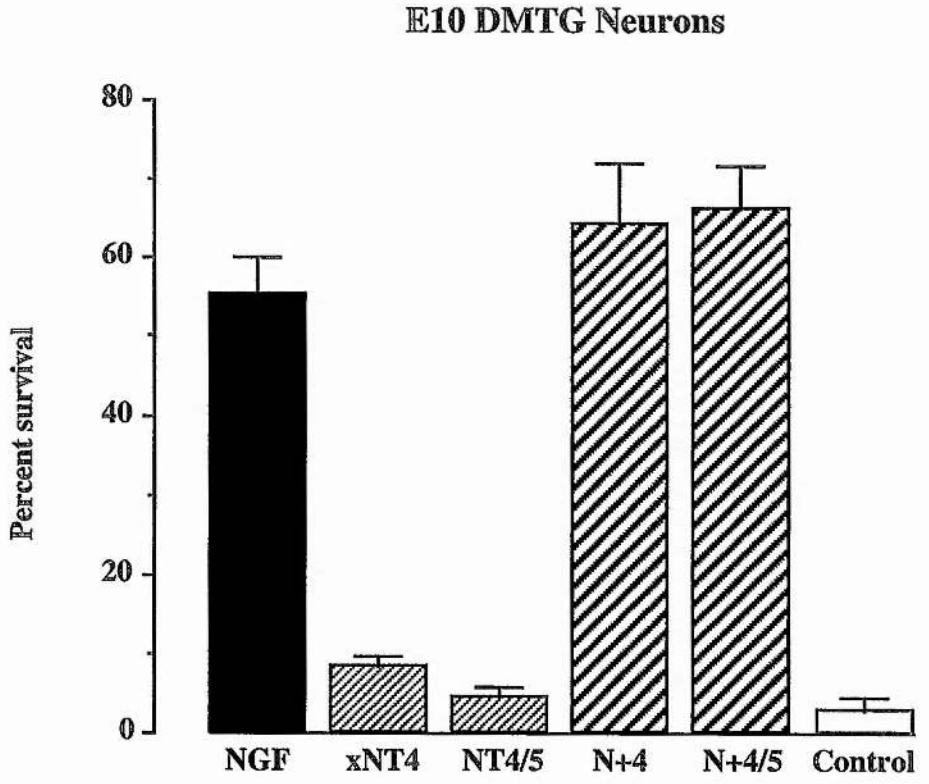


Figure 3.6 Survival of E10 chick DMTG neurons in response to neurotrophins.

The bar graph shows the percentage of neurons surviving after 48 hours in low density, dissociated, glia-free cultures containing no neurotrophic factors (control), 50 ng/ml of xNT-4 or NT-4/5, 2 ng/ml of NGF or combinations of these factors. The mean ± SEM of triplicate cultures are shown (N = NGF, 4 = xNT-4, 4/5 = NT-4/5).

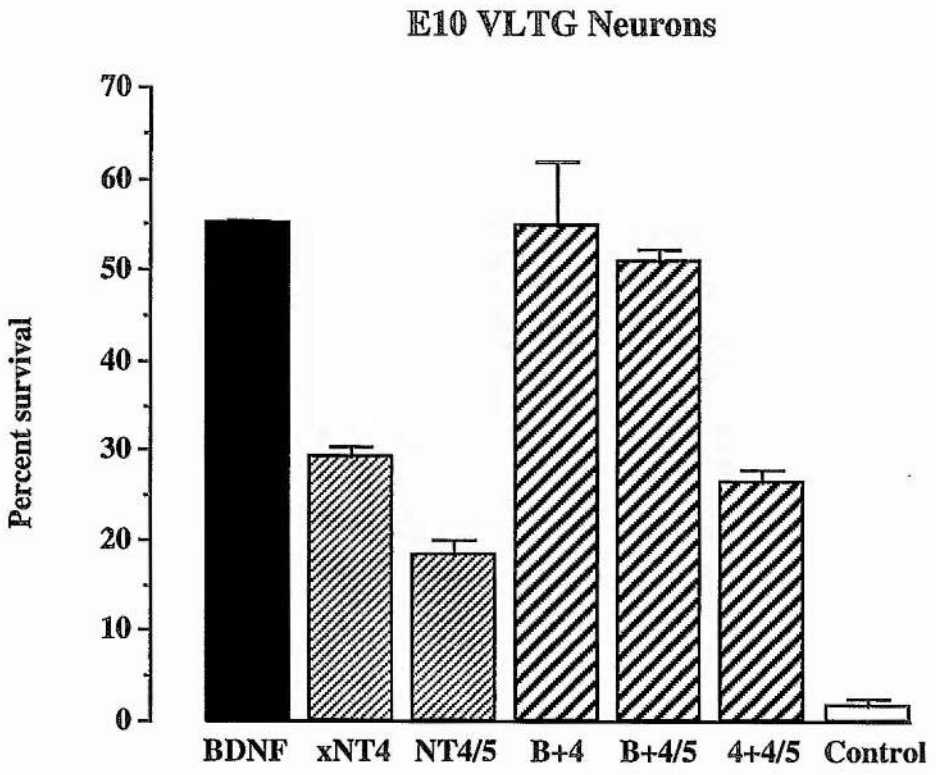


Figure 3.7 Survival of E10 chick VLTG neurons in response to neurotrophins.

Bar graph showing the percentage of neurons surviving after 48 hours in low density, dissociated, glia-free cultures containing no neurotrophic factors (control) or 50 ng/ml of xNT-4 or NT-4/5, 2 ng/ml of BDNF, or combinations of these factors. The mean ± SEM of triplicate cultures from a representative experiment are shown. (B = BDNF, 4 = xNT-4, 4/5 = NT-4/5)

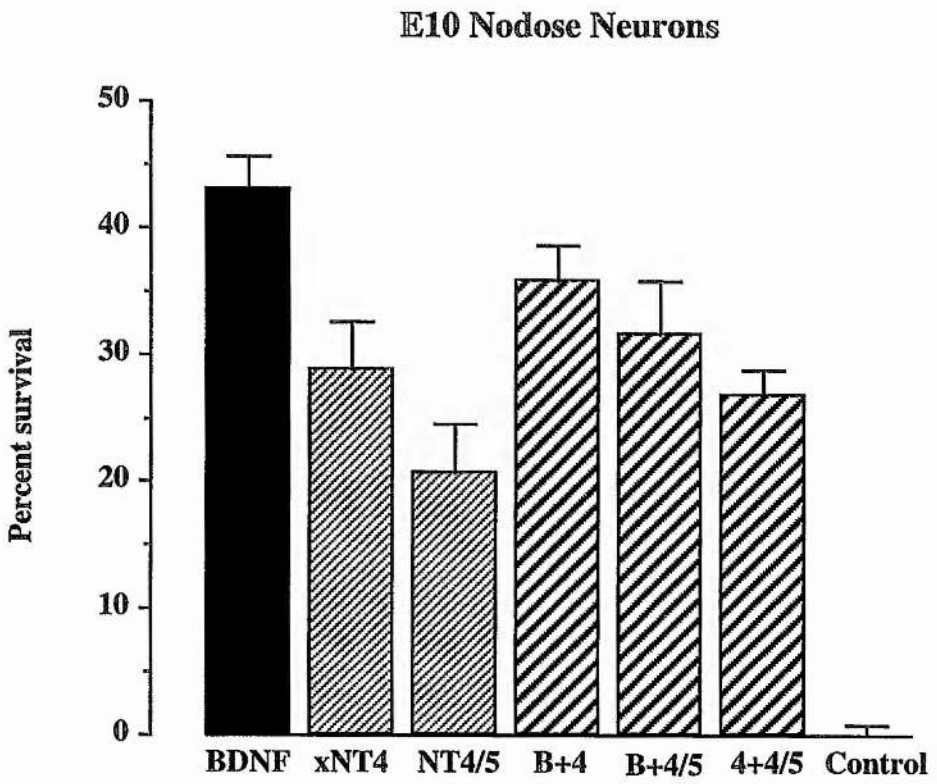


Figure 3.8 Survival of E10 chick nodose neurons in response to neurotrophins.

The bar graph shows the percentage of neurons surviving after 48 hours in low density, dissociated, glia-free cultures containing no neurotrophic factors (control), or 50 ng/ml of xNT-4 or NT-4/5, 2 ng/ml of BDNF or combinations of these factors. The mean ± SEM of triplicate cultures from a representative experiment are shown. (B = BDNF, 4 = xNT-4, 4/5 = NT-4/5)

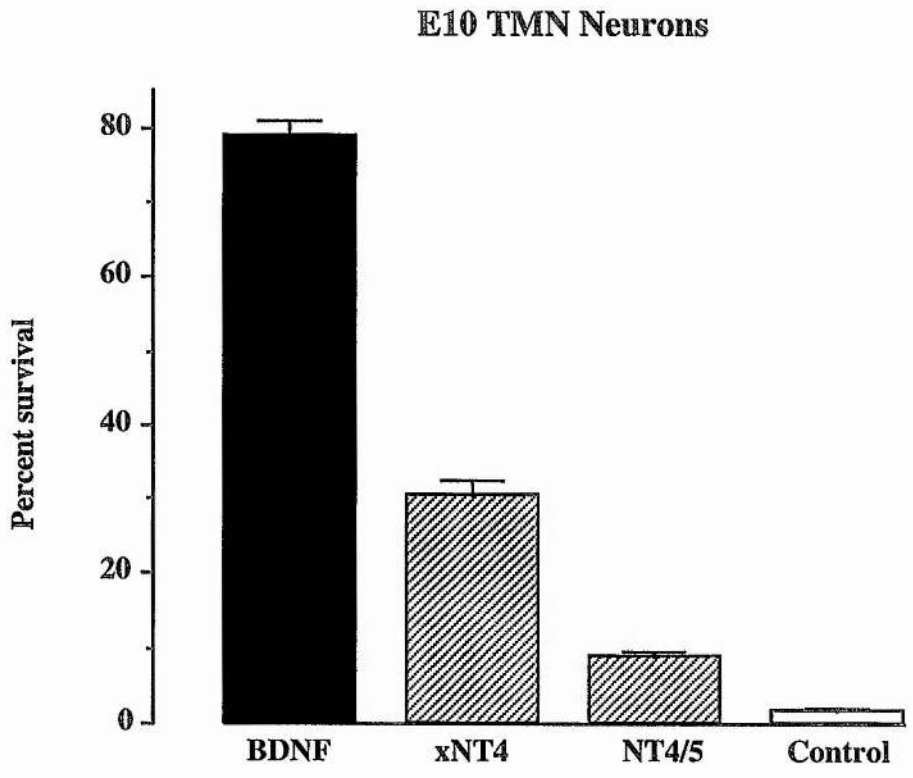


Figure 3.9 Survival of E10 chick TMN neurons in response to neurotrophins.

Bar graph showing the percentage of neurons surviving after 48 hours in low density, dissociated, glia-free cultures containing no factors (control), or 50 ng/ml of xNT-4, NT-4/5, or 2 ng/ml of BDNF. The mean ± SEM of triplicate cultures are shown. (B = BDNF, 4 = xNT-4, 4/5 = NT-4/5)

Although E10 chick neurons generally did not respond well to xNT-4 or NT-4/5, E6 DMTG neurons, which are BDNF-dependent at this age (Buj-Bello *et al.*, 1993), do respond to 50 ng/ml of these factors. I therefore compared the responses of these neurons to the three factors (Fig. 3.10). Although a similar number of neurons survived in the presence of saturating concentrations of either xNT-4, NT-4/5 or BDNF, xNT-4 and NT-4/5 were far less potent than BDNF. For BDNF, the effective concentration at half-maximal survival (EC_{50}) was 8 pg/ml, while for xNT-4, the EC_{50} was 220 pg/ml, and for NT-4/5, half-maximal survival was obtained at 8 ng/ml (calculated by interpolation of the data). This data suggests that xNT4 is an order of magnitude, and NT-4/5 three orders of magnitude less potent than BDNF as a survival factor for E6 DMTG neurons. Similar dose-response experiments were conducted using E10 TMN neurons, also revealing striking differences in the efficacy of these neurotrophins (see Fig. 3.11). In this case, NT-4/5 was virtually ineffective as a survival factor for E10 TMN neurons, and the maximal survival obtained with xNT-4 was ~ 60% of that obtained with BDNF.

Thus, unlike other neurotrophins, NT-4/5 can discriminate between mouse and chick embryonic neurons. Furthermore, although all the BDNF-responsive neurons I tested in the mouse embryo are supported equally well by NT-4/5, certain populations of BDNF-responsive neurons are more effectively supported by NT-4/5 than others in the chick embryo. Furthermore, I have shown that xNT-4 is more effective in promoting the survival of BDNF responsive chick embryo neurons than mammalian NT-4/5.

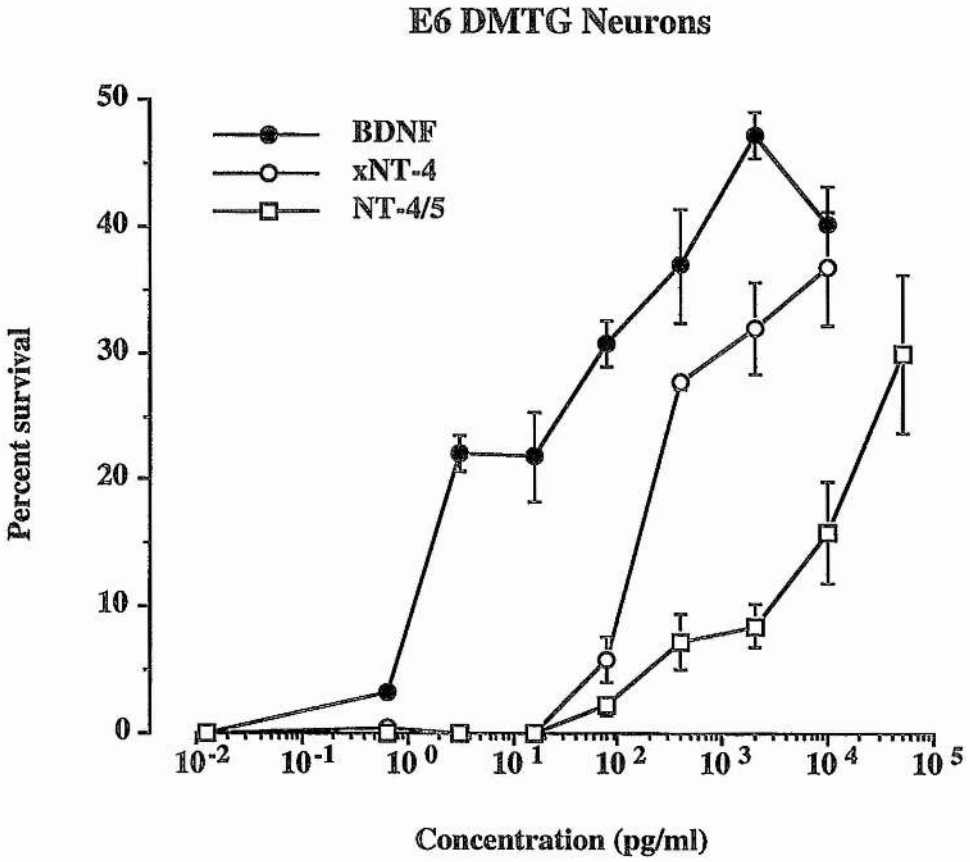


Fig.ure 3.10 Dose-responses of embryonic chick DMTG neurons to BDNF, xNT-4 and NT-4/5.

The number of E6 DMTG neurons surviving after 48 hrs incubation with different concentrations of BDNF (filled circles), xNT-4 (open circles) or NT-4/5 (open squares), are expressed as a percentage of the number of neurons identified 6 to 9 hours after plating. The mean ± SEM of triplicate cultures from representative experiments are shown.

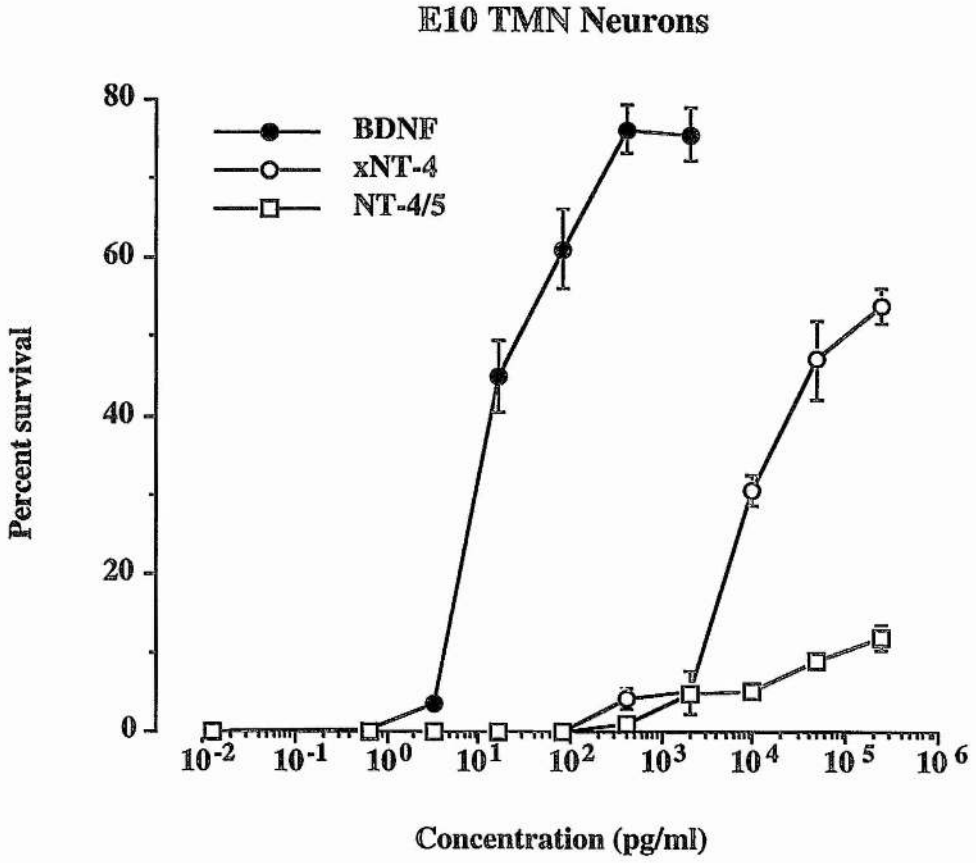


Fig.ure 3.11 Dose-responses of embryonic chick TMN neurons to BDNF, xNT-4 and NT-4/5. The number of E10 TMN neurons surviving after 48 hrs incubation with different concentrations of BDNF (filled circles) or xNT-4 (open circles) are expressed as a percentage of the number of neurons identified 6 to 9 hours after plating. The mean ± SEM of triplicate cultures from representative experiments are shown. The effective concentration at half-maximal survival was; 26 pg/ml for BDNF and 7 ng/ml for xNT-4 (NT-4/5 - not done).

3.3 Discussion.

I have quantitatively determined the ability of NT-4/5 to promote the survival of sensory and autonomic neurons of mouse and chick embryos in low-density, glia-free cultures. Furthermore, I have extended this study to compare the survival promoting effects of mammalian NT-4/5 with *Xenopus* NT-4.

NT-4/5 promoted the survival of mouse E11 trigeminal neurons, which are transiently dependent on BDNF at this developmental stage, and of mouse E11 to E18 nodose neurons, which also depend on BDNF (Fig 3.1). NT-4/5, at the concentration tested, had negligible effect on the survival of mouse E15 trigeminal neurons which are NGF-dependent at this embryonic stage. Surprisingly, although NT-4/5 and BDNF were equipotent in promoting the survival of mouse neurons, NT-4/5 was 3 orders of magnitude less potent than BDNF as a survival factor for homologous populations of chick embryo neurons. Furthermore, NT-4/5 had negligible effect on the survival of some populations of chick neurons. Thus, NT-4/5 appears to be a species-specific neurotrophin. It displays the same activities as BDNF on mammalian cranial neurons and can discriminate between different BDNF-responsive chick neurons.

One explanation for the specificity of NT-4/5 among vertebrates is that the structure of NT-4/5 was not as conserved between mammals and birds as that of other neurotrophins (Ip *et al.*, 1992a). I therefore compared the responses of human NT-4/5 with *Xenopus* NT-4, which bear 65% sequence homology at the amino acid level (Ip *et al.*, 1992a). Interestingly, the *Xenopus* derived NT-4 is more effective at promoting the survival of BDNF-responsive chick neurons than mammalian NT-4/5. There remains the possibility that an avian homologue of NT-4/5 exists which is more closely related to xNT-4, however at

this juncture, none has been identified. Thus, the increased potency of xNT-4 may arise from a greater affinity for the avian *trkB* receptor than that of NT-4/5.

Although NT-4/5 is a highly potent neurotrophin for certain populations of embryonic mouse neurons, its neuronal specificity is not unique. In this study of different populations of neurons at stages throughout their early development, NT-4/5 displayed identical neuronal specificity and potency to BDNF. In contrast, the neuronal specificity of NGF and NT3 , although partially overlapping with that of BDNF and NT-4/5 on early trigeminal neurons, was clearly different (Fig 3.1; Hohn *et al.*, 1990; Rosenthal *et al.*, 1990; Buj-Bello *et al.*, 1993). The apparent redundancy in function of NT-4/5 observed here could be explained in several ways. First, NT-4/5 may possess unique activities on neuronal populations that have not been tested in this study. Second, although BDNF and NT-4/5 promote the survival of identical neuronal populations in culture, they may each have distinct activities *in vivo* due to modulation by accessory factors. Third, it is possible that the only difference between BDNF and NT-4/5 is their distinct tissue distribution. If correct, this possibility implies that amino acids which are not conserved between the two proteins are not essential for function.

The common neuronal specificity of BDNF and NT-4/5 in mouse embryos may be a consequence of the binding of these neurotrophins to the same cell surface receptor. Two classes of neurotrophin receptors have been identified: p75 and the *trk* family of tyrosine kinases of which three members, *trkA, trkB* and *trkC*, have been identified (Chao, 1992; Meakin and Shooter, 1992). Considerable evidence suggests that *trk* receptors are directly involved in neurotrophin signal transduction and ligand discrimination. Neurotrophins promote rapid autophosphorylation of *trk* tyrosine kinases (Kaplan *et al.*, 1991a, 1991b; Klein *et al.*, 1991a; 1991b; Soppet *et al.*, 1991) and elicit responses in oocytes (Nebreda *et al.*, 1991), cell lines (Cordon-Cardo *et al.*, 1991; Glass *et*

al., 1991; Lamballe *et al.*, 1991b; Loeb *et al.*, 1991; Squinto *et al.*, 1991) and embryonic neurons transfected or microinjected with *trk* cDNAs (Allsopp *et al.*, 1993a). In contrast, p75 is probably not a functional neurotrophin receptor alone (Bothwell, 1991) and binds NGF, BDNF, NT-3 and *Xenopus* NT-4 with similar affinity (Sutter *et al.*, 1979; Rodriguez-Tébar and Barde, 1990; Hallböök *et al.*, 1991; Rodriguez-Tebar *et al.*, 1992).

Using proliferation (Ip *et al.*, 1992a) and tyrosine phosphorylation (Berkemeier *et al.*, 1991) assays in 3T3 fibroblasts expressing either *trkA* or *trkB*, NT-4/5 was shown to be a potent activator of *trkB* and a weak activator of *trkA* . Like NT-4/5, BDNF also promotes rapid phosphorylation of *trkB* and elicits a mitogenic response in *trkB*-expressing fibroblasts (Glass *et al.*, 1991; Klein *et al.*, 1991b; Soppet *et al.*, 1991; Squinto *et al.*, 1991) but is inactive on *trkA* and *trkC* (Lamballe *et al.*, 1991b). Thus, it seems likely that BDNF and NT-4/5 exert their common effects on the survival of embryonic mouse neurons by acting via *trkB*.

NT-4/5 at 50 ng/ml did not show any survival promoting effects on either E18 SCG neurons or E15 trigeminal neurons, both of which were supported by NGF (Fig. 3.1). Therefore, although NT-4/5 at high concentrations can activate the *trkA* high affinity NGF receptor (Hempstead *et al.*, 1991; Kaplan *et al.*, 1991a, 1991b; Klein *et al.*, 1991a; Lamballe *et al.*, 1991b; Meakin *et al.*, 1992) in fibroblasts (Berkemeier *et al.*, 1992; Ip *et al.*, 1992a), this activation does not appear to have any physiological relevance for the survival of developing mouse neurons.

Previous studies of the biological activity of NT-4/5 reported that it promoted the survival of substantial numbers of embryonic chick DRG (Ip *et al.*, 1992a), and sympathetic (Berkemeier *et al.*, 1991) neurons grown in dissociated culture. The discrepancy between these findings and my results may have been due to differences in the purity, processing or concentration of NT-

4/5 used. The previous studies used partially purified conditioned media from mammalian cell lines that contained an unknown concentration of NT4/5 and could possibly carry aggregated or truncated forms of this protein. In contrast, in this study I used highly purified recombinant NT-4/5 and xNT-4 at known concentrations that were produced by *Eschericia coli.*

Although BDNF and NT-4/5 display identical specificity and potency as survival factors for mouse neurons, they differ in their ability to prevent the death of chick embryo neurons. NT-4/5, at a concentration of 50 ng/ml, promoted the survival of E6 DMTG neurons but was an ineffective survival factor for E10 TMN neurons. In contrast, BDNF displays similar potency in promoting the survival of both of these neuronal populations and is more potent than NT-4/5. Thus, NT-4/5 and BDNF differ in both specificity and potency as survival factors for chick embryo neurons. Although E6 DMTG neurons respond to NGF (Buj-Bello *et al.*, 1993) whereas E10 TMN neurons are unresponsive to NGF (Davies *et al.*, 1987), it is unlikely that NT-4/5 exerts its effects on E6 DMTG neurons via a regular *trkA* receptor because it does not support the survival of other NGF-dependent neurons. Alternatively, it is possible that E6 DMTG and E10 TMN neurons express different forms of the *trkA* or *trkB* receptors or of p75-like accessory molecules. Multiple *trkB* transcripts and at least three distinct *trkB* receptors that differ in their cytoplasmic domains have already been identified (Klein *et al.*, 1989; Klein *et al.*, 1990; Middlemas *et al.*, 1991; Allen *et al.*, 1994). Recently, the developmentally regulated expression of truncated *trkB* isoforms was demonstrated in trigeminal sensory neurons (Ninkina *et al.*, 1996). These isoforms of *trkB* are up-regulated concomitant with down-regulation of the fully functional *trkB* receptor, at a time when trigeminal neurons lose their responsiveness to BDNF (Ninkina *et al.*, 1996). It therefore seems unlikely that differences in the specificity of xNT-4 or NT-4/5 are conferred by differential activation of the truncated *trkB* isoforms. The extracellular region of the Trk

receptors has previously been implicated in discrimination between ligands, and several studies have suggested that either the immunoglobulin-like domains of *trkB* or alternatively the leucine-rich motifs of the receptor are involved in this discrimination (Urfer *et al.*, 1995; Windisch *et al.*, 1995a; Windisch *et al.*, 1995b;Windisch *et al.*, 1995c; Ninkina *et al.*, 1997). Another recent study has shown that a long and a short isoform of chick *trkB* exist which differ in the extracelluar domain (Ströhmaier *et al.*, 1996). Whereas the long and short *trkB* isoforms bind BDNF equally well, the short isoform has a markedly reduced binding capacity for xNT-4 and NT-4/5 (Ströhmaier *et al.*, 1996). Furthermore, differences in the binding capacity of these isoforms were observed which suggest that *Xenopus* NT-4 has greater affinity than mammalian NT-4/5 (Ströhmaier *et al.*, 1996).

I therefore propose that the observations I have made in the latter part of this study can be accounted for in two ways. First, that differences in the survival promoting effects of BDNF, xNT-4 and NT-4/5 that I observed on cultured BDNF-responsive neurons, are due to the different binding affinities of these proteins for *trkB*, thus; BDNF > xNT-4 > NT-4/5. Second, that the higher concentrations of xNT-4 required to support the survival of E10 TMN neurons compared with E6 DMTG neurons (see Figs. 3.10 and 3.11) can be accounted for by proposing that the later stage TMN neurons express greater levels of a *trkB* isoform that binds xNT-4 and NT-4/5 with reduced affinity.

Given more time, I would have extended the scope of this study to analyse the expression of chick *trkB* receptor isoforms in different avian cranial sensory ganglia at different stages of their development.

CHAPTER 4

NGF BINDING TO P75 ENHANCES THE SENSITIVITY OF SENSORY AND SYMPATHETIC NEURONS TO NGF AT DIFFERENT STAGES OF DEVELOPMENT.

4.1 Introduction.

The sensitivity of neurons to neurotrophins is primarily controlled by the expression of their receptors, TrkA, TrkB, TrkC and p75. The Trk family of receptor tyrosine kinases are signalling receptors that undergo rapid transphosphorylation following neurotrophin binding. Expression studies in cell lines have shown that TrkA is a receptor for NGF, TrkB is a receptor for BDNF and NT4/5 and TrkC is a receptor for NT3, although NT3 can also bind and signal less efficiently via TrkA and TrkB (Bothwell, 1995). The finding that the distinctive neuronal deficiencies in mice with null mutations in the *trkA*, *trkB* and *trkC* genes are similar to those observed in mice with null mutations in the NGF, BDNF and NT3 genes, respectively, suggests that Trks play a key role in mediating the specific survival-promoting actions of neurotrophins (Davies, 1994b; Snider, 1994; Lewin and Barde, 1996).

p75 is a transmembrane glycoprotein that binds all neurotrophins with the same affinity but with different rate constants. Although p75 is not essential for the survival response of neurons to neurotrophins, considerable evidence suggests that it plays an important role in modulating the sensitivity of neurons to different neurotrophins during development. The finding that embryonic sensory and postnatal sympathetic neurons, but not embryonic sympathetic neurons, from p75$^{-/-}$ mice are less sensitive to NGF than wild type neurons suggests that p75 selectively enhances the sensitivity of neurons to NGF at certain stages of development (Davies *et al.*, 1993b; Lee *et al.*, 1994). Although reductions in the sensitivity of p75-deficient PNS neurons to other neurotrophins

has not been observed, sympathetic neurons from postnatal p75-deficient mice are more sensitive to NT3 than sympathetic neurons from wild type animals (Lee *et al.*, 1994). Likewise, the ability of NT3 to activate TrkA in the PC12 pheochromocytoma cell line is enhanced when NT3 binding to p75 to prevented by function-perturbing antibodies or when p75 expression is very low (Benedetti *et al.*, 1993; Clary and Reichardt, 1994), suggesting that p75 reduces the ability of NT3 to signal via TrkA. Furthermore, studies with mutated BDNF and NT4/5 proteins that bind TrkB normally but fail to bind p75 suggest that p75 plays a role in TrkB ligand discrimination. Whereas the BDNF mutant activates TrkB as effectively as wild type BDNF, the NT4/5 mutant activates TrkB less effectively than wild type NT4/5 (Ryden *et al.*, 1995). How p75 selectively modulates neuronal responsiveness to neurotrophins is not understood. In particular, since p75 and TrkA cluster independently of NGF in cultured cells (Ross *et al.*, 1996), it is unclear whether p75 affects NGF signalling by a direct interaction between p75 and TrkA that is independent of NGF binding to p75.

Recently, an additional level of complexity has been added to the controversy, involving a novel p75 signalling mechanism mediated by sphingomyelin (reviewed by Chao, 1995). Sphingomyelin is a ubiquitous sphingolipid residing in the outer leaflet of the cell membrane, which upon hydrolysis by a sphingomyelinase enzyme, produces the second messenger ceramide (Chaterjee, 1994). The second messenger function of ceramide is known to influence both cell growth and gene activation by the transcription factor NF-κB (Carter *et al.*, 1996). Ceramide has been shown to induce apoptosis in the leukaemic U937 cell line (Obeid *et al.*, 1993), in the neuron-like PC12 cell line (Hartfield *et al.*, 1997), and in primary cultures of rat embryo mesencephalic neurons (Brugg *et al.*, 1996). In contrast, ceramide has also been implicated in neuronal survival, and has been shown to rescue cultured sympathetic neurons from cell death induced by NGF deprivation (Ito and Horigome, 1995). The production of ceramide initiated by NGF through the p75

receptor, has recently been demonstrated in the T9 glioma cell line (Dobrowsky *et al.*, 1994). Ceramide induction through p75 is specifically dependent on NGF binding and is mediated by the intracellular domain of the receptor (Dobrowsky *et al.*, 1994).

Several studies have implicated the cytoplasmic domain of p75 in intracellular signalling. For example, when PC12 cells expressing receptor chimeras with an EGF extracellular domain fused with the p75 cytoplasmic region are treated with EGF, these cells show a response similar to that seen when p75 expressing cells are treated with NGF (Yan *et al.*, 1991). EGF treatment of T9 glioma cells expressing these receptor chimeras has been shown to mediate an increase in sphingomyelin hydrolysis (Dobrowsky *et al.*, 1994) and addition of NGF, BDNF, or NT-3 to p75 expressing NIH 3T3 cells also activates this pathway (Dobrowsky *et al.*, 1995). Furthermore, the transcription factor NF-κB is activated in p75 expressing Schwann cells after treatment with NGF, an effect which is mimicked by the addition of cell permeable ceramide analogs (Carter *et al.*, 1996).

To clarify the role of p75 in enhancing the survival response of sensory and sympathetic neurons to NGF at certain stages of development, I have compared the response of these neurons to NGF and an NGF mutant that binds TrkA normally, but has negligible binding to p75. I demonstrate here that sensory and sympathetic neurons are less responsive to the NGF mutant than wild type NGF at the same stages of development at which sensory and sympathetic neurons from $p75^{-/-}$ mice are less responsive to wild type NGF than neurons from wild type embryos. This confirms, by an alternative approach, the stage-specific modulation of NGF sensitivity by p75 in developing sensory and sympathetic neurons and demonstrates that this effect requires NGF binding to p75. Furthermore, the demonstration that there are similar levels of p75 and TrkA mRNAs expressed at the stages of maximal NGF sensitivity has

implications for the formulation of models to explain how p75 modulates NGF signalling via TrkA. In addition, I have compared the survival responses of NGF and the NGF mutant in the presence of a cell permeable analogue of the lipid second messenger ceramide, demonstrating that the p75 modulated survival response is independent of this lipid second messenger pathway.

4.2 Production of the NGF mutant and binding to TrkA and p75

Mutagenesis, expression and protein characterisation of the NGF mutant was performed as described elsewhere (Shih *et al.*, 1994). Oligonucleotide-directed mutagenesis of human NGF (Künkel, 1985) was modified according to the BioRad Mutagene kit (BioRad). NGF and the mutant protein concentration was determined by two independent enzyme-linked immunoassays (ELISA). One of these assays utilised a purified rabbit anti-human NGF polyclonal antibody and the other utilised a purified murine monoclonal antibody to NGF. Each assay was repeated at least three times for NGF and the NGF mutant and the results varied by less than 20% in a single transfection. Between separate transfections, the concentration of NGF and the mutant ranged between 3 and 8 mg/ml. Parallel metabolic labelling experiments (Shih *et al.*, 1994) indicated that >90% of the NGF or NGF mutant was represented in the fully processed 14 kDa form.

It has been shown previously that mutations within variable region 1 (residues 25-35) of murine NGF can result in significant loss of p75 binding while binding to TrkA is minimally affected (Ibañéz *et al.*, 1992). To explore the role of p75 in modulating the survival response of developing neurons to NGF, I used a bacterially synthesised and expressed mutant form of human NGF in which Lys 32, Lys 34 and Glu35 were replaced by Ala (K32A, K34A, E35A).

The affinity of this mutant for TrkA and p75 was determined by competition binding of [^{125}I]NGF to TrkA and p75 immunoadhesion proteins (Fig. 4.1).

The binding of the NGF mutant relative to wild type NGF was determined by competition displacement of [^{125}I]NGF from TrkA and p75 receptor immunoadhesion proteins (Shelton *et al.*, 1995). Briefly, detachable 96-well strips (Corning) were assembled and coated overnight at 4^{o}C with 100 ng of goat F(Ab')$_2$ fragments of anti-human IgG$_1$ Fc domain (Chappel) diluted in 0.1 M Tris, pH9. After washing with PBS, 10 ng of *trkA*-IgG or p75-IgG immunoadhesion proteins (gifts of Drs. David Shelton and Avi Ashkenazi, Genentech) comprising the Fc portion of human IgG$_1$ Human [^{125}I]NGF and the extracellular receptor domains of TrkA or p75, respectively, were incubated in each well for 90 minutes at room temperature in binding buffer (L15 medium, 20 mM HEPES, pH7.2, 1 mg/ml Fraction V BSA (Sigma) and 0.5 mg/ml cytochrome C). The plate was then washed with PBS and increasing concentrations of NGF or NGF mutant diluted in binding buffer were added to each well together with 25 pM [^{125}I] human NGF. Binding was carried out at room temperature for 90 minutes. The plates were then washed with PBS plus 0.05% Tween 20, after which the wells were detached and counted. Human [^{125}I]NGF was iodinated by the lactoperoxidase-Enzymobead method (Escandon *et al.*, 1993); the specific activity was approximately 60 mCi/mg as determined by TCA precipitation. The relative 50% inhibitory concentrations (IC_{50}) for human NGF and TrkA (39 $\pm$ 11 pM, n = 3) or human NGF and p75 (205 $\pm$ 85 pM, n = 3) are within a factor of 2 to 3 of the IC_{50} and Kd values for mouse NGF binding to these receptors reported previously (Sutter *et al.*, 1979; Klein *et al.*, 1991a; Kahle *et al.*, 1992; Vröegop *et al.*, 1992).

Production of the NGF mutant and the binding studies referred to in this study were carried out by Dr. John Winslow of Genentech Inc. USA, and are reproduced by permission.

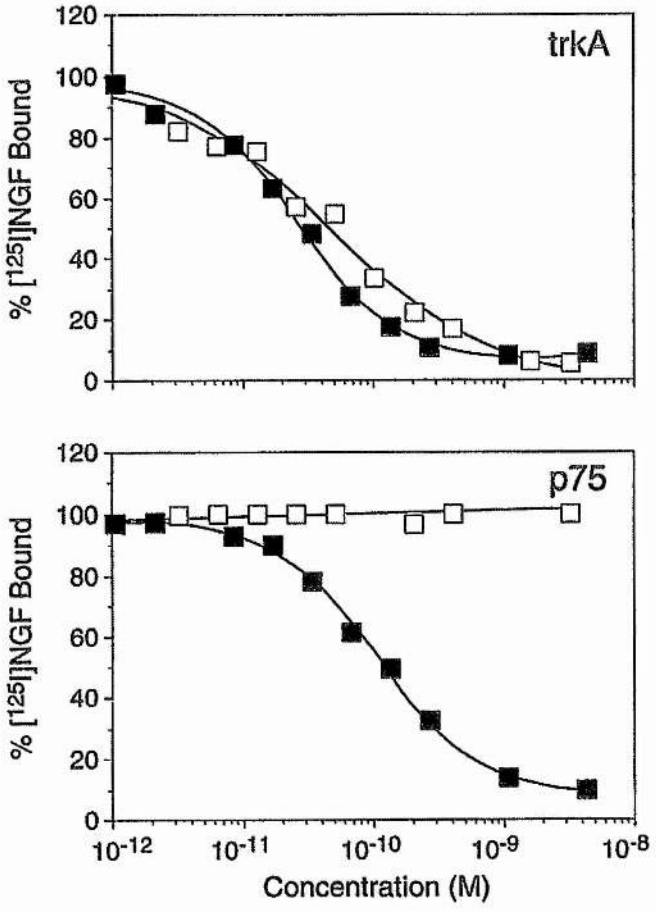


Figure 4.1 Receptor binding of human NGF and the NGF mutant to TrkA and p75. The relative affinity of NGF (solid squares) and the mutant (open squares) for TrkA (top panel) and p75 (bottom panel) receptor immunoadhesion proteins are compared by competitive binding of [^{125}I]NGF. The data presented is one representative experiment out of three. Each data point was determined in duplicate and the error is between 2-7% of the mean values shown. In this particular experiment, the IC_{50} (concentration of NGF or mutant that results in 50% inhibition of [^{125}I]NGF binding) for NGF is 28 pM whereas the IC_{50} for the mutant is 45 pM. For p75 binding, the IC_{50} for NGF is 106 pM while there is no IC_{50} for the mutant even at the highest concentration (4,500 pM).

(Courtesy of Dr. John Winslow, Genentech Inc.)

4.3 Results

To determine if the NGF mutant differs in its effectiveness in promoting neuronal survival compared with NGF, I compared the dose-responses of two populations of NGF-responsive neurons of developing mice to wild type NGF and the NGF mutant. Trigeminal neurons were studied at E14, the age at which p75 deficient trigeminal neurons undergo a shift in survival responsiveness, requiring higher concentrations of NGF to support their survival (Davies *et al.*, 1993). Similarly, sympathetic neurons were studies at two ages, E17 and P4, ages at which p75 deficient neurons show differing responses to NGF. Virtually all neurons (> 99%) in control (neurotrophin-free) cultures died by 48 hours of incubation.

4.3.1 Influence of the NGF mutant on sensory neuron survival

The dose response of E14 trigeminal neurons to the mutant NGF was consistently shifted to higher concentrations compared with the dose response of these neurons to NGF (Fig. 4.2). Interpolation of the data from three separate dose response experiments on E14 trigeminal neurons showed that there was a four-fold difference between the concentration of NGF that elicited half-maximal survival (36 pg/ml) and the concentration of the NGF mutant that elicited half-maximal survival (146 pg/ml).

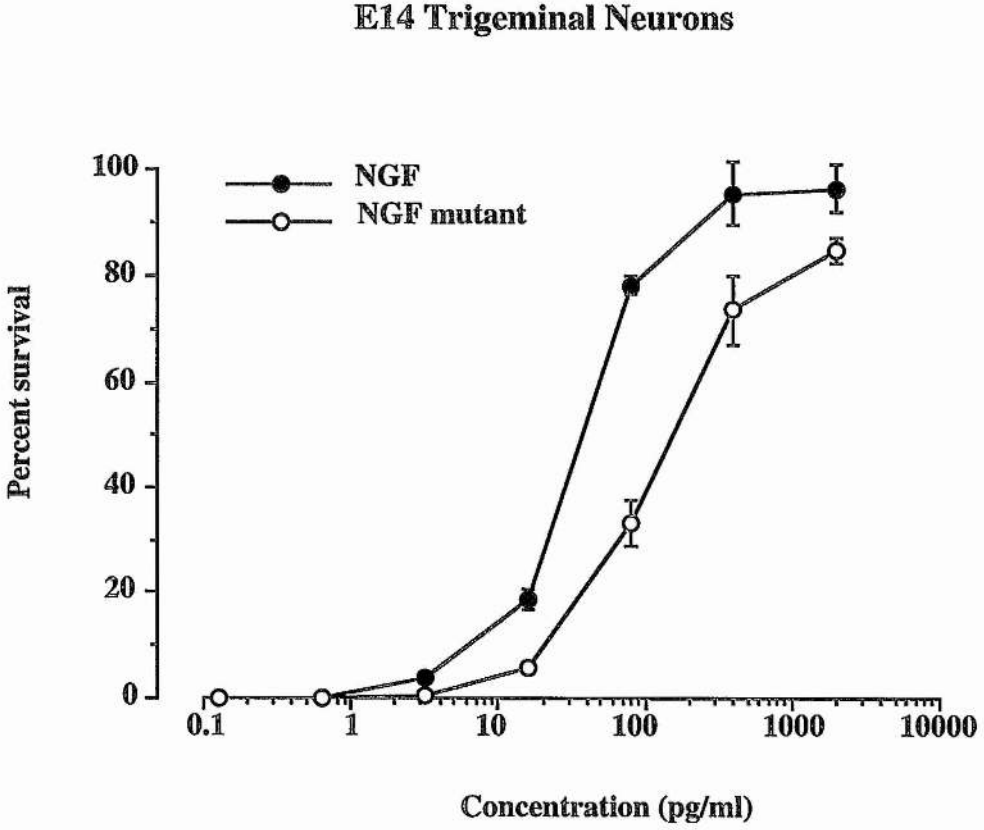


Figure 4.2 Graph of the survival response of E14 mouse trigeminal ganglion neurons to NGF and the NGF mutant. The means and standard errors of the percentage survival after 48 hours. The combined dose data from three separate experiments (n = 9) are shown.

4.3.2 Influence of the NGF mutant on sympathetic neuron survival

In contrast to trigeminal neurons, E17 SCG neurons responded similarly to the NGF mutant and NGF (Fig.4.3). The dose response curves for the NGF mutant and NGF were very close together. Interpolation of the data from three separate dose response experiments on E17 SCG neurons showed that the concentrations of NGF and the NGF mutant that elicited half-maximal survival were very similar (78 pg/ml and 94 pg/ml, respectively).

Although embryonic SCG neurons responded equally well to the NGF mutant and NGF, by the fourth postnatal day the NGF mutant was less effective in promoting the survival of SCG neurons than NGF (Fig. 4.4). Interpolation of the data from three separate dose response experiments on P4 SCG neurons showed that there was a five-fold difference between the concentration of NGF that elicited half-maximal survival (44 pg/ml) and the concentration of the NGF mutant that elicited half-maximal survival (234 pg/ml).

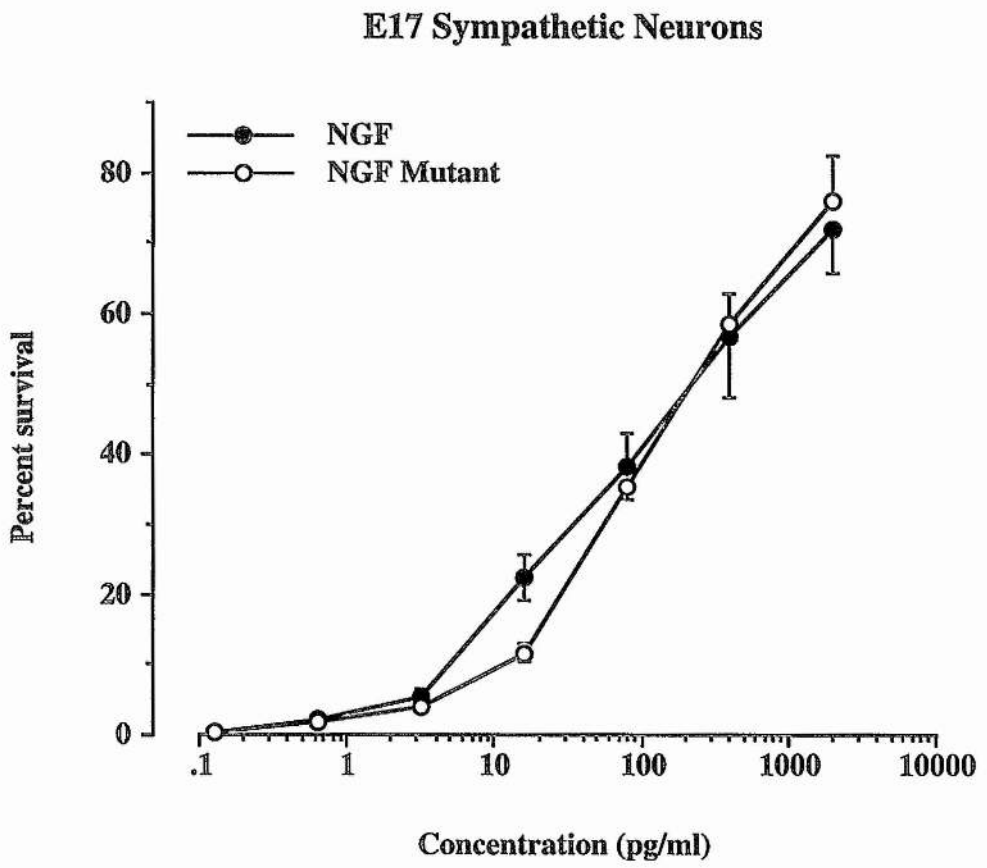


Figure 4.3 Graph of the survival response of E17 mouse SCG neurons to NGF and the NGF mutant. The means and standard errors of the combined dose data from three separate experiments are shown (n = 9).

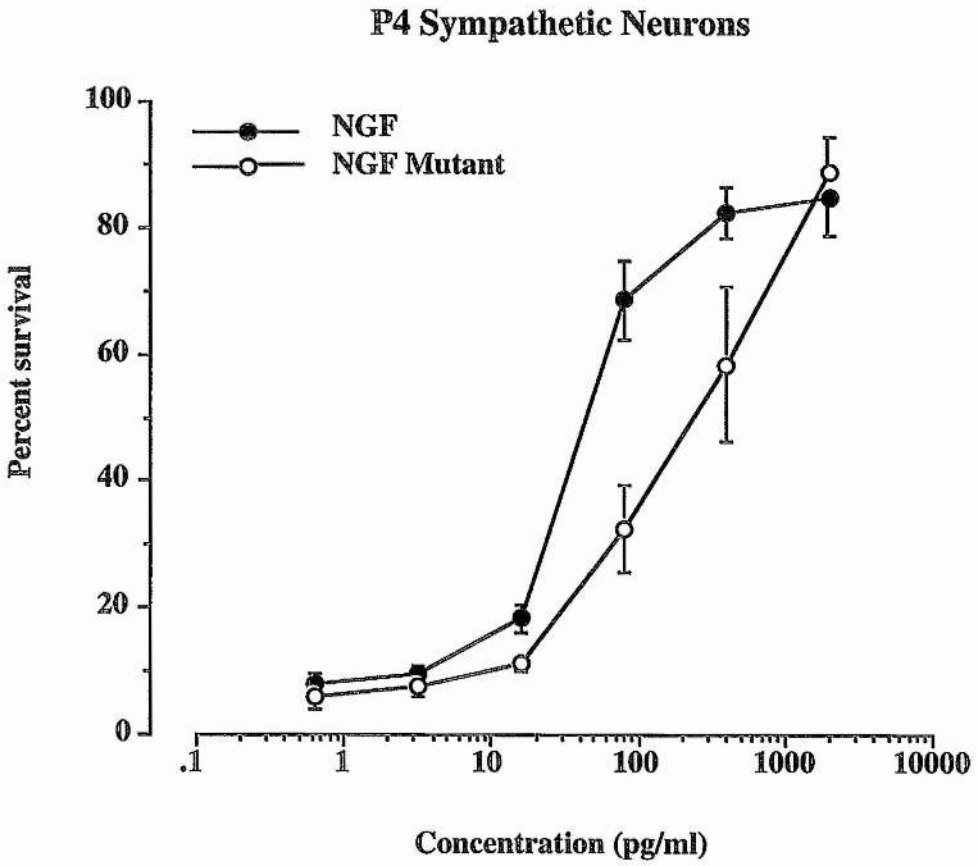


Figure 4.4 Graph of the survival response of P4 mouse SCG neurons to NGF and the NGF mutant. The means and standard errors of the combined dose data from three separate experiments are shown (n = 9) .

4.3.3 Influence of the NGF mutant on chicken sensory and sympathetic neuron survival.

In addition to comparing the effects of wild type NGF and the NGF mutant on the survival response of developing mouse sensory and sympathetic neurons, I carried out similar studies using embryonic chicken neurons. Like E14 embryonic mouse trigeminal ganglion neurons, the NGF-dependent neurons of the dorsomedial part of the E10 embryonic chicken trigeminal ganglion (DMTG neurons) were less sensitive to the mutant NGF than wild type NGF (Fig. 4.5). A three-fold difference in the dose response curve was observed in the mid-concentration range between wild type NGF (concentration at half-maximal survival = 26 pg/ml) and the NGF mutant (concentration at half-maximal survival = 75 pg/ml). Like mouse SCG neurons, the neurons of the chicken lumbar sympathetic chain showed an age-related change in the survival response to the NGF mutant (Figs. 4.6 -4.8). At E10 the dose responses of these neurons to wild type NGF and the NGF mutant were very similar (the concentrations of NGF and the NGF mutant that promoted half-maximal survival were 6.3 pg/ml and 7.4 pg/ml respectively) (Fig. 4.6). Similar results were obtained in chicken sympathetic neuron cultures set up at E12 (concentration at half-maximal survival = 16 pg/ml for NGF, and 20 pg/ml for the NGF mutant) (Fig. 4.7). By E14, the NGF mutant was less effective in promoting the survival of these neurons compared with wild type NGF. The concentrations of NGF and the NGF mutant that promoted half-maximal survival were 17 pg/ml and 130 pg/ml, respectively (Fig. 4.8). These results suggest that the age-related modulation of the NGF survival response by p75 is conserved between the two classes of vertebrates.

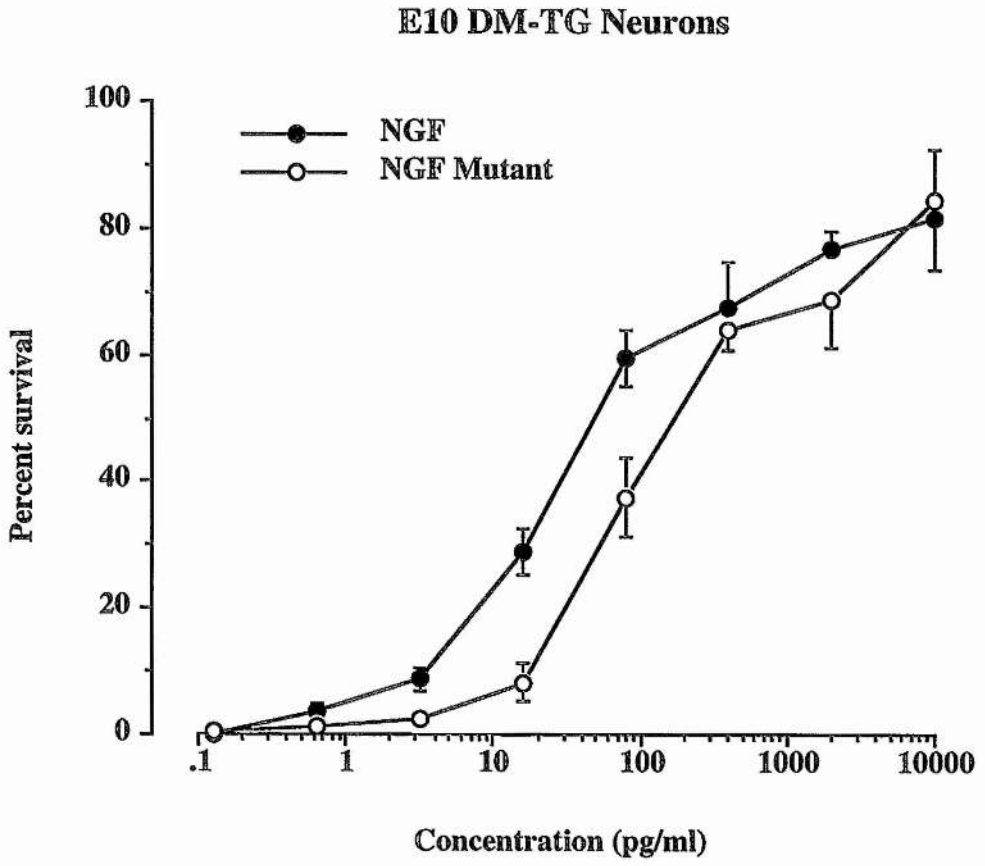


Figure 4.5 Graph of the survival response of E10 chicken DMTG neurons to NGF and the NGF mutant. The means and standard errors of the combined dose data from three separate experiments (each set up in triplicate) are shown (n = 9).

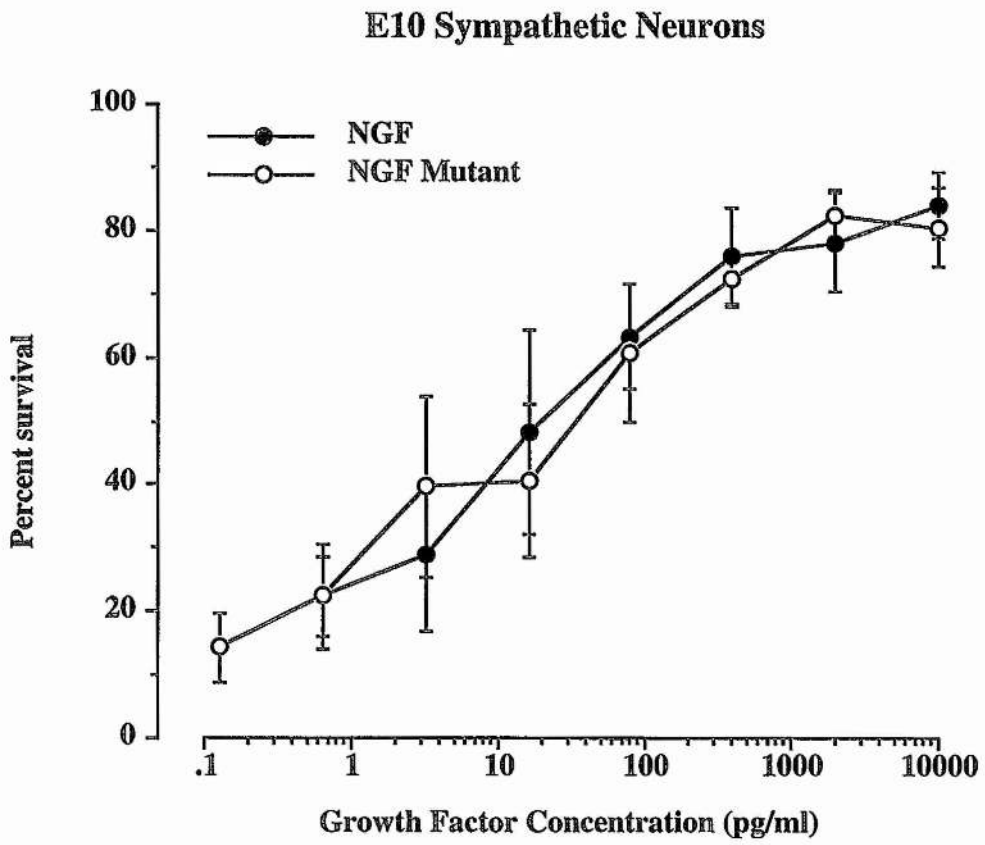


Figure 4.6 Graph of the survival response at 48 hours of E10 chicken lumbar sympathetic neurons to NGF and the NGF mutant. The means and standard errors of the combined dose data from two separate experiments (each set up in triplicate) are shown (n = 6).

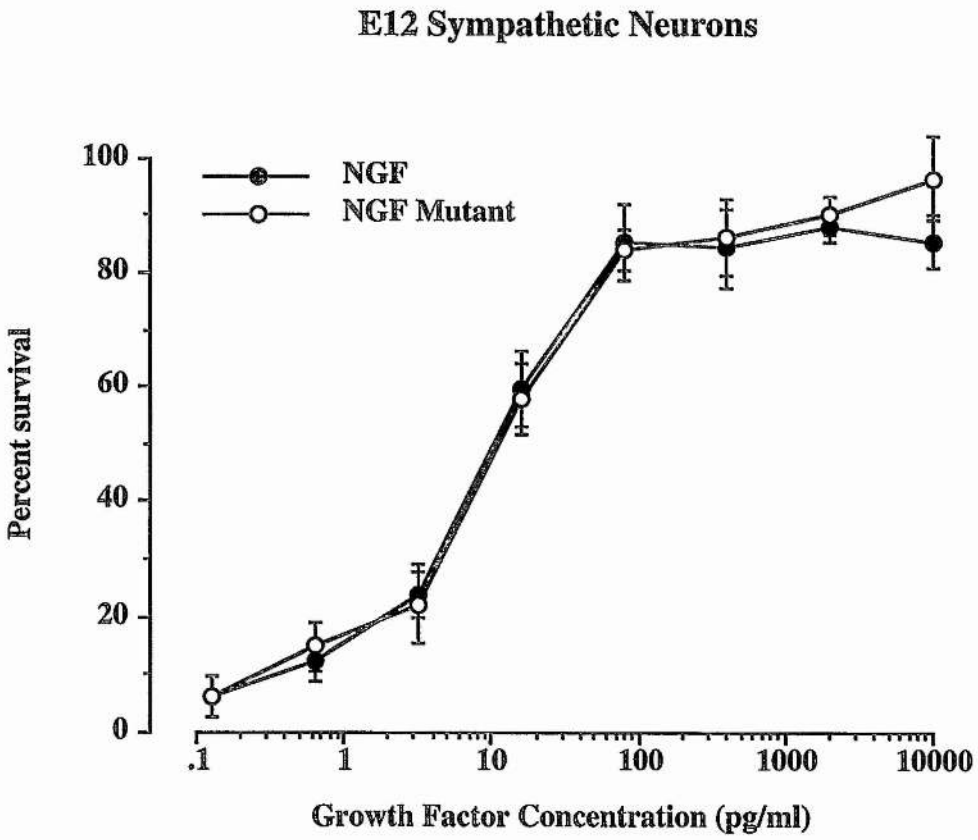


Figure 4.7 Graph of the survival response at 48 hours of E12 chicken lumbar sympathetic neurons to NGF and the NGF mutant. The means and standard errors of the combined dose data from two separate experiments (each set up in triplicate) are shown (n = 6).

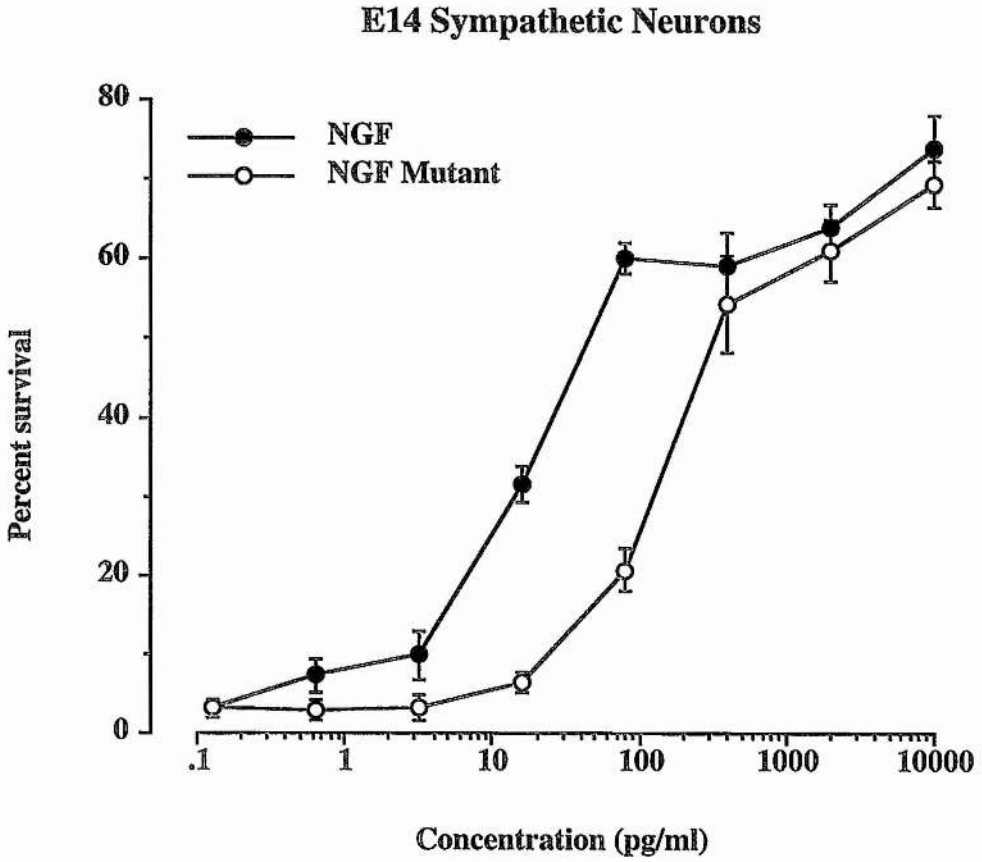


Figure 4.8 Graph of the survival response at 48 hours of E14 chicken lumbar sympathetic neurons to NGF and the NGF mutant. The means and standard errors of the combined dose data from three separate experiments (each set up in triplicate) are shown (n = 9).

4.3.4 Influence of the lipid second messenger ceramide on the p75 modulated survival response.

Since the p75 receptor is known to initiate sphingomyelin hydrolysis to produce ceramide upon binding to NGF (Dobrowsky *et al.*, 1994), I investigated whether the cell permeable ceramide analogue *N*-acetylsphingosine (C_2-ceramide) can influence the survival response of E10 DMTG neurons (Fig. 4.9). A concentration of 10μM ceramide was used in these experiments, as this has previously been shown to be within the effective concentration range for mimicking the effects of NGF on cultured T9 glioma cells (Dobrowsky *et al.*, 1994). Interpolation of the combined data from two separate dose-response experiments indicated that C_2-ceramide does not significantly enhance the survival response of trigeminal neurons in the presence of the NGF mutant (the concentration at half-maximal survival for the NGF mutant and the NGF mutant + C_2-ceramide = 76 pg/ml and 79 pg/ml respectively). Interestingly, when dose response experiments were carried out combining NGF with C_2-ceramide, the half-maximal survival response was enhanced 1.7 fold (concentration at half-maximal survival for NGF and NGF + C_2-ceramide = 33 pg/ml and 19 pg/ml respectively). Similar results were obtained using E14 mouse trigeminal neurons (data not shown), suggesting an additional level of complexity is involved in the p75 modulated survival response to NGF.

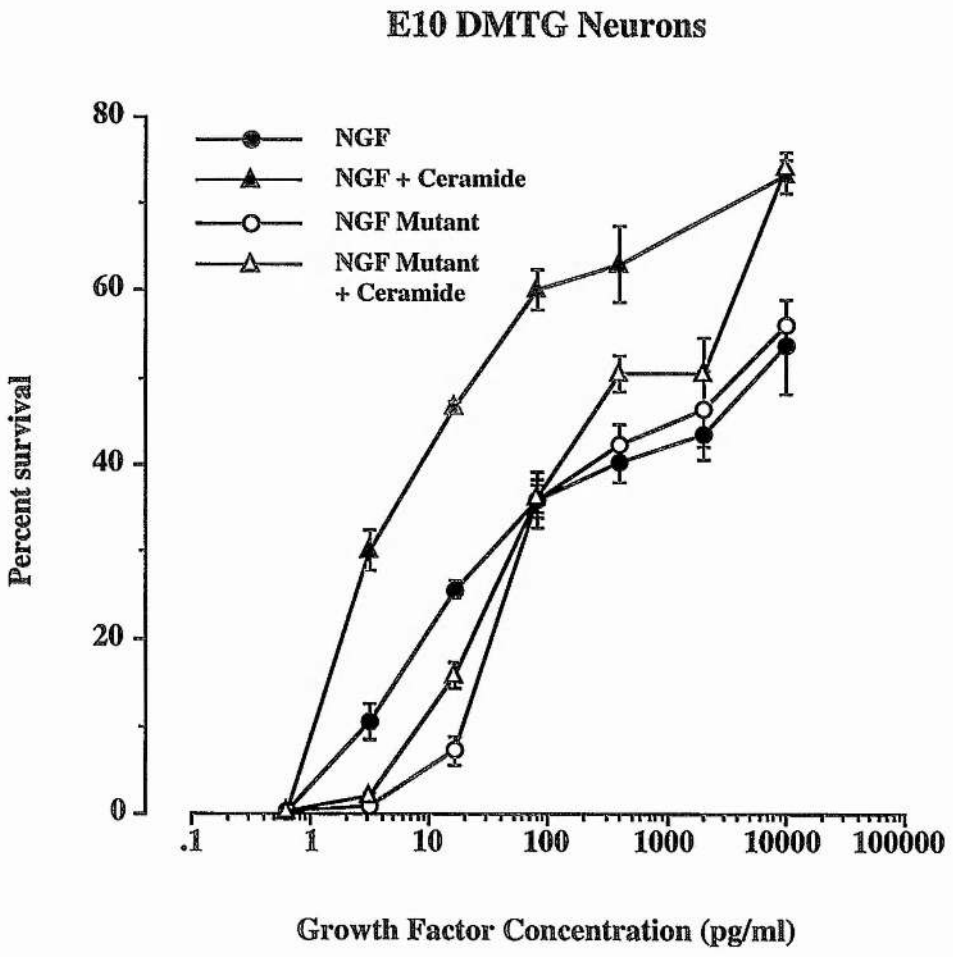


Figure 4.9 Graph of the survival response at 48 hours of E10 chicken DMTG neurons to NGF and the NGF mutant ± C_2-ceramide. The means and standard errors of the combined dose data from two separate experiments (each set up in triplicate) are shown (n = 6).

4.4 Discussion

I have studied the function of the p75 receptor in developing sensory and sympathetic neurons by comparing the survival response of these neurons to NGF and an NGF mutant that binds TrkA normally, but shows negligible binding to p75.

The binding affinity of human NGF to p75, reported here is slightly higher than previously reported values of mouse NGF binding to p75 (Kd = 1 to 2 X 10^{-9}M), was observed. However, similar results have also been reported for human NGF binding to p75 (Vröegop *et al.*, 1992). Regardless of the absolute affinity within the binding assays, in three separate binding experiments, the NGF mutant displayed minimal decrease in TrkA binding relative to the NGF control (1.44 +/- 0.22-fold). In contrast, no inhibition of [^{125}I]NGF binding to p75 occurred with concentrations of the same mutant as high as 10 nM, indicating at least a 100- to 1,000-fold loss of affinity. Similar relative binding affinities for human NGF and the mutant were observed in cell lines expressing full-length TrkA and p75 (data not shown). These data suggest that any loss of potency or efficacy of neuronal responses in the presence of the NGF mutant can be ascribed to loss of interaction with the p75 receptor (J. Winslow, personal communication).

In this study, saturating concentrations of the NGF mutant were as effective as NGF in promoting neuronal survival, indicating that p75 is not essential for NGF signal transduction. This result contrasts with studies using tumour cell lines transfected with p75 which suggested that p75 is necessary for NGF responsiveness (Hempstead *et al.*, 1989; Matsushima and Bogenmann, 1990; Pleasure *et al.*, 1990; Berg *et al.*, 1991; Hempstead *et al.*, 1991). However, this data supports a growing body of evidence that signalling via TrkA is sufficient for eliciting responses to NGF. In addition to the demonstration that

NGF elicits a mitogenic response in fibroblasts expressing TrkA without p75 (Klein *et al.*, 1991a), previous studies with NGF mutants that bind to TrkA but not p75 have shown that saturating levels of these mutants elicit typical NGF responses from PC12 cells and sympathetic neurons (Drinkwater *et al.*, 1991; Ibañéz *et al.*, 1992).

To assess whether p75 affects neuronal survival at limiting ligand concentrations, I compared the dose responses of sensory and sympathetic neurons to NGF and the NGF mutant. Although the dose responses of embryonic mouse sympathetic neurons to NGF and the NGF mutant were not significantly different, the dose responses of postnatal sympathetic and embryonic sensory neurons to the NGF mutant were consistently shifted to higher concentrations compared with the dose responses of these neurons to NGF. Both embryonic sensory and postnatal sympathetic neurons were 4- to 5-fold less sensitive to the NGF mutant compared with NGF in the mid concentration range. These findings are strikingly consistent with the NGF response of sensory and sympathetic neurons obtained from mice with a null mutation in the p75 gene (Davies *et al.*, 1993b; Lee *et al.*, 1994). Whereas the p75 mutation does not affect the dose response of embryonic sympathetic neurons to NGF, p75-deficient embryonic sensory and postnatal sympathetic neurons are less sensitive to NGF than wild type neurons. Remarkably, p75-deficient embryonic sensory and postnatal sympathetic neurons were 4- to 5-fold less sensitive to NGF than wild type neurons. Thus, my results demonstrate by a complementary experimental approach that p75 enhances the survival response of embryonic sensory and postnatal sympathetic neurons to NGF.

The data presented here show that the presence of p75 does not in itself increase the survival response of neurons to NGF. It is clear that the modulation of the NGF survival response by p75 necessitates direct binding of NGF to p75. Although p75 and TrkA appear to cluster independently of NGF in cultured cells

(Ross *et al.*, 1996), an interaction between these receptors is not sufficient to enhance NGF responsiveness, because the dose response of neurons co-expressing p75 and TrkA to the NGF mutant is highly similar to that of p75-deficient neurons to wild type NGF.

In addition to showing developmental changes in the responsiveness of mouse sympathetic neurons to NGF and the NGF mutant, I have observed a similar phenomenon in developing chicken sympathetic neurons. Between E10 and E14, chicken sympathetic neurons become less responsive to the NGF mutant compared with wild type NGF. Also, like embryonic mouse trigeminal neurons, mid-embryonic chicken DMTG neurons were less sensitive to the NGF mutant than wild type NGF. These results indicate that p75 plays a similar role in modulating the survival response of sensory and sympathetic neurons to NGF during development in both classes of vertebrates.

Because neurotophin binding to p75 results in the production of ceramide (Dobrowsky *et al.*, 1994), I tested whether this lipid second messenger had the ability to enhance the survival responses of cultured chick embryo DMTG neurons at sub-saturating concentrations of NGF and the NGF mutant. A cell permeable analogue of ceramide (C_2 ceramide), did not significantly enhance the survival of these cells when cultured in the presence of sub-saturating concentrations of the NGF mutant. This result indicates that at this stage of neuronal development, modulation of neuronal survival by p75 is not dependent upon activation of the sphingomyelin pathway. Additionally, since the survival response of E10 DMTG neurons was moderately enhanced by C_2 ceramide in the presence of sub-saturating levels of NGF, the sphingomyelin pathway may involve further signalling interactions, the investigation of which are beyond the scope this study.

To explain the age-related change in the role of p75 in enhancing the NGF survival response in sympathetic neurons the relative levels of p75 and

TrkA mRNA in embryonic and postnatal mouse sympathetic neurons have been measured (Fig. 4.10). In neurons purified from embryonic sympathetic ganglia at the stage when p75-deficient neurons and wild type neurons respond similarly to NGF (Davies *et al.*, 1993b), and the dose responses of wild type neurons to NGF and the NGF mutant are similar, the level of p75 mRNA is five-fold lower than TrkA mRNA. However, by the fifth postnatal day when p75-deficient neurons are less responsive to NGF than wild type neurons (Lee *et al.*, 1994) and the NGF mutant is less effective than NGF in promoting the survival of wild type neurons, the relative levels of p75 and TrkA mRNA are similar. The relative levels of these mRNAs are also similar in purified E14 trigeminal neurons, which, like postnatal sympathetic neurons, are less responsive to the NGF mutant than wild type NGF. These measurements of the relative levels of p75 and TrkA mRNA in purified neurons obtained from trigeminal and sympathetic ganglia are similar to the relative levels of the mRNAs measured in whole ganglia (Wyatt and Davies, 1993; Wyatt and Davies, 1995), suggesting that the neurons of these ganglia largely account for the expression of p75 and TrkA.

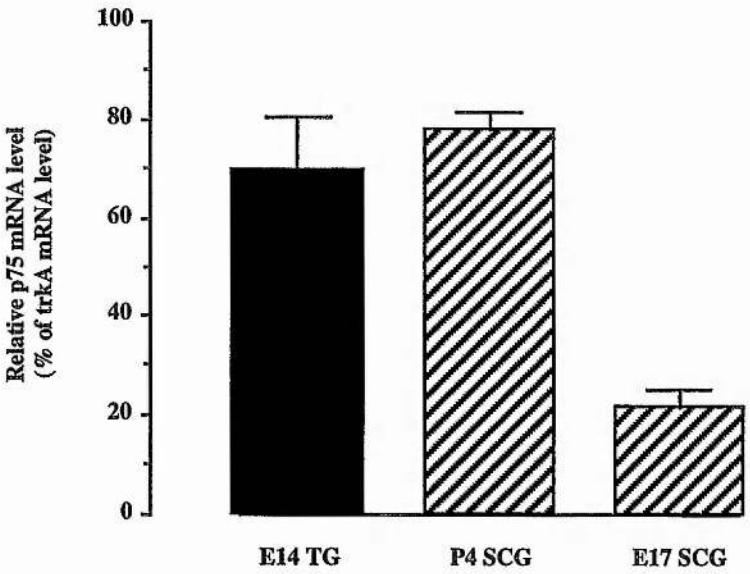


Figure 4.10 Bar chart of the relative levels of p75 mRNA in purified E14 trigeminal ganglion neurons, E17 SCG neurons and postnatal day 4 SCG neurons expressed as percentage of the level of TrkA mRNA in these neurons. In each of three separate preparations of neurons there was less than 1% contamination by satellite cells. The mean ± SEM for three separate estimations of p75 mRNA and TrkA mRNA in purified neurons are plotted.

(Courtesy of Dr. Sean Wyatt, St. Andrews University)

Although estimates of mRNA levels may not necessarily reflect p75 and TrkA mRNA receptor levels, due to the rate of protein translation, prior estimates of the p75:TrkA ratio are in the range of 6-20:1 for PC12 cells (Verdi *et al., 1994*; Verdi and Anderson, 1994), and primary sympathetic neurons (Sutter *et al*, 1979). However, these estimates have been largely determined from the ratio of low to high affinity NGF binding sites which may not truly represent the true ratio of p75:TrkA. For example, high and low affinity neurotrophin binding has been determined for the Trk receptors alone (Klein *et al*, 1991a; Tsoulfas *et al*, 1993, Dr. John Winslow, personal communication) and therefore binding affinity alone may not be sufficient for the quantitative determination of co-expressed TrkA and p75 receptors.

Quantitative mRNA determination suggests that the p75:TrkA ratio approaches one in primary neurons during the period of programmed cell death, at a time when p75 appears to modulate TrkA function. This ratio may be effective in enhancing neuronal survival by increasing TrkA activation or signalling through TrkA substrate proteins. Increased TrkA activation may occur at higher ratios of p75:TrkA, as observed in transfected MAH cells (Verdi *et al.*, 1994), although in this context mitogenesis and not survival is enhanced. Thus, although the higher p75:TrkA ratios produced by gene transfer in cell lines can result in enhanced TrkA responsiveness, modulation of the NGF neuronal survival response by p75 *in vivo* occurs over a much narrower range, and when the ratio is close to 1:1.

How p75 enhances the survival response of NGF-dependent neurons to NGF is not clear. The finding that the binding of NGF to TrkA in PC12 cells is reduced by disrupting NGF binding to p75 by an anti-p75 antibody or by excess BDNF, suggests that p75 enhances the binding of NGF to TrkA (Barker and Shooter, 1994). Furthermore, co-expression of TrkA and p75 in fibroblasts results in the formation of a class of high-affinity receptors that is not apparently

present in cells expressing TrkA alone (Battleman *et al.*, 1993), and expression of high levels of p75 in MAH cells enhances NGF-induced TrkA phosphorylation compared with cells expressing TrkA alone (Verdi *et al.*, 1994). Recently, p75 has been shown to accelerate TrkA-mediated signalling in cultured PC12 cells (Canossa *et al.*, 1996). In addition, p75 forms a complex with TrkA on the cell surface (Wolf *et al.*, 1995; Ross *et al.*, 1996) and is immunoprecipitated in a complex with TrkA in the presence of [^{125}I]NGF (Huber and Chao, 1995). These studies suggest that p75 enhances binding of NGF to TrkA which in turn increases the efficiency of TrkA signalling.

Several models have been proposed to explain how p75 might increase the efficiency of TrkA signalling. For example, p75 may act to concentrate NGF locally in the microenvironment surrounding cell surface TrkA receptors (Barker and Shooter, 1994). However, in this model, TrkA receptors are envisioned to be surrounded by larger numbers of p75, and it has not been demonstrated that there is a large excess of p75 molecules available to surround TrkA molecules at the cell surface. In addition to modulating TrkA signalling by interacting directly or indirectly with TrkA, it is possible that p75 may modulate the survival response of TrkA-expressing neurons by activating certain intracellular signalling pathways directly. For example, NGF has recently been shown to promote sphingomyelin hydrolysis (Dobrowsky *et al.*, 1994) and NF-kB activation (Carter *et al.*, 1996) by binding to p75 in certain non-neuronal cells that do not express Trks. In addition, p75 signalling activated by NGF binding in cells lacking TrkA can promote apoptosis in early retinal cells (Frade *et al.*, 1996) and in cultured oligodendrocytes (Casaccia-Bonnefil *et al.*, 1996). The occurrence of p75-dependent signalling events in neurotrophin-dependent neurons and their significance in promoting the survival of sensory neurons has not been established. Furthermore, the results of experiments where the lipid analog C_2-ceramide was administered to cultured trigeminal sensory neurons

(Fig. 4.10) argue against a direct involvement of this pathway in the p75 mediated survival response of sensory neurons.

In conclusion, I have demonstrated that p75 modulates the survival response of sensory and sympathetic neurons to NGF at the stages of their development when these neurons express similar levels of p75 and TrkA. Furthermore, I have shown that modulation of the NGF survival response by p75 is dependent on NGF binding to p75 rather than on interactions that occur between p75 and TrkA independent of NGF binding. The modulation of NGF sensitivity by p75 is only observed at low, limiting concentrations of NGF. This together with the fact that p75 exerts this effect during the period of naturally occurring neuronal death in the trigeminal (Davies and Lumsden, 1984) and sympathetic ganglia (Wright *et al.*, 1983) suggests that p75 expression plays an important role in governing the number of neurons that survive to maturity.

CHAPTER 5

CYTOKINES PROMOTE THE SURVIVAL OF CRANIAL SENSORY NEURONS AT DIFFERENT STAGES OF THEIR DEVELOPMENT.

5.1 Introduction.

Different neurotrophins can co-operate in regulating the survival of a particular population of neurons by either acting on neurons at the same stage during development or by acting sequentially. Examples of concurrent neurotrophin action include BDNF and NT3 supporting the survival of the proprioceptive neurons of the trigeminal mesencephalic nucleus during the period of naturally occurring neuronal death (Davies *et al.*, 1986a; Hohn *et al.*, 1990; Ernfors *et al.*, 1994a, 1994b; Jones *et al.*, 1994) and NGF and NT3 acting together on sympathetic neurons during an equivalent stage of their development (Zhou and Rush, 1995, Wyatt and Davies, 1997). The clearest evidence for the sequential action of neurotrophins in regulating the survival of developing neurons comes from studies of the embryonic mouse trigeminal ganglion. In culture, early trigeminal ganglion neurons show a transient survival response to BDNF and NT3 before becoming dependent on NGF for survival (Buchman and Davies, 1993; Paul and Davies, 1995). Neuronal apoptosis is markedly elevated in the early trigeminal ganglia of mice that are homozygous for a null mutation in the *trkB* gene, which encodes the BDNF receptor tyrosine kinase, whereas neuronal apoptosis is markedly elevated later in development in the trigeminal ganglia of mice that are homozygous for a null mutation in the *trkA* gene, which encodes the NGF receptor tyrosine kinase (Piñón *et al.*, 1996). Neuronal apoptosis is also markedly elevated in the early trigeminal ganglia of $NT3^{-/-}$ mice (Wilkinson *et al.*, 1996), although the loss of neurons in the early trigeminal ganglia may be due in part to the death of progenitor cells (ElShamy and Ernfors, 1996).

In addition to the neurotrophins, several other proteins have been shown to promote the survival of neurons during development. Ciliary neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), oncostatin-M (OSM) and interleukin-6 (IL-6), comprise a family of cytokines that although showing less than 15% amino acid sequence identity, share several characteristic structural features (Bazan, 1991; Robinson *et al.*, 1994; McDonald *et al.*, 1995) and signal via oligomeric receptor complexes that have one or more components in common (Stahl and Yancopoulos, 1994; Wollert *et al.*, 1996). These cytokines have multiple actions on cells of the nervous system, including promoting survival of some neurons. For example, CNTF promotes the survival of sympathetic, parasympathetic, motorneurons and some sensory neurons in vitro (Barbin *et al.*, 1984; Arakawa *et al.*, 1990; Oppenheim *et al.*, 1991; Burnham *et al.*, 1994), and is involved in promoting the survival of some adult motorneurons *in vivo* (Masu *et al.*, 1993). LIF promotes the survival of motorneurons, sympathetic neurons and some sensory neurons in vitro (Martinou *et al.*, 1992; Murphy *et al.*, 1993; Kotzbauer *et al.*, 1994; Thaler *et al.*, 1994) and plays a role in promoting the survival of embryonic motorneurons in vivo (Li *et al.*, 1995). CT-1 promotes the survival of cultured ciliary ganglion neurons, midbrain dopaminergic neurons and motorneurons (Pennica *et al.*, 1995b; Pennica *et al.*, 1996). IL-6 enhances the in vitro survival of a proportion of forebrain cholinergic and midbrain catecholaminergic neurons (Hama *et al.*, 1989; Kushima *et al.*, 1992). OSM promotes the survival of a subset of late embryonic DRG neurons in culture (Ware *et al.*, 1995).

Studies of the neurotrophic actions of individual cytokines have been restricted to one or just a few stages of development. The purpose of the present study was to carry out a comprehensive, comparative study of the neurotrophic effects of cytokines on sensory neurons at closely staged developmental intervals to determine when these factors exert their effects, and to determine the relationship between cytokine and neurotrophin responsiveness. Cutaneous

sensory neurons of the trigeminal ganglion, which switch their survival requirements from BDNF/NT-3/NT-4/5 to NGF during the early stages of target field innervation (Davies *et al.*, 1993a; Buchman and Davies, 1993; Paul and Davies, 1995; Piñón *et al.*, 1996), and enteroceptive neurons of the nodose ganglion, which retain dependence on BDNF throughout embryonic development, were chosen for this study (Davies *et al.*, 1993a; Buj-Bello *et al.*, 1994). I show that whereas nodose neurons survive in response to CNTF, LIF, OSM and CT-1 throughout development, trigeminal neurons do not begin to respond to these factors until the late embryonic period. Nodose neurons additionally show a survival response to IL-6 in the late embryonic period. These findings indicate that cytokines are able to influence the survival of different populations of sensory neurons at different stages in their development. Together with earlier studies, my results indicate that trigeminal neurons pass through several sequential stages during which they have different neurotrophic factor survival requirements: an early period of BDNF/NT3 dependence, followed by a phase of NGF dependence and a late stage of cytokine responsiveness.

5.2 Results.

5.2.1 Nodose neurons

The survival of the great majority of nodose neurons was promoted by BDNF in cultures set up at stages from E11 to E19, whereas in control cultures very few neurons were surviving by 48 hours (Figs. 5.1-5.4). A clear survival response to CNTF, LIF, OSM and CT-1 was observed at all ages studied. The proportion of neurons responding to these factors increased from between 20% and 40% at E11 (after subtraction of the small number of neurons surviving in control cultures) to between 50% and 80% at E19. In E11 (Fig. 5.1) and E14 (Fig 5.2) cultures, there were two to three times more neurons surviving with BDNF compared with these cytokines, whereas in E17 (Fig 5.3) and E19 (Fig. 5.4) cultures, similar numbers of neurons survived with BDNF and these cytokines. Although there was no difference in the number of neurons in control cultures and cultures supplemented with IL-6 at E11 and E14, there was a clear survival response to IL-6 in E17 cultures, and the number of neurons surviving with IL-6 had risen to over 40% in E19 cultures. These results show that nodose neurons respond to CNTF, LIF, OSM and CT-1 throughout development and exhibit a late survival response to IL-6. Because the serum-free medium used in these experiments was not conducive for the survival of non-neuronal cells, the survival of neurons was unlikely to be influenced by neurotrophic factors other than those added to the cultures.

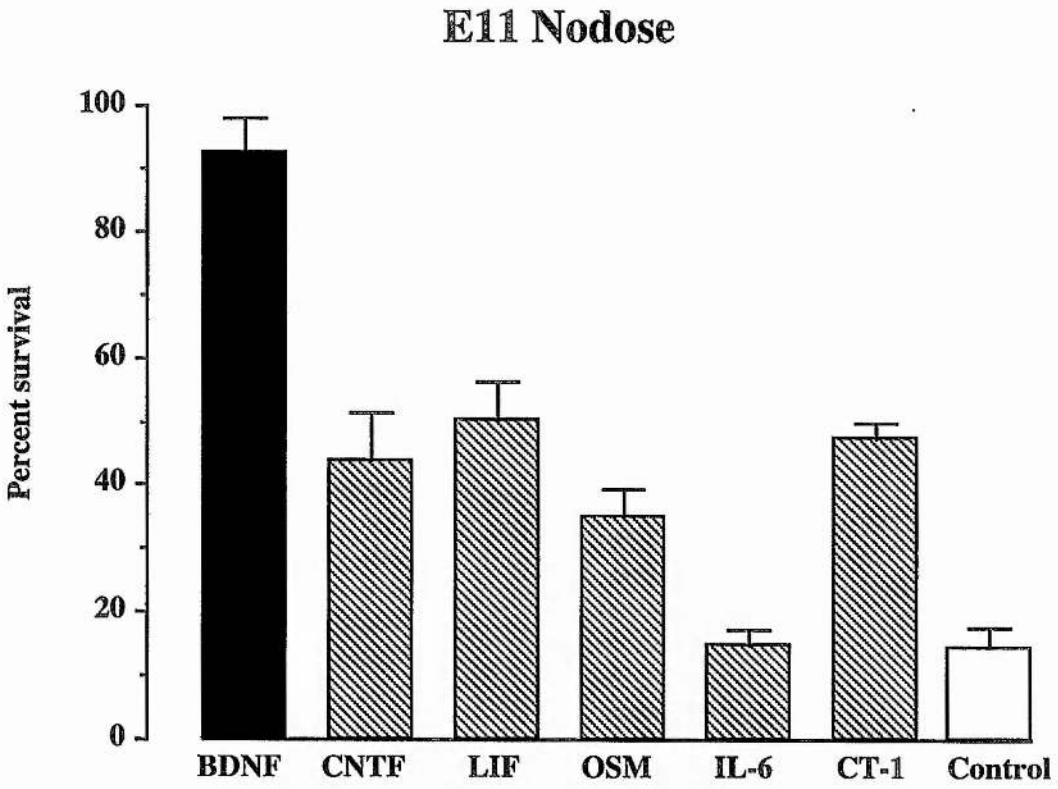


Figure 5.1. Bar chart of the percentage survival of E11 nodose ganglion neurons.

Neurons were cultured for 48 hours with saturating concentrations of BDNF (10 ng/ml), CNTF, LIF, OSM, IL-6 or CT-1 (50 ng/ml). Control = no added neurotrophic factors. The number of neurons surviving at 48 hours is expressed as a percentage of the number of neurons identified 6-9 hours after plating. The mean and standard errors of two experiments are shown (n = 6).

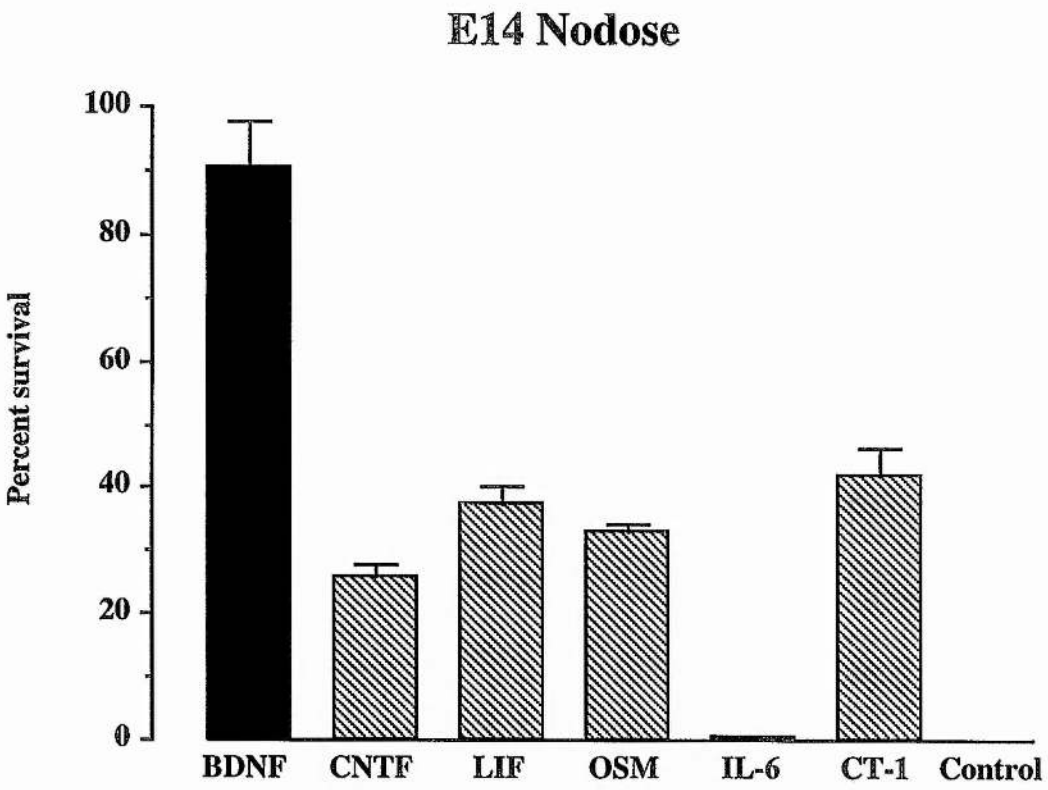


Figure 5.2. Bar chart of the percentage survival of E14 nodose ganglion neurons.

Neurons were cultured for 48 hours with saturating concentrations of BDNF (10 ng/ml), CNTF, LIF, OSM, IL-6 or CT-1 (50 ng/ml). Control = no added neurotrophic factors. The mean and standard errors of two experiments are shown (n = 6).

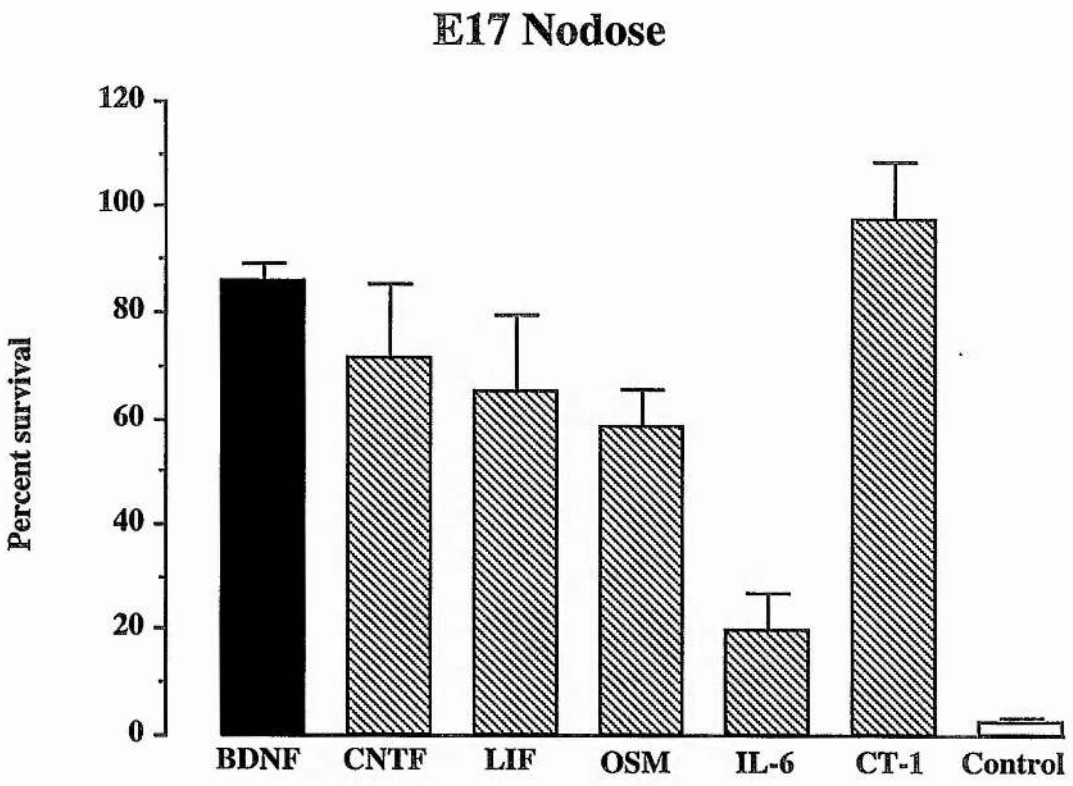


Figure 5.3 Bar chart of the percentage survival of E17 nodose ganglion neurons.

Neurons were cultured for 48 hours with saturating concentrations of BDNF (10 ng/ml), CNTF, LIF, OSM, IL-6 or CT-1 (50 ng/ml). Control = no added neurotrophic factors. The mean and standard errors of two experiments are shown (n = 6).

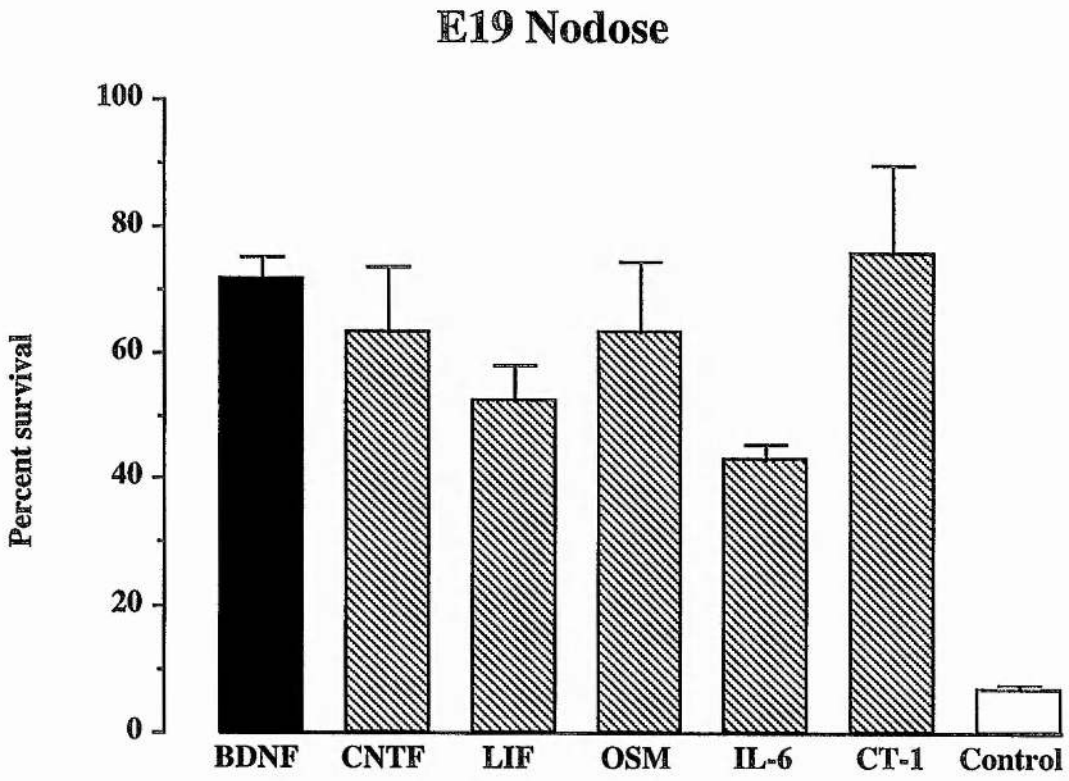


Figure 5.4 Bar chart of the percentage survival of E19 nodose ganglion neurons.

Neurons were cultured for 48 hours with saturating concentrations of BDNF (10 ng/ml), CNTF, LIF, OSM, IL-6 or CT-1 (50 ng/ml). Control = no added neurotrophic factors. The mean and standard errors of two experiments are shown (n = 6).

When nodose neurons were cultured with saturating concentrations of different neurotrophic factors in combination the overall survival did not increase at either E14 or E19 (Fig. 5.5). This indicates that the neurons which respond to cytokines comprise a subset of those that respond to BDNF early on, whereas BDNF, CNTF, LIF, OSM and CT-1 act on the same neurons later in development. The lack of any additional survival in E14 cultures containing two different cytokines also shows that these cytokines act on the same subset of neurons at this age. Interestingly, in E14 cultures there were fewer neurons surviving with BDNF plus cytokines compared with BDNF alone. Statistical analysis of the data revealed that whereas the survival response of BDNF compared with BDNF and IL-6 in combination did not show a significant difference ($P = 0.364$, Student's *t*-test), BDNF in combination with other cytokines does display a significantly reduced survival response ($P = 0.009$ for BDNF + CNTF, 0.013 for BDNF + LIF, 0.041 for BDNF + CT-1, Student's *t*-test).

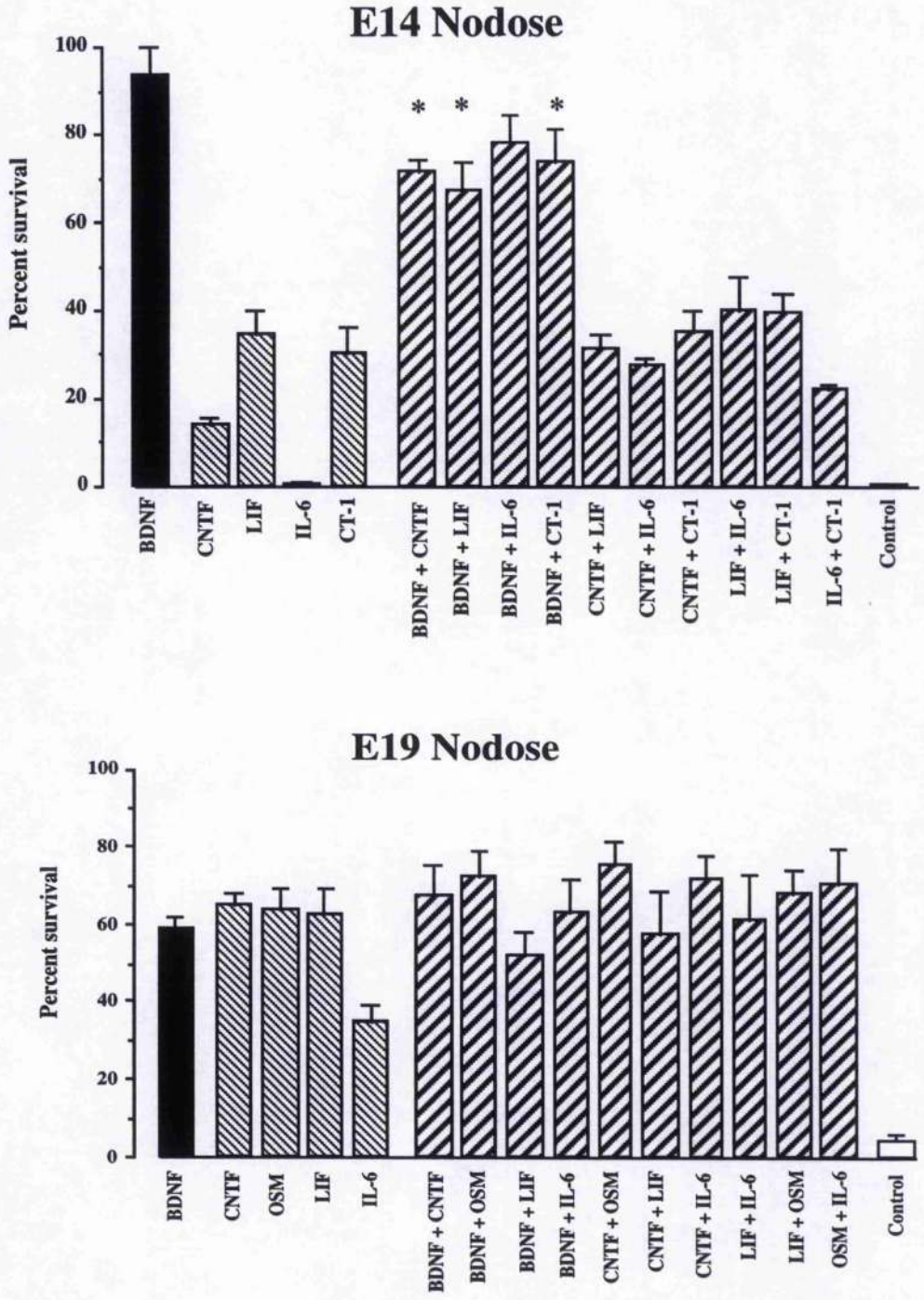


Figure 5.5. Bar charts of the percentage survival of E14 and E19 nodose ganglion neurons. Neurons were cultured for 48 hours with saturating concentrations of BDNF and cytokines alone and in combination. Control = no added neurotrophic factors. In each case, the mean and standard errors of two experiments are shown (n = 6).

Dose response analysis (Fig. 5.6) showed that BDNF was the most potent neurotrophic factor for nodose neurons at E19. The concentration of BDNF that elicited half maximal survival (EC_{50}) was estimated at 181.5 ± 23.3 pg/ml by interpolating the data from 3 separate experiments. CNTF, LIF, OSM and CT-1 were slightly less potent; EC_{50} values: 205 ± 13.2 pg/ml (9.0 pM), 462 ± 5 pg/ml (10.2 pM), 357 ± 73 pg/ml (12.7 pM) and 462 ± 99 pg/ml (21.5 pM), respectively. IL-6 was the least potent of the cytokines; EC_{50} = 822 ± 96 pg/ml (31.6 pM).

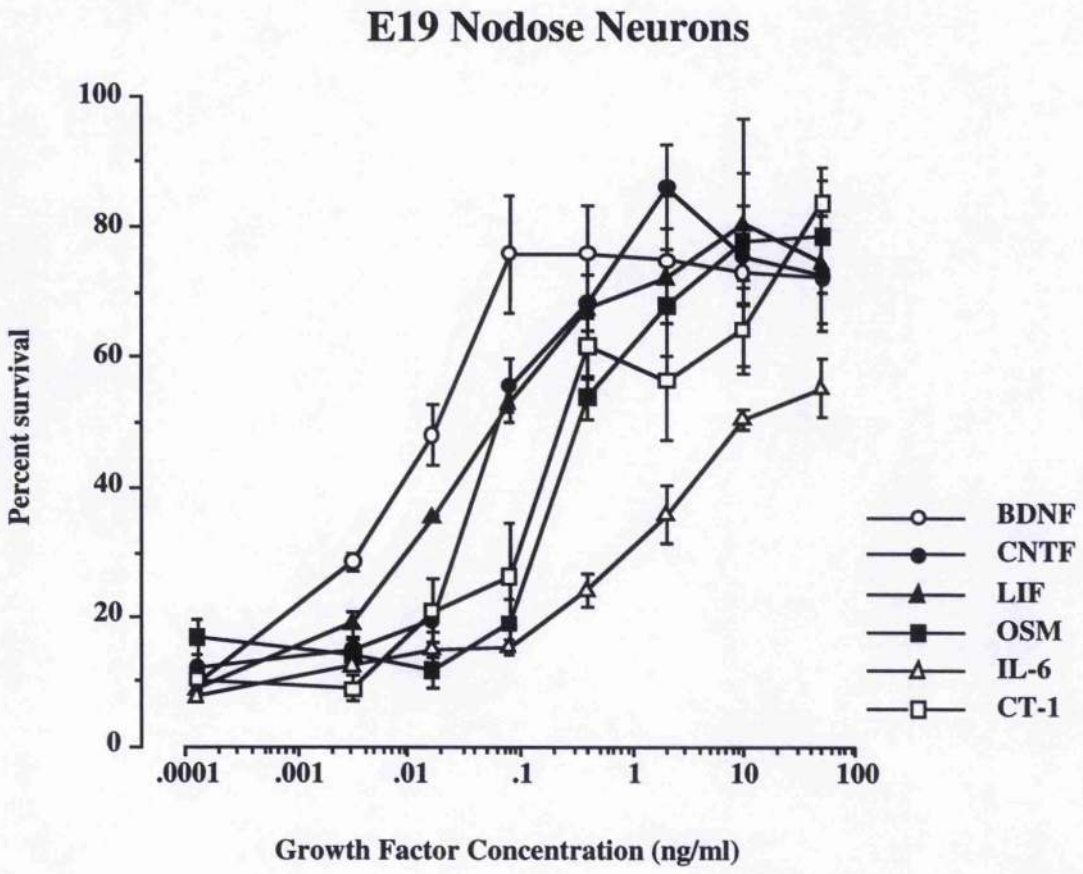


Figure 5.6 Graph of the dose responses of E19 nodose neurons to BDNF and cytokines.

Concentrations ranging from 3.2 pg/ml to 50 ng/ml were used. The combined mean and standard errors of three experiments are shown (n = 9).

5.2.2 Trigeminal neurons

The influence of cytokines on the survival of trigeminal ganglion neurons was compared with that of neurotrophins at stages throughout development. Because the survival dependence of trigeminal neurons switches from BDNF to NGF during the early stages of target field innervation (Buchman and Davies, 1993), E11 cultures were grown with BDNF and E14 and older neurons were grown with NGF. In the presence of these neurotrophins, the majority of neurons survived for 48 hours (Figs. 5.7 - 5.10). The reason for the overall increase in the number of identified neurons in E11 cultures between 6 and 48 hours incubation in the presence of BDNF was due to the enhanced survival of neurons that differentiate from progenitor cells in these early cultures (Paul and Davies, 1995). Cytokines had no effect on neuronal survival in E11 and E14 cultures (Figs 5.7 and 5.8). At these ages virtually all neurons were dead in cytokine-supplemented cultures by 48 hours incubation. In E17 cultures (Fig. 5.9) a small proportion of the neurons were supported by CNTF, LIF, OSM and CT-1, and this increased to approximately 40% by E19 (Fig. 5.10). There was no response to IL-6 throughout development, although 5% of the neurons were surviving with this factor in E19 cultures. There was no additional survival in cultures supplemented with NGF and cytokines (Figure 5.11), indicating that a proportion of NGF-responsive neurons acquire responsiveness to cytokines during the late embryonic period.

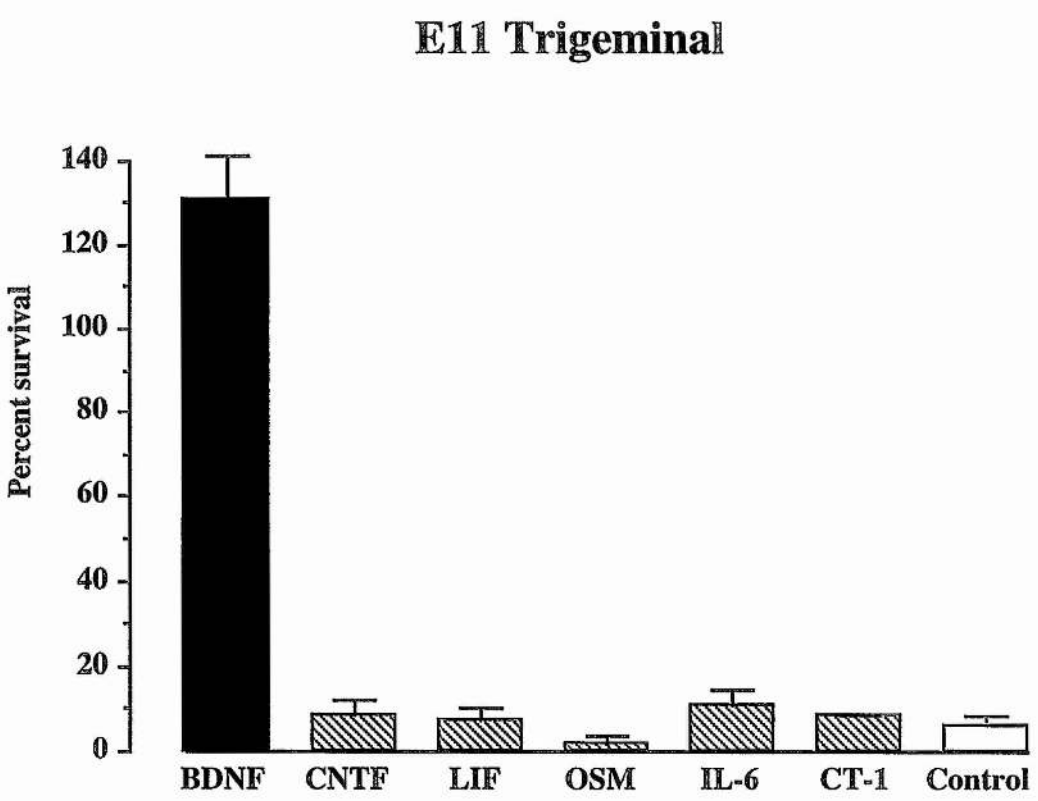


Figure 5.7 Bar chart of the percentage survival of E11 trigeminal ganglion neurons.

Neurons were cultured for 48 hours with saturating concentrations of BDNF (10 ng/ml), CNTF, LIF, OSM, IL-6 or CT-1 (50 ng/ml). Control = no added neurotrophic factors. The number of neurons surviving at 48 hours is expressed as a percentage of the number of neurons identified 6-9 hours after plating. The mean and standard errors of two experiments are shown (n = 6).

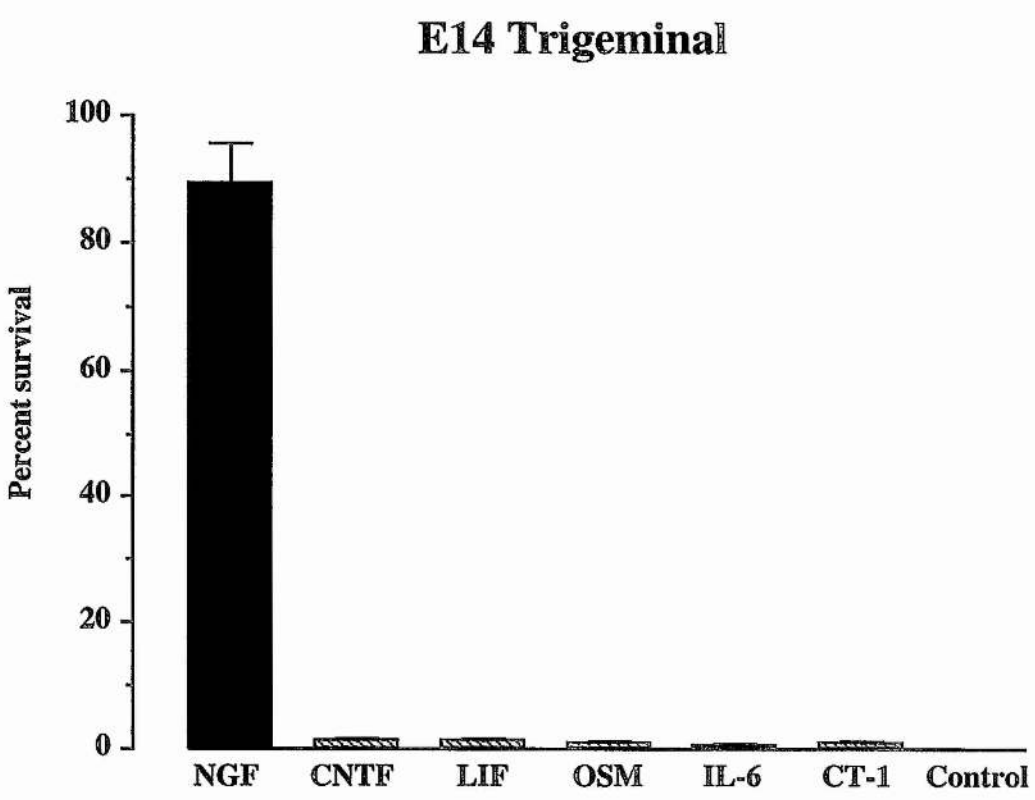


Figure 5.8 Bar chart of the percentage survival of E14 trigeminal ganglion neurons.

Neurons were cultured for 48 hours with saturating concentrations of NGF (10 ng/ml), CNTF, LIF, OSM, IL-6 or CT-1 (50 ng/ml). Control = no added neurotrophic factors. The mean and standard errors of two experiments are shown (n = 6).

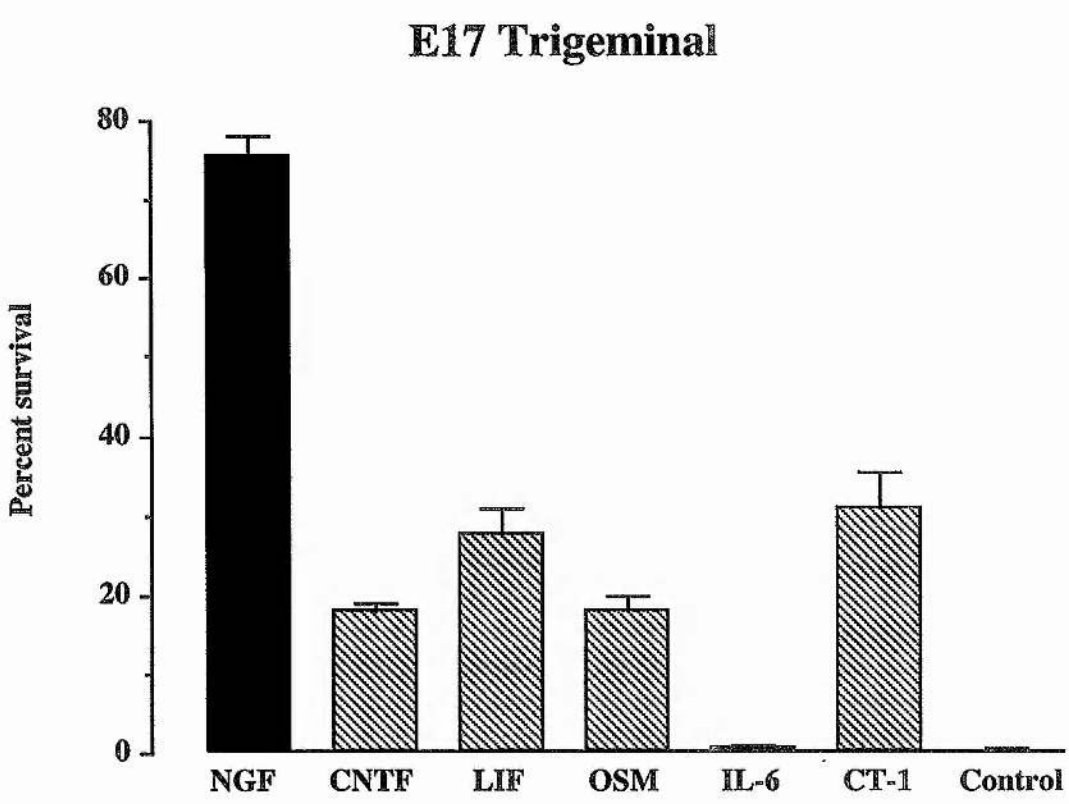


Figure 5.9 Bar chart of the percentage survival of E17 trigeminal ganglion neurons

Neurons were cultured for 48 hours with saturating concentrations of NGF (10 ng/ml), CNTF, LIF, OSM, IL-6 or CT-1 (50 ng/ml). Control = no added neurotrophic factors. The mean and standard errors of two experiments are shown (n = 6).

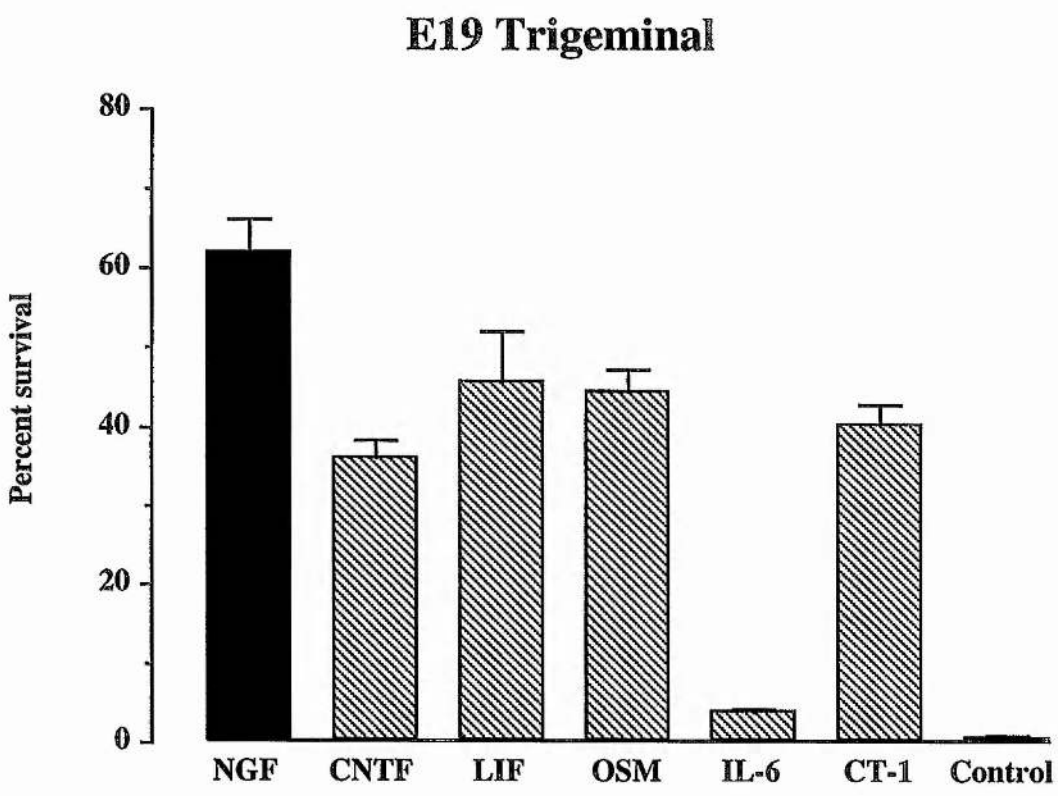


Figure 5.10 Bar chart of the percentage survival of E19 trigeminal ganglion neurons.

Neurons were cultured for 48 hours with saturating concentrations of NGF (10 ng/ml), CNTF, LIF, OSM, IL-6 or CT-1 (50 ng/ml). Control = no added neurotrophic factors. The mean and standard errors of two experiments are shown (n = 6).

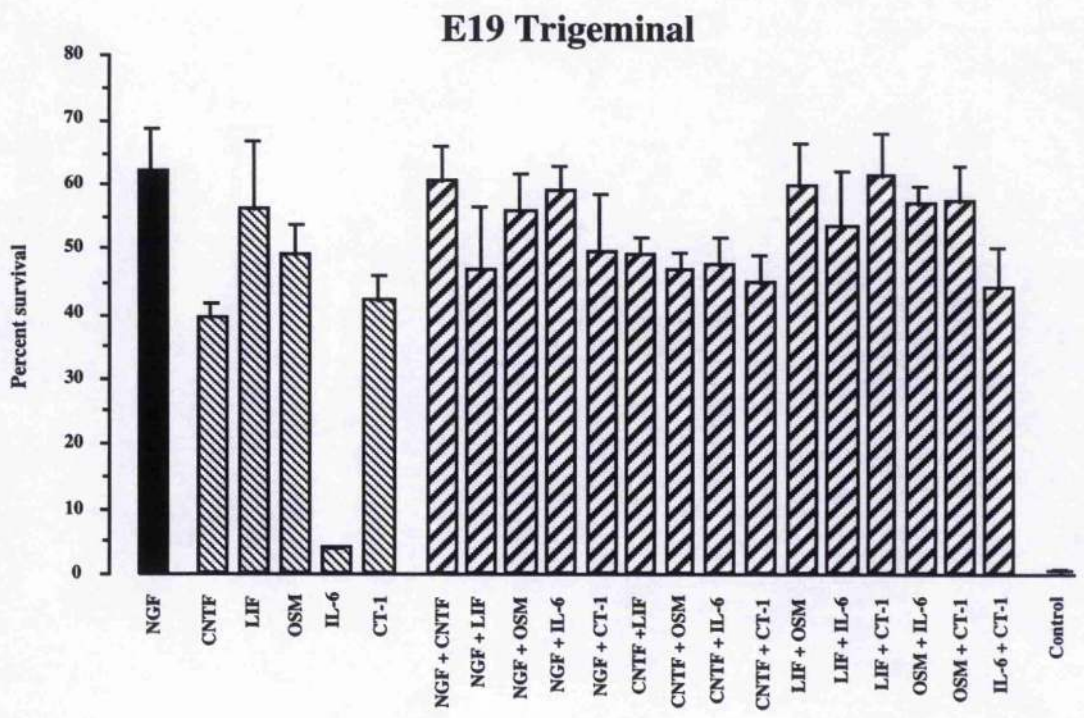


Figure 5.11. Bar chart of the percentage survival of E19 trigeminal ganglion neurons.

Neurons were cultured for 48 hours with saturating concentrations of NGF and cytokines both alone and in combination. Control = no added neurotrophic factors. The number of neurons surviving at 48 hours is expressed as a percentage of the number of neurons identified 6-9 hours after plating. The mean and standard errors of two experiments are shown (n = 6).

5.4 Discussion.

Previous work has shown that the neurotrophin survival requirements of embryonic trigeminal ganglion neurons switch from BDNF and NT3 to NGF early in development and that they retain dependence on NGF throughout the period of naturally occurring neuronal death (Buchman and Davies, 1993; Paul and Davies, 1995a; Piñón *et al.*, 1996; Wilkinson *et al.*, 1996). Here I demonstrate that trigeminal neurons acquire survival responses to CNTF, LIF, OSM and CT-1 after the majority of these neurons have become dependent on NGF for survival. Because there is no additional survival in cultures containing cytokines plus NGF compared with NGF alone, the cytokine-responsive neurons do not constitute a separate population of trigeminal neurons but comprise a major subset of the NGF-responsive neurons. Since the cytokine survival response is evident by E17 and the period of naturally occurring neuronal death is not over until birth (Davies and Lumsden, 1984), it is possible that these cytokines play a role in regulating the final number of neurons in the trigeminal ganglion or are important for sustaining the survival of trigeminal neurons after selection of the appropriate number of neurons has taken place as a result of competition for target-derived NGF (Davies *et al.*, 1987). Together with previous work, my results demonstrate that the responsiveness of trigeminal neurons to neurotrophic factors passes through several phases during development. After a brief period of neurotrophin independent survival, the neurons have a transient dependence on BDNF and NT3 before becoming NGF dependent, and subsequently acquire responsiveness to cytokines.

In contrast to the late survival response of trigeminal neurons to cytokines, I have shown that nodose ganglion neurons respond to CNTF, LIF, OSM and CT-1 as early as E11, and show a marked survival response to these cytokines throughout development. Although a previous in vitro study of rat nodose ganglion neurons at a single developmental stage (E16.5) showed that a

proportion of these neurons survive in response to CNTF and LIF, it was reported that these cytokines and BDNF have a partially additive effect on survival, suggesting that they act in part on different subsets of neurons (Thaler *et al.*, 1994). In contrast, I found no additive effects of BDNF in combination with CNTF, LIF, OSM or CT-1 at either E14 or E19, suggesting that BDNF-responsive and cytokine-responsive neurons are completely overlapping. In addition, my study has revealed that a subset of nodose neurons acquire a survival response to IL-6 at late embryonic stages. Although the IL-6 receptor shares one component in common with the receptors for CNTF, LIF, OSM or CT-1 (namely, gp130), it also comprises a specific receptor component termed IL-6Ra. Thus, it is likely that the late response of nodose neurons to IL-6 is a consequence of the expression of IL-6Rα at this stage of development.

In vitro studies of mouse dorsal root ganglion (DRG) neurons have shown that a small percentage of these neurons respond to LIF with enhanced survival in the late embryonic period and that the majority are LIF-responsive postnatally (Murphy *et al.*, 1993). The finding that there is a significant reduction in the number of DRG neurons in mice that are homozygous for a targeted null mutation in the *lifrβ* gene, which encodes a LIF receptor component that is essential for signalling, suggest that the *in vitro* response of DRG neurons to LIF is physiologically relevant (P. Bartlett, personal communication). Because LIFRβ is also a signalling component of the oligomeric receptors for CNTF, OSM and CT-1 (Stahl and Yancopoulos, 1994; Wollert *et al.*, 1996), it is unclear which of these cytokines is the physiologically relevant LIFRβ ligand required for the survival of DRG neurons and the cranial sensory neurons examined in the present study. Although analysis of mice that have targeted mutations in the genes encoding these cytokines may resolve this issue, this is already additional circumstantial evidence that LIF may play a role in promoting the survival of sensory neurons in vivo. LIF mRNA in skin and gut from E15 onwards (Murphy *et al.*, 1993), and the retrograde transport of

iodinated LIF from the periphery (Hendry *et al.*, 1992) raises the possibility that LIF may function as a target-derived neurotrophic factor for late embryonic and newborn DRG. Alternatively, because LIF mRNA is also expressed within embryonic DRG (Murphy *et al.*, 1993; Fan and Katz, 1993), it is possible that it may also act locally on these neurons.

Several investigators have focused on the ability of various cytokines to influence the neuropeptide and neurotransmitter expression in sympathetic neurons. CNTF, LIF, and CT-1 have been observed to alter the expression of neurotransmitters in sympathetic neurons (Fan and Katz, 1993; Pennica *et al.*, 1995b). These factors can induce cholinergic function concomitant with a reduction of noradrenergic expression, and can also influence the expression of several neuropeptides, including substance P, somatostatin, CGRP and VIP (Rao *et al.*, 1990; Pennica *et al.*, 1995b). Previous in vitro studies have also demonstrated that both LIF and CNTF can function to regulate neurotransmitter synthesis in rat sensory neurons (Nawa *et al.*, 1990; Ip *et al.*, 1991; Murphy *et al.*, 1991; Rao *et al.*, 1992, Fan and Katz, 1993; Thaler, 1994; Ware *et al.*, 1995). LIF and CNTF were shown to partially inhibit the expression of the catecholamine-synthesising enzyme, tyrosine hydroxlyase (TH), in low density cultures of E16.5 rat trigeminal neurons (Fan and Katz, 1993). In contrast, a comparison of TH-immunostained nodose and trigeminal ganglia from E13.5, E17.5 and E18.5 wild type (WT) and LIFR$\beta^{-/-}$ mutant mice revealed no obvious differences in the numbers of catecholaminergic neurons (Ware *et al.*, 1995).

In a recent study, Qiu and co-workers have suggested that iodinated LIF (^{125}I-LIF) binds to trigeminal and nodose ganglia of the developing rat (Qiu *et al.*, 1994). Furthermore, they suggest that binding to different types of sensory ganglia is detectable in varying magnitudes at different developmental stages. Although the results produced were semi-quantitative, they suggest that the magnitude of bound ^{125}I-LIF increases between the ages E15/16 and E18/P0. In

addition, this study demonstrated differences in the amount of bound ^{125}I-LIF between E18 sensory and sympathetic ganglia, suggesting that the magnitude of binding is lower in sympathetic ganglia. This finding is consistent with experiments I conducted using E19 SCG neurons, which did not survive in the presence of any of the cytokines tested (data not shown). Likewise, E21 sympathetic neurons could not be supported by either CNTF or LIF, but after 5 days in culture with NGF could be supported by either of these factors on NGF withdrawal (Kotzbauer *et al.*, 1994). P6 sympathetic neurons also survive in the presence of LIF and CNTF in dissociated cultures (Kotzbauer *et al.*, 1994). Taken together these results indicate that cytokine regulated cell survival may be dependent upon the timing of receptor synthesis in cytokine responsive neurons.

In summary, my work has demonstrated clear differences in the responses of different populations of cranial sensory neurons to cytokines during development. Whereas trigeminal neurons survive in response to CNTF, LIF, OSM or CT-1 late in development, most nodose neurons respond to these cytokines throughout the greater part of their embryonic development and additionally acquire responsiveness to IL-6 at late embryonic stages. These results suggest complex interactions between neurotrophins and cytokines in regulating the survival of different kinds of sensory neurons during development.

Conclusions

The data presented in this thesis show that during development, different populations of sensory neurons can respond differently to survival factors. Whereas certain populations of neurons can respond to several factors from the early stages of target innervation, other populations undergo a developmental switch in their trophic factor requirements.

I have demonstrated that the enteroceptive neurons of the nodose ganglion of the mouse survive in the presence of both neurotrophins, BDNF and NT-4/5 and the structurally related cytokines CNTF, LIF, OSM, and CT-1 from the earliest stages of target encounter throughout development and at later stages respond to an additional cytokine, IL-6 (see Fig. 6).

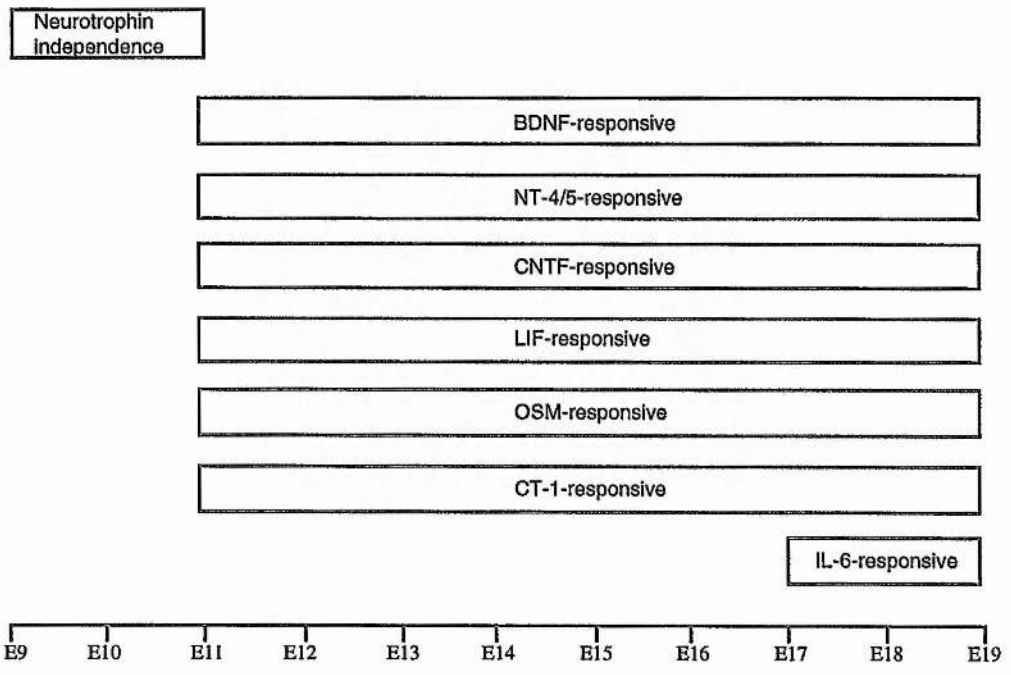


Figure 6. Summary of the developmental responsiveness of mouse nodose ganglion neurons to different neurotrophic factors shown in this study. Developmental time is represented from left to right in the diagram.

The cutaneous sensory neurons of the mouse trigeminal ganglion show different neurotrophic factor survival requirements to those of the nodose ganglion. After their initial period of neurotrophin independence, these neurons initially respond transiently to BDNF and NT4/5 (these neurons are also transiently responsive to NT-3 during this period (Buchman and Davies, 1993)). Trigeminal neurons then undergo a switch in their survival requirements, becoming NGF responsive and finally, begin to respond to the structurally related cytokines much later in development (see Fig. 7).

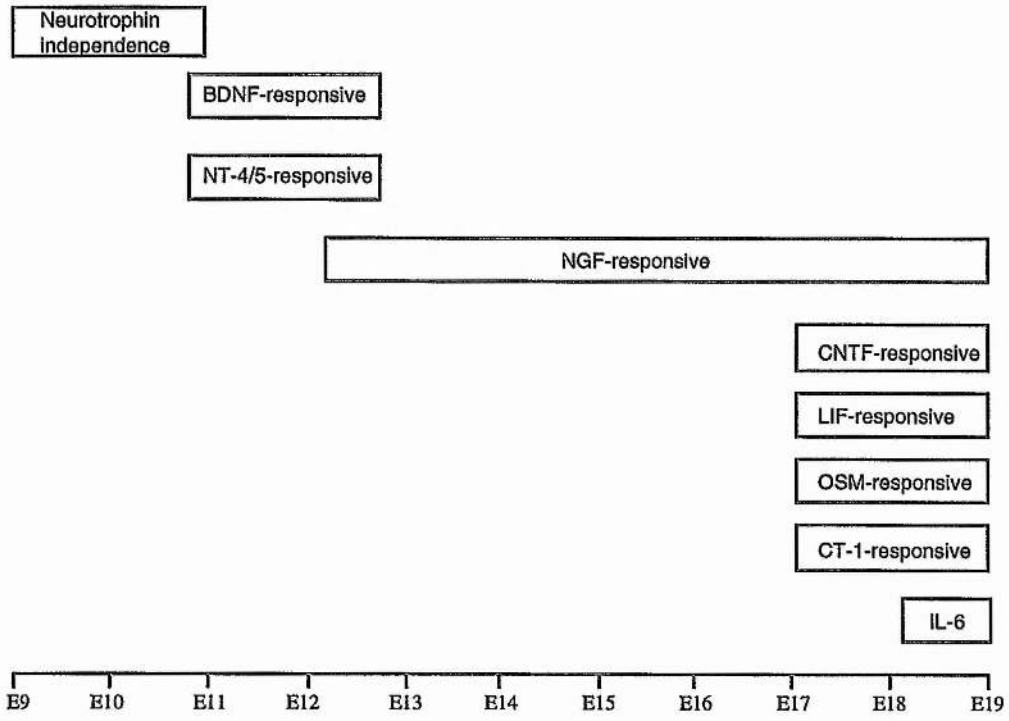


Figure 7. Summary of the developmental changes in neurotrophic factor responsiveness through which trigeminal ganglion neurons pass. Developmental time is represented from left to right in the diagram.

Studies of neurons from $p75^{-/-}$ mice have shown that during the mid-embryonic period, the common low affinity receptor p75 enhances the survival responses of trigeminal sensory neurons to NGF (Davies *et al.*, 1993b).

Similarly, p75 enhances the sensitivity of neurons from the superior cervical sympathetic ganglion to NGF, however in this case these neurons show the enhanced response postnatally (Lee *et al.*, 1994). In this study I have investigated the p75 modulated NGF survival response using an alternative approach. Using a mutated form of NGF that does not significantly bind p75, I have shown that p75 also plays a role in mediating the reponse of embryonic trigeminal and postnatal superior cervical sympathetic neurons to NGF. The reduced survival response of these neurons is not enhanced by addition of cell permeable analogues of the lipid second messenger ceramide, which is generated by NGF binding to p75 in other cell types (Dobrowsky *et al.*, 1994). Measurement of the relative levels of p75 and *trkA* mRNA suggest that endogenous p75 enhances the NGF survival response when the levels of p75 and *trkA* mRNA are similar.

BDNF responsive neurons from chick embryos are not as effectively supported by mammalian NT-4/5 or *Xenopus* NT-4, as with BDNF due to the lack of evolutionary conservation of NT-4/5 compared with other neurotrophins. The different activity of NT4/5 among different populations of chick neurons may be due to the expression of different isoforms of trkB.

In conclusion, the differing developmental survival responses displayed by NGF-dependent and BDNF-dependent neurons may be consequence of the functional segregation of the different populations of neurons studied.

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Appendix I

MEDIA AND SOLUTIONS

Liebovitz's L-15

This is a medium used for the purpose of dissection only. It is air buffered, and is made up from a powdered stock solution supplied by Gibco (Gibco Cat. No. 41300-021). 1L of medium is made up from the powdered stock using double distilled de-ionized water (ddH_2O), and to this add:

100mg Streptomycin sulphate
60mg Penicillin G

The pH is adjusted to 7.3 using HCl or NaOH and the medium is then filter sterilised using a 0.22-μm filter.

Ham's F-12

This is an air buffered general purpose medium supplemented with L-glutamine which is used for rinsing cells and dishes. The medium is supplied by Gibco as a powdered concentrate (Gibco Cat. No. 21700-026). 1L of medium is made up from powdered stock using ddH_2O to this add:

100mg Streptomycin sulphate
60mg Penicillin G
Heat Inactivated Horse Serum (Gibco 26050-039) to 10%

The pH is adjusted to 7.3 using HCl or NaOH the medium is then filter sterilised using a 0.22-μm filter.

Ham's F-14

This is a specially formulated bicarbonate buffered growth medium used for culturing embryonic sensory neurons . The formulation of this medium is based on that of Ham's F-12 but contains several modifications. The constituents of the medium are as follows.

Amino Acids	**mg/l**
L-Alanine	17.70
L-Arginine HCl	422.00
L-Asparagine H_2O	30.01
L-Aspartic Acid	26.50
L-Cysteine HCl.H_2O	70.32
L-Glutamic Acid	29.30
L-Glutamine	292.00
Glycine	14.90
L-Histidine HCl.H_2O	41.96
L-Isoleucine	7.94
L-Leucine	26.10
L-Lysine HCl	72.90
L-Methionine	8.88
L-Phenylalanine	9.96
L-Proline	68.90
L-Serine	20.90
L-Threonine	23.90
L-Tryptophan	4.04
L-Tyrosine	15.58
L-Valine	23.30

Vitamins	**mg/l**
Ascorbic Acid	15.00
d-Biotin	0.00073
D-Ca Pantothenate	0.48
Choline Chloride	13.96
Folic Acid	1.30
i-Inositol	18.00
Lipoic Acid	0.21
Nicotinamide	0.037
Pyridoxine HCl	0.062
Riboflavin	0.038
Thiamine HCl	0.34
Vitamin B_{12}	1.36

Inorganic Salts	**mg/l**
$CaCl_2.2H_2.O$	283.22
$CuSO_4.5H_2.O$	0.000249
$FeSO_4.7H_2.O$	0.834
KCl	372.80
$MgSO_4.6H_2.O$	174
$MgSO_4.7H_2.O$	37.00
NaCl	7599.00
$NaHCO_3$	1176.00

Na_2HPO_4	142.04
$ZnSO_4.7H_2.O$	0.863
Other Componenets	**mg/l**
D-Glucose	1981.80
Hypoxanthine	4.00
Linoleic Acid	0.084
Phenol Red	1.20
Putrescine 2HCl	0.161
Sodium Pyruvate	220.00
Thymidine	0.73

Source: Barde *et al.* (1980)

The medium is supplied by Imperial Laboratories (Cat No. 3-791-35) as a powdered concentrate and is made up to 1L (from a 10L stock preparation) using ddH_2O, and is stored at -40°C in 50ml aliquots.

To 500ml of F-14 add:

50mg Streptomycin sulphate

30mg Penicillin G

Alternatively, these antibiotics can be added to the stock preparation.

To make up 500ml F-14 add to the 50ml stock:

450ml ddH_2O

1g $NaHCO_3$

Heat Inactivated Horse Serum to 10%

The pH is adjusted to ~7.0 using CO_2 (dry ice) and the medium is filter sterilised using a 0.22-μm filter.

SATO

SATO medium is a specially formulated defined medium used for culturing embryonic neurons.

To make up a 222ml stock solution add the following:

Pathocyte-4-BSA (ICN Biomedicals Cat. No. 810101)	100ml
Putrescine (160mg/100ml H_2O) (1g) (Sigma P-7505)	100ml
Progesterone (1mg/1.6ml EtOH) (1mg) Sigma P-6149	1ml
L-Thyroxine (4mg/10ml EtOH) (4mg) Sigma T- 0397	10ml
Sodium selenite (1mg/2.6ml PBS) (1mg) Sigma S-9133	1ml
Tri-iodo-thyronine (16.8mg/50ml EtOH) (100mg) Sigma T-6397	10ml

The above ingredients are aliquoted in 11.1ml aliquots, stored at -40°C. Sato aliquots (11.1 ml) are added to 500ml Ham's F-14 medium containing 1g $NaHCO_3$ and 5ml of 200mM glutamine (Gibco Cat. No. 25030-024). The medium is then equilibrated to a pH of ~7.0 using dry ice. Source Sato, (1985).

General Solutions

Borate buffer (0.15M)

4.6g boric acid (Sigma) are dissolved in 500ml ddH2O (pH 8.4 with NaOH). This solution is used to dissolve the polyornithine.

HBSS

Hank's balanced salt solution (Gibco, Cat No. 1410-070) is supplied Ca and Mg free. This solution is used to dilute aliquots of laminin and trypsin, and is used to trypsinize and suspend dissociated cells prior to placing them on a dropping funnel.

KOH

5M KOH 140g KOH pellets (Sigma) in 500ml ddH2O. This solution is used for making tungsten wire needles.

Laminin

(Sigma L2020) This is a basement membrane protein required for cell attatchment. Stored at -70°C. Remove 1ml from freezer and defrost in fridge for 1hr. Aliquot 20ml in 1.5ml eppendorfs . This solution is reconstituted with 1ml of medium and used to coat 35 mm culture dishes prior to neuronal culture.

Polyornithine (P-ORN)

(Sigma P-8638) Stored at 4°C. 250mg are added to 500ml borate buffer. Filter sterilise into 100ml bottles, with 0.22-μm filter. This solution is used for coating 35 mm tissue culture dishes, prior to adding laminin.

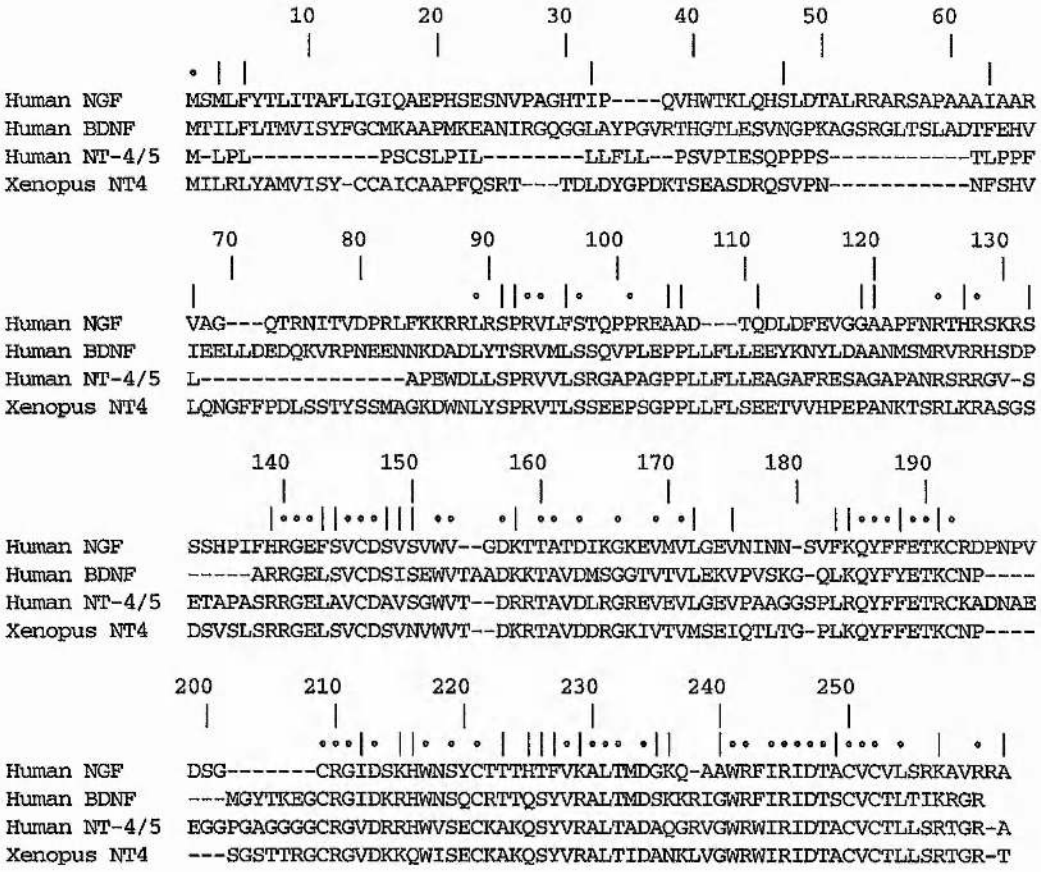
Trypsin

Supplied by Worthington Inc. 50mg are added to 5ml of Ca/Mg free HBSS (Gibco). Filter sterilise using a 0.22- μm filter. This solution is used for the enzymatic dissociation of ganglionic tissues and is inactivated using Ham's F-12 + 10% HIHS.

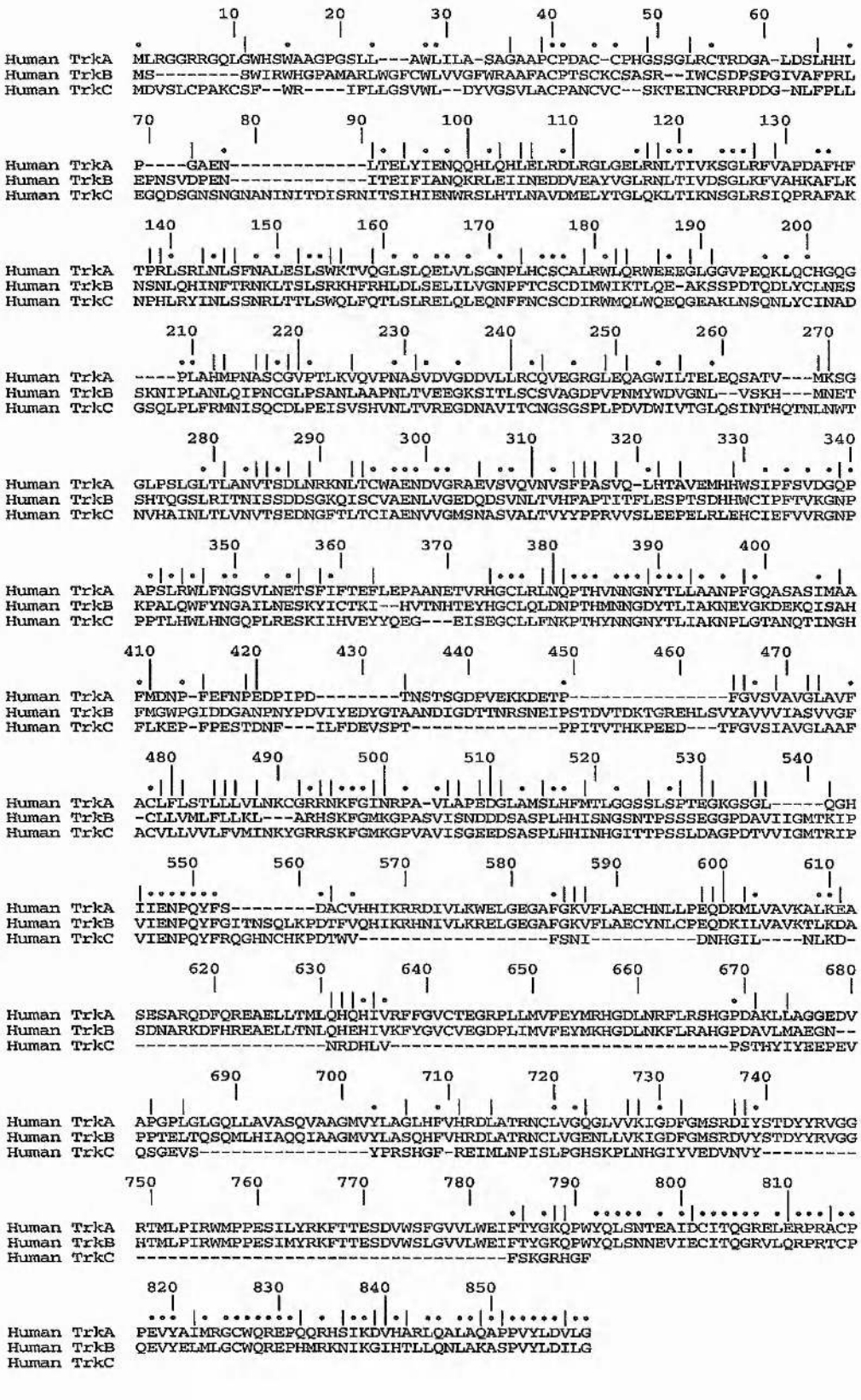
Appendix II

SEQUENCE ALIGNMENTS OF THE NEUROTROPHIC FACTORS AND THEIR RECEPTORS

Ⅰ Protein sequence comparisons between the different members of the neurotrophin family used in this study.



II Protein sequence comparisons between the different members of the neurotrophin receptor tyrosine kinase family of proteins.



III Protein sequence comparisons between the neurotrophic cytokines used in this study.