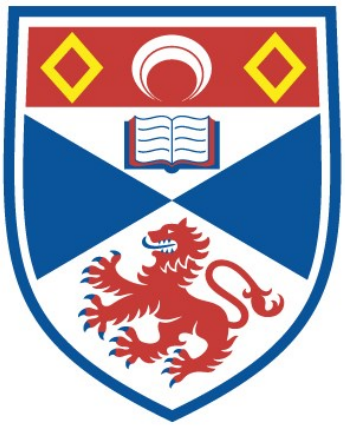
NEUROTROPHIN SWITCHING IN DEVELOPING SENSORY NEURONS

Luzia Giraldez Pereira Piñón

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



1997

"Since the full-grown forest turns out to be impenetrable, why not to revert to the study of the young wood in the nursery stage?"

Santiago Ramón y Cajal

I dedicate this Thesis to my parents and to Kumar

NEUROTROPHIN SWITCHING IN DEVELOPING SENSORY NEURONS

A Thesis submitted to the University of St. Andrews for the Degree of Doctor of Philosophy (PhD)

by

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October, 1996.

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I, Luzia Giraldez Piñón, here by notify that this thesis, which is approximately 52, 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 23/10/96 Signature of candidate

I was admitted as a research student at St. George's Hospital and Medical School, University of London, in January 1992 and as a candidate for the degree of PhD in November 1993; the higher study for which this is a record was carried out in St. George's Hospital Medical School and the University of St. Andrews between January 1992 and October 1996.

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Abstract

The main aim of this project was to define the neurotrophin survival requirements of sensory neurons during the early stages of their development both *in vivo* and *in vitro*. The *in vitro* survival of neural crest-derived but not placode-derived cranial sensory neurons is promoted by several different neurotrophins early in their development. Neural crest-derived neurons subsequently lose responsiveness to all neurotrophins except NGF. Loss of responsiveness of neural crest-derived sensory neurons to BDNF and NT3 is associated with a marked shift in the dose responses of these neurons to higher neurotrophin concentrations. Analysis of the timing of cell death in the trigeminal ganglia of mouse embryos that are homozygous for null mutations in the *TrkA, TrkB* and *TrkC* genes which encode high affinity receptors for NGF, BDNF and NT3 respectively, show that there is an early peak of apoptosis in *TrkB* and *TrkC* knockouts which is consistent with the early survival response of trigeminal neurons to BDNF and NT3 *in vitro*. The elevated peak of apoptosis in *TrkA* knockouts occurs at the same development stages as in wild type embryos which is consistent with the later response of trigeminal neurons to NGF *in vitro*. Furthermore, there is a high level of expression of TrkC mRNA in early trigeminal neurons which accords with the early survival response of these neurons to NT3. It is also shown that subsets of trigeminal neurons discriminate between neurotrophins at very high concentrations during the period of cell death, indicating that neurotrophin responses can be far more highly specific than previously thought. Taken together, these results show that neurotrophin switching is a physiologically relevant phenomenon in certain populations of developing sensory neurons.

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Abbreviations

BDNF Brain-derived neurotrophic factor

BSA Bovine serum albumin

ChAT Choline acetyl transferase

CIAP Calf intestinal alkaline phosphatase

CMF-PBS Calcium and magnesium-free phosphate-buffered isotonic saline

CNS Central Nervous System

CNTF Ciliary neurotrophic factor

ddNTP Dideoxynucleotide triphosphate

DEPC Diethylpyrocarbonate

DMTG dorsomedial trigeminal ganglia

DNase I Deoxyribonuclease 1

dNTP Deoxynucleotide triphosphate

DRG Dorsal root ganglia

DTT Dithiothreitol

E Embryonic day

EDTA Ethylenediaminetetraacetic acid

F12 Ham nutrient mixture F-12

F14 Ham nutrient mixture F-14

FGF Fibroblast growth factor

GDNF Glial cell line-derived neurotrophic factor

HBSS Hanks buffered salt solution

HIFCS Heat-inactivated foetal calf serum

HIHS Heat-inactivated horse serum

IL-6 Interleukin-6

IMS Industrial methylated spirits

IPTG Isopropyl-ß-D-thiogalactopyranoside

Kb Kilobase

kDa Kilodaltons

Kd Dissociation constant

L15 Leibovitz's L15 nutrient mixture

LB/LA Luria broth/Luria agar

LIF Leukaemia inhibitory factor

LMP Low melting point

MOPS 4-Morpholinepropanesulfonic acid

mRNA Messenger Ribonucleic Acid

NGF Nerve growth factor

NT3 Neurotrophin 3

NT4 Neurotrophin 4

NT5 Neurotrophin 5

NT6 Neurotrophin 6

OD Optical density

P-ORN Poly-DL-ornitine

PBS Phosphate buffered saline

PEG Polyethylene glycol

PNS Peripheral Nervous System

RNase Ribonuclease

RT-PCR Reverse Transcription-polymerase chain reaction

SCG Superior cervical sympathetic ganglion

SDS Sodium duodecyl sulphate

SH2 Src homology 2

STE Sodium-Tris-EDTA

TAE buffer Tris-acetate-EDTA buffer

TBE buffer Tris-boric acid-EDTA buffer

TE Tris EDTA

TEMED N' Tetramethylethylenediamine

TGF-ß (1,2,3) Transforming growth factor-ß (1,2,3)

TH Tyrosine hydroxylase

TMN Trigeminal mesencephalic nucleus

VLTG Ventrolateral trigeminal ganglion

VRC Vanadyl-ribonucleoside complex

X-gal 5-Bromo-4chloro-3-indoyl-ß-D-galactopyranoside

Chapter 1

INTRODUCTION

1.1. OVERVIEW

In the developing vertebrate nervous system neurons are generated in excess. Once differentiated, the neurons extend processes to their target fields. During the period of target field innervation a substantial number of neurons die. This phenomenon is called naturally occurring neuronal death (Oppenheim, 1991; Davies, 1994a for review). The purpose of this process is to match the number of neurons to the requirements of their target fields and eliminate superfluous and inappropriately connected neurons. The subsequent discovery of nerve growth factor (NGF), a protein which promotes the survival of certain kinds of neurons and is produced in the target fields of these neurons, led to our current understanding of how target fields regulate their innervation density. Several homologous proteins have been discovered subsequently, and constitute the NGF family of neurotrophins. The neurotrophins exert their biological activity by binding to cell surface receptors. Two kinds of receptors have been identified, namely, gp75 and members of the trk family of tyrosine kinases (Bothwell, 1995 for review). gp75 is a common receptor for all neurotrophins and binds them with low affinity. In contrast, the trk receptors are specific for different neurotrophins which they bind with high affinity. To understand how neurotrophins regulate the size and the connections of neuronal populations, it is essential to study neurons at successive stages of their development. Sensory neurons are particularly advantageous for these studies because these neurons

and their progenitors cells can be easily obtained for *in vitro* studies at different stages throughout their development. Another advantage of sensory neurons is that a substantial amount of information is available concerning their developmental history (Davies and Lumsden, 1986). To ascertain the neurotrophin requirements of sensory neurons during the early stages of development, I have studied the responsiveness of various populations of cranial sensory neurons to neurotrophins *in vitro* during embryonic development. These studies have shown that some populations of cranial sensory neurons switch their survival requirements from one set of neurotrophins to another during the early stages of target field innervation. To determine the physiological relevance of my *in vitro* observations, I have studied the timing of neuronal death in mice with targeted null mutations in the *TrkA, TrkB* and *TrkC* genes. Subsequently, I have studied the specificity of binding of neurotrophins to their respective receptors in developing chicken cranial sensory neurons *in vitro.* Finally, to clarify the role of high affinity receptor expression in sensory neuron development, I have carried out a detailed investigation of the expression of TrkC mRNA, which is the preferred receptor for Neurotrophin-3 (NT3). The switch in neurotrophin dependence was correlated with changes in receptor mRNA expression. The General Background and the results of all these studies are presented in the next five chapters.

1.2. GENERAL BACKGROUND

1.2.1. Development of the Vertebrate Nervous System

The nervous system of vertebrates is derived from the ectoderm. In a process known as neurulation, a broad central region of the ectoderm thickens, rolls up into a tube, and pinches off from the rest of the ectoderm. This tube, the neural tube, forms the brain and the spinal cord. Along the line where the neural tube pinches off from the future epidermis, a number of ectodermal cells break loose from the epithelium and migrate through the mesoderm. These neural crest cells form almost all of the peripheral nervous system (PNS), including autonomic neurons, most sensory neurons, sympathetic ganglion neurons and all of the support cells of the PNS, the Schwann cells and satellite cells.

1.2.2. Sensory Neurons

Sensory neurons are extremely useful for studying neuronal differentiation and development in the vertebrate nervous system because these neurons and their progenitor cells are accessible to experimental manipulation from the earliest stages of their development. Sensory neurons differentiate from progenitor cells that migrate from two regions of the embryonic ectoderm, the neural crest and neurogenic placodes (Le Douarin, 1986). The term placode was used by Von Kuppfer (1894) to designate the epithelial thickenings appearing in the cephalic ectoderm of the early vertebrate embryo. Epithelial neuron progenitor cells detach from the "neurogenic" placodes and migrate to certain

developing cranial sensory ganglia where they differentiate (D'Amico-Martel and Noden, 1983). The neural crest at the lateral margins of the neural plate gives rise to the sensory neurons associated with spinal and some cranial nerves. Neuronal differentiation is recognised by the emergence of central and peripheral axonal processes and by the expression of neuron-specific proteins such as neurofilament proteins. In all but one case, the cell bodies lie in anatomically discrete aggregates termed sensory ganglia. Dorsal root ganglia (DRG) are located on the dorsal roots of spinal nerves and cranial sensory ganglia are found on 5 of the 12 pairs of cranial nerves. The exception is a population of neural crest-derived sensory neurons located in the midbrain that constitute the trigeminal mesencephalic nucleus (TMN).

The contributions to populations of cranial sensory neurons from neural crest and placodes in avian embryos have been mapped by grafting quail (LeDouarin, 1973) or radiolabelled chick primordia into unlabelled chick hosts (Weston, 1963). These findings are summarised in Table 1.1. All of the ganglion supporting cells and Schwann cells are derived from the neural crest (D'Amico-Martel and Noden, 1983).

Cranial Nerve	Neuronal Populations	Origin	
		Neural Crest	Placodes
V	Trigeminal (Dorsomedial)	√	
V	Trigeminal (Ventrolateral)		√
V	Trigeminal Mesencephalic	√	
VII	Geniculate		√
VIII	Vestibulo-acoustic		√
Distal IX	Petrosal		√
Proximal X	Jugular	√	
Distal X	Nodose		√

Table 1.1. Origin of chicken cranial sensory neurons

1.2.3. Naturally Occurring Neuronal Death

Once early sensory neurons differentiate in developing sensory ganglia their axons grow to their peripheral and central target fields. The peripheral target field of a population of sensory neurons comprises those tissues to which they extend their peripheral axons, whereas the central target field comprises those populations of second order neurons with which its central axons synapse. For example, the peripheral target fields of dorsal root ganglia comprise a heterogeneous collection of tissues including skin, muscle and viscera. The peripheral target fields of many populations of cranial sensory neurons, however, consist of just one kind of tissue.

During the phase of target field innervation appropriate connections are established. A remarkable feature of this phase of development (establishment of connection with the target field) is the death of a substantial number of the innervating neurons. During the formation of the nervous system, nerve cells are produced in excess. The final number of cells is attained by a phase of cell death that occurs shortly after innervation of target fields (Oppenheim, 1991 for review). In the case of sensory neurons, the magnitude of cell death ranges from 25% in the vestibular and cochlear ganglia (Ard and Morest, 1984) to over 75% in the TMN (Rogers and Cowen, 1974) and generally takes place over a period of 3 to 6 days.

Neuroembryologists have understood for several decades that the number of neurons in developing vertebrates is influenced by cellular mechanisms that depend on feedback from neuronal targets. The currently accepted view of such

regulation is that targets elaborate trophic signals on which neurons are selectively dependent for their survival. There is also some evidence that afferents play a role in maintaining neuronal survival (Oppenheim, 1991 for review). Thus, the number of neurons that survive to maturity is governed by interactions with their targets and afferents.

Natural neuronal cell loss occurs by the process of programmed cell death or apoptosis. This differs in many respects from necrosis which is typical of pathological conditions (Kerr, Wyllie and Curie, 1972; Altman, 1992). General (though not universal) characteristics of apoptosis are well established (Wyllie *et al*, 1980). Distinctive morphological changes occur that include nuclear condensation and degradation of DNA to oligosomal fragments. In some cases this process is dependent on protein synthesis (a striking illustration of the active nature of the process). Apoptosis typically affects scattered single cells and is identified histologically by the presence of small, round or oval cytoplasmic fragments, some of which contain pyknotic nuclear remnants (Kerr *et al*, 1972).

Despite considerable progress, it has proven difficult to identify the molecules responsible for apoptosis by conventional biochemical and molecular approaches in mammalian systems. Fortunately, there is increasing evidence that apoptosis occurs by a mechanism that has been at least partially conserved throughout animal evolution. In the nematode *Caenorhabditis elegans,* 14 genes have been shown to function in programmed cell death. Three of these genes affect the execution of all somatic cell deaths: *ced-3, ced-4* and *ced-9* (ced stands for cell death defective). The activity of *ced-3* and *ced-4* is required for cell death to occur, and *ced-9* protects cells that should survive from undergoing programme cell death (Hengartner *et al*, 1992).

Overexpression of the vertebrate homologues of *ced-3* , *ICE* (interleukin 1B converting enzyme), in mammalian cells causes apoptosis (Wang *et al*, 1994), suggesting that either *ICE* , or a related cysteine protease may function in mammalian cell death. Likewise, the vertebrate homologue of ced-9, bcl-2, has been shown to be a suppressor of apoptosis in the vertebrate nervous system. The human bcl-2 gene was identified at the breakpoint site of the t(14;18) chromosomal translocation that is associated with follicular lymphoma (Cleary *et al*, 1986). Bcl-2 is thought to contribute to oncogenesis by suppressing signals that induce apoptotic cell death (Williams *et al*, 1990; Vaux *et al*, 1992). Overexpression of bcl-2 *in vitro* can prevent haematopoietic and neural apoptosis that has been induced by growth factor withdrawal (Nuñez *et al*, 1990; Garcia *et al*, 1992; Allsopp *et al*, 1993).

Bcl-2 is the founder member of an expanding family of genes that regulate apoptosis, which now includes *bclx-l, bax, bad, bak, mcl-1* and *A1*. *Bax* (Oltvai *et al*, 1993), *bad* (Yang *et al*, 1995) and *bak* (Farrow *et al*, 1995; Chittenden *et al*, 1995; Klefer *et al*, 1995) have been identified as effector proteins in the cell death program, whereas *bcl-2* (Garcia *et al*, 1992) and *bclx-l* (Boise *et al*, 1993) are putative repressors of neuronal death (for review Nuñez and Clarke, 1994). Proteins of the *bcl-2* family contain a stretch of hydrophobic amino acids at their C-termini which appear to be important for attachment to intracellular membranes (Nguyen *et al*, 1993; Tanaka *et al*, 1993), but not important for cell death preventing functions.

Genetic analysis of the process of naturally occurring cell death in *Drosophila melanogaster* has led to the isolation of a gene, *reaper*, that is capable of integrating information from different signalling pathways to activate the apoptotic program (White *et al*, 1994; White *et al*, 1996). The mechanism by

which reaper induces apoptosis is not clear. The *reaper* gene encodes a small polypeptide of 65 amino acids with no significant similarity to other known proteins.

1.2.4. Importance of Neurotrophic Factors in the Regulation of Neuronal Survival

The discovery of NGF by Rita Levi-Montalcini 40 years ago led to the widely accepted hypothesis that neuronal target fields play an important role in regulating the number of neurons that survive by their production of limited quantities of NGF, or other so called neurotrophic factors, which the innervating neurons require for their survival. This hypothesis is based on two main assumptions. First, the survival of developing vertebrate neurons depends on specific neurotrophic factors secreted by the target cells that the neurons innervate. Second, many types of neurons are produced in excess, so that only a proportion gets enough neurotrophic support from their target cells to survive. Thus, the level of trophic factor production in the target field directly influences the size of the innervating populations of neurons (Thoenen and Barde, 1980; Davies, 1988; Barde, 1989).

The most important evidence for the neurotrophic hypothesis has come from the demonstration that developing neurons whose survival is promoted by NGF *in vitro*, namely sympathetic and certain sensory neurons, also depend on NGF *in vivo*. Anti-NGF antibodies eliminate these neurons during the early stages of target field innervation, whereas exogenous NGF rescues neurons that

would otherwise die (Levi-Montalcini, 1987). Likewise, mice lacking a functional NGF gene have severely reduced numbers of sensory and sympathetic neurons and exhibit marked reductions in response to nociceptive stimuli (Crowley *et al*, 1994). NGF is synthesised in the target fields of NGF-dependent neurons in proportion to their innervation density both in the adult (Korsching and Thoenen, 1983; Heumann *et al*, 1984; Shelton and Reichardt, 1984) and during development (Harper and Davies, 1990), and is conveyed from these tissues to the cell bodies of the innervating neurons by rapid axonal transport (Hendry *et al, 1974*; Korshing and Thoenen, 1983).

Studies of the site and timing of NGF synthesis and NGF receptor expression during development gave further support to the neurotrophic hypothesis. The demonstration that NGF synthesis commences in the target fields of NGF-dependent sensory (Davies *et al*, 1987) and sympathetic (Korsching and Thoenen, 1988) neurons with the onset of target field innervation, and that NGF receptors are not expressed until the neurons innervate their targets (Davies *et al*, 1987) showed that NGF does not affect these neurons at earlier stages of development.

NGF is now known to be only one member of a family of homologous neurotrophic proteins, called neurotrophins, which includes brain derived neurotrophic factor (BDNF) (Barde *et al*, 1982), Neurotrophin-3 (NT3) (Hohn *et al*,1990; Maisonpierre *et al*, 1990; Rosenthal *et al*,1990; Jones *et al*, 1990; Ernfors *et al*, 1990), Neurotrophin-4 (NT4) cloned in Xenopus, mammalian Neurotrophin 4/5 (Berkemeier *et al*,, 1991; Ip *et al*, 1992), and the recently cloned Neurotrophin 6 (NT6) (Gotz *et al*, 1994).

Nerve Growth Factor

For over four decades, NGF provided the only basis for the concept that trophic molecules are necessary for neuronal survival. An important source for the initial purification and characterisation of NGF was from the mouse submandibular salivary gland (Cohen, 1960). NGF isolated from this source is an acidic protein complex with a molecular weight of 130 kDa consisting of three subunits, alpha, beta and gamma, that are present in the ratio 2:1:2, respectively (Varon *et al* 1968). The biological activity of this molecular complex is due to a dimer consisting of two identical 118 amino acid chains. Therefore, NGF exists as a 26 kDa homodimer of 13 kDa polypeptides, referred to as ßNGF. Although the association of the chains of the dimer is non-covalent, the interaction is stable even at physiologically low NGF concentrations (Bothwell and Shooter, 1977).

Sequencing of the mouse submandibular gland NGF ß subunit (Angeletti and Bradshaw (1971) provided invaluable information about its primary structure and led to the subsequent cloning of the mouse (Scott *et al*, 1983), human (Ullrich *et al*, 1983), bovine (Meier *et al*,1986) and chick (Ebendal *et al*, 1986) NGF genes which exhibit a high degree of homology. The NGF gene codes for a precursor polypeptide of 307 amino acid residues which, upon cleavage, gives rise to the 118 amino acid mature NGF protein (Scott *et al*, 1983; Berger and Shooter, 1977; Edwards *et al*, 1988a, b). The mouse gene spans 45 kb and has several small 5' exons (Selby *et al*, 1987). At least four different mRNA transcripts can be produced by differential splicing, although the functional difference of these various transcripts is not yet fully understood. Most of the pre-pro NGF coding sequence, including the full sequence of the biologically active processed NGF, is located in one exon at the 3' end of the gene. Gene transfer studies in various cell lines have established that the two primary

translation products (27 or 34 kDa corresponding to the two major NGF mRNA transcripts) give rise to the precursor protein (Edwards *et al*, 1988a, b). The trypsin-like enzymes necessary for the correct processing of NGF are found in many cells (Edwards *et al*, 1988). This is in agreement with *in vivo* data indicating that NGF is made by a variety of cells, such as epidermal cells, smooth muscle cells or hippocampal neurons. There are indications that, depending on the tissue or the cell transfected, NGF can be stored in vesicles and released either by calcium-dependent mechanism or via the 'constitutive' pathway (Barth *et al*, 1984; Edwards *et al*, 1988b).

Although crystals of NGF have been available for some time, they have only recently been successfully analysed by X-ray diffraction methods to produce a three dimensional structure of the mature NGF molecule to 2.3 A° resolution. The structure is characterised by a new protein folding that is rich in ß-strands (McDonald *et al*, 1991).

The amino terminus of NGF is susceptible to proteolytic cleavage. A comparison of the bioactivity of highly purified full length (1-118) recombinant human NGF (rhNGF) and a NH_2-terminal truncated (10-118) rhNGF revealed lower potency of the truncated rhNGF with regard to early NGF responses in neuron-like PC12 cells. It was found that, whereas the truncated form of rhNGF (10-118) had a 300-fold less affinity for TrkA (the high affinity receptor for NGF) than the full length molecule (1-118). The amino-terminal truncation of NGF changed its affinity for the low affinity receptor (gp75) only slightly (5-10 fold). These observations suggest that amino acids 1-9 of NGF are important for binding to the signal transducing receptor TrkA (Kahle *et al*, 1992).

Mice that are homozygous for null mutations in NGF gene failed to respond to noxious mechanical stimuli, and histological analysis revealed profound cell loss in both sensory and sympathetic ganglia. Within dorsal root ganglia, effects of the mutation appeared to be restricted to small and medium peptidergic neurons. In contrast, within the central nervous system, basal forebrain cholinergic neurons differentiate and continue to express phenotypic markers for the life span of the null mutant mice. Thus, differentiation and initial survival of central NGF-responsive neurons can occur in the absence of NGF (Crowley et al, 1994)

Brain Derived Neurotrophic Factor

BDNF is a 12.3 kDa protein that was purified from pig brain using an assay based on sensory neuron survival (Barde *et al*, 1982). Using partial sequence data, a pig BDNF cDNA was isolated that has an open reading frame coding for a precursor protein of 252 amino acids (Leibrock *et al*, 1989). A striking feature of the primary structure of mature BDNF is its similarity to NGF. Fifty one amino acids are common to BDNF and NGF. However, most of the cleaved precursor sequence is unrelated to that of NGF. Subsequently, full length human (Jones and Reichardt, 1990; Maisonpierre *et al*, 1991), mouse (Hofer *et al*, 1990) and rat BDNF (Maisonpierre *et al* 1991) were isolated as well as partial cDNA clones for monkey, chicken and Xenopus BDNF (Isackson *et al*, 1991; Hallbok *et al*, 1993).

BDNF mRNA is predominantly expressed in the brain and spinal cord but is also found in a number of non-neuronal cell types like human platelets (Leibrock *et al.*, 1989; Yamamoto and Gurney, 1990), fibroblasts and sciatic nerve Schwann cells (Acheson *et al*, 1991). BDNF mRNA expressing cells are widely distributed in the rat brain, with high levels in the hippocampus, the internal and external pyramidal layers of the cerebral cortex and the claustrum (Wetmore *et al*, 1990). In the peripheral nervous system, BDNF mRNA is expressed in rat dorsal root ganglion neurons (Ernfors *et al*, 1990). BDNF mRNA has also found in early chicken DRG neurons (Wright *et al*, 1992). Since early DRG neurons mature when grown in single cell culture and antisense BDNF oligonucleotides, but not sense oligonucleotides, inhibit maturation, it is possible that BDNF promotes maturation by an autocrine route at this stage of development (Wright *et al*, 1992).

Expression of BDNF mRNA in spinal cord and superior colliculus supports the idea that BDNF is a target-derived neurotrophic factor, as these regions contain the target cells of neurons previously shown to respond to BDNF (Lindsay *et al*, 1985; Davies *et al*, 1986; Johnson *et al*, 1986).

Although the neurotrophic theory was formulated on the basis of work done on NGF, it holds true for other members of the neurotrophin family. In a similar way to NGF, BDNF promotes survival of certain neuronal populations during development. BDNF supports the survival *in vivo* of placode and neural-crest- derived sensory neurons (Lindsay *et al*, 1985; Hofer and Barde, 1988) and increases the survival and differentiated functions of rat septal cholinergic neurons in culture (Alderson *et al*, 1990). BDNF also promotes the survival and differentiated phenotype of both dopaminergic and GABAergic neurons of the forebrain and substantia nigra (Hyman *et al*,1994; Knusel *et al*, 1991).

Embryonic retinal ganglion cells respond to BDNF *in vitro* from E11 onwards, a time corresponding to target field innervation *in vivo* (Johnson *et al*, 1986; Rodriguez-Tebar *et al*, 1989). Although not required for the survival of adult retinal ganglion cells, BDNF stimulates re-elongation from these cells and can rescue them from degeneration (Thanos *et al*, 1989). Chicken sensory neurons like dorsal root, trigeminal mesencephalic, jugular and geniculate neurons, are responsive to BDNF *in vitro* (Lindsay *et al*, 1985; Davies *et al*, 1986). BDNF is also a neurotrophic factor for dopaminergic neurons of the substantia nigra, increasing their *in vitro* survival, including those cells that degenerate in Parkinson's disease (Hymann *et al*, 1991).

Homozygous mutant BDNF mice (-/-) are reduced in size as compared to their litter mates and most die within the first three postnatal weeks. However, the heterozygous animals (-/+) are viable but consistently intermediate in size (Carrol *et al* 1994). There is an increased level of cell death within DRG neurons in the mutant animals. In addition, BDNF (-/-) mutants were found to have reduced numbers of inner ear vestibular and nodose neurons (Ernfors *et al*, 1994, Jones *et al*, 1994).

Neurotrophin 3

Taking advantage of sequence identities between NGF and BDNF, a third member of the neurotrophin family, NT3, was originally cloned from a mouse genomic template by PCR using degenerate oligonucleotides. Mature NT3 consists of 119 amino-acids of 13.6 kDa (Relative molecular mass of 13.625, pI

9.3). Like BDNF and NGF, recombinant human NT3, exists as a dimer at physiological relevant concentrations (Radziejewski *et al*, 1992).

In every respect, NT3 structure resembles that of NGF and BDNF. The primary translation product of NT3 contains a putative signal sequence of 18 amino-acids followed by a pro-sequence of 121 amino-acids. Such pro-sequences, presumably involved in the folding and correct formation of the disulphide bridges of these proteins, have also been found in mouse NGF (103 amino-acids) and mouse BDNF (112 amino-acids). Almost 50% of the structure is conserved among the three proteins. All six cysteine residues, which in NGF and BDNF are involved in the formation of disulphide bridges, are among the conserved residues.

The sequences of the mammalian NT3 species characterised so far are entirely identical within the region encoding the mature biologically active NT3 protein (Maisonpierre *et al*, 1991). The chicken NT3 sequence contains only one amino acid change from its mammalian counterparts (Hallbook *et al*, 1993).

In the brain, Northern blotting analysis has shown that the highest NT3 mRNA level is present in the cerebellum and hippocampus (Ernfors *et al*, 1990) and the overall distribution of NT3 mRNA is very different from that of NGF and BDNF in adult mouse tissues (Hohn *et al*, 1990). It can be detected in many adult tissues such as brain, heart, skin and muscle (Hohn *et al*, 1990; Maisonpierre *et al*, 1990; Rosenthal *et al*, 1990; Maisonpierre *et al*, 1990b) suggesting that it may act as a target derived factor for central and peripheral projections. NT3 mRNA has also been found in adult rat peripheral tissues such as kidney, secretory cells in the male rat submandibular gland and epithelial cells

in the ovary (Ernfors *et al*, 1990). The expression of NT3 in the central and peripheral nervous system is described in more detail in Chapter 5

Unlike BDNF, NT3 does not have neurotrophic activity on central cholinergic or dopaminergic neurons (Johnson *et al*, 1986; Knusel *et al*, 1991; Hohn *et al*, 1990; Maisonpierre *et al*, 1990). However, it prevents the degeneration of noradrenergic neurons of the locus coeruleus *in vivo* (Arenas and Persson, 1994). In addition to promoting survival, NT3 induces neurite outgrowth from a subset of neural crest and placode-derived neurons. NT3 has also been shown to induce a mitogenic response in neural crest cells grown as homogeneous cultures. This effect is larger when migrating neural crest cells were grown together with some somite cells (Kalcheim *et al*, 1992). Using an anti-NT3 antibody in early stages of development Gaese *et al* (1994) have demonstrated that NT3 plays an essential role during gangliogenesis. Further evidence that NT3 prevents the death of proliferating sensory precursors cells during neurogenesis has been obtained from NT3 knockout animals (ElShamy and Ernfors, 1996, a, b). Excessive cell death in DRG and trigeminal neurons of $NT3^{-/-}$ mice occurs in a period that precedes the programmed cell death period and results in the reduction in the number of proliferating precursors (El Shamy and Ernfors, 1996a, b). It has also been shown that NT3 stimulates the differentiation of motoneurons from avian neural tube progenitor cells but has no significant effect on the survival of differentiated avian motoneurons (Averbuch-Heller *et al*, 1994).

The mutant mice for NT3 have severe movement defects of the limbs, and most died shortly after birth. Substantial portions of peripheral sensory and sympathetic neurons are lost while motor neurons are not affected. Spinal proprioceptive afferents and their peripheral sense organs (muscle spindles and

Golgi tendon organs) are completely absent in homozygous mutant mice. The number of muscle spindles in heterozygous mutant mice is half of that in control mice, indicating that NT3 is present at limiting concentrations in the embryo. No gross abnormalities are seen in sensory receptors such as Pacinian corpuscles, cutaneous afferents containing substance P and calcitonin-related peptide, and deep nerve fibers in the joint capsule and tendon. These mutants demonstrated that NT3 is required for the development and survival of subpopulations of dorsal root ganglia, trigeminal and nodose ganglia neurons *in vivo*, as well as mesencephalic trigeminal nucleus neurons. These observations also confirm tissue culture studies which have shown that NT3 promotes the survival of neurons from these ganglia *in vitro* (Ernfors *et al*, 1990; Hohn *et al*, 1990; Maisonpierre *et al*, 1990; Rosenthal *et al*, 1990; Avila *et al*, 1993; Ibanez *et al*, 1993).

Neurotrophin 4/5

NT4 was originally isolated from *Xenopus* and viper after an evolutionary conservation study of members of the NGF family. The NT4 gene encodes a precursor protein of 236 amino acids which is processed into a 123 amino acid mature NT4 protein with 50 to 60% amino acid identity to NGF, BDNF and NT3 (Hallbook *et al*, 1991; Berkemeier *et al*, 1991 and Ip *et al*, 1992).

Northern analysis of different tissues from Xenopus has shown that NT4 mRNA is present only in the ovary. *In situ* hybridisation has detected NT4 mRNA in the developing whisker follicles and skin of embryonic rats, regions where the other neurotrophins are also present although in different cellular

locations (Ibañez *et al*, 1993). NT4 has been shown to stimulate neurite outgrowth in cultured peripheral sensory neurons (Ibañez *et al*, 1993).

Using degenerated oligonucleotides derived from conserved regions between NGF, BDNF and NT3, a novel NGF-related protein has been cloned from human placental DNA by PCR and named NT5 (Berkemeier *et al*, 1991). From the screening of a human genomic library a full length precursor human NT5 protein (hNT5) has been isolated that is 22.4 kDa and contains 210 amino acids. The mature hNT5 protein is basic (pI 10.3) and has 123 amino acids. Similar to other neurotrophins, the hNT5 open reading frame possesses a consensus sequence for N-glycosylation just upstream of a dibasic cleavage site that could generate the mature polypeptide. The overall homology of hNT5 with other neurotrophins is 66% with Xenopus NT4, 55% with hNT3, 56% with hBDNF and 50% with hNGF. NT5 mRNA has been detected in rat embryos from embryonic day 12. In adult rats, NT5 mRNA is expressed in thymus, lung, ovary, brain, heart, stomach and kidney (Berkemeier *et al*, 1991). It has been demonstrated that NT5 is well conserved between species and that NT5 is the mammalian homologue of Xenopus NT4 (Ip *et al*, 1992). Therefore they are currently considered the same molecule and named NT4/5.

NT4/5 is as potent as BDNF in supporting the survival of early mice trigeminal and nodose ganglion neurons, but does not have any survival effect on chicken trigeminal mesencephalic or ventrolateral trigeminal ganglia neurons, which are BDNF dependent (Davies *et al*, 1993).

NT4 deficient mice are long-lived and show no obvious neurological defects. Nodose and petrosal sensory neurons are dramatically reduced in the

absence of NT4 gene. Motor neurons are not affected in the absence of NT4 (Conover *et al*, 1995).

Neurotrophin 6

NT6 is the most recently isolated neurotrophin. It was cloned from a genomic library of the teleost platyfish Xiphophorus maculatus (Gotz *et al*, 1994). So far it has not been found as a soluble protein in the medium surrounding cells that synthesised it. Analysis of NT6 gene expression by Northern blotting has shown that a 1.4 kilobase transcript is expressed from organogenesis onwards in the embryonic vavulla cerebelli (a rostral protrusion of the teleostean cerebellum under the midbrain tectum). Expression persists in some adult tissues, such as gill, liver and eye, with a weak expression in skin, spleen, heart and skeletal muscle (Gotz *et al*, 1994).

Recombinant purified NT6 has a spectrum of actions similar to NGF on chick sympathetic and sensory neurons like dorsal root ganglia, although with lower specific activity than NGF. It had no survival effect on ciliary and nodose neurons. Therefore, the spectrum of responsive neurons seems similar to that of NGF (Gotz *et al*, 1994).

1.2.5. Neurotrophic Factor Receptors

Binding studies using iodinated NGF or BDNF have revealed two kinds of receptor for each factor: low affinity receptors that have a dissociation constant of 10^{-9}M and high affinity receptors that have a dissociation constant of 10^{-11}M (Sutter *et al* 1979; Rodriguez-Tebar and Barde, 1988). A cDNA encoding a transmembrane glycoprotein with a relative molecular mass of 75000 (gp75) that binds NGF with low affinity has been cloned (Johnson *et al*, 1986; Radeke *et al*, 1987). Competition binding studies and gene transfection experiments have shown that gp 75 NGF receptor also binds BDNF and NT3 with low affinity (Rodriguez-Tébar *et al*, 1990). In contrast, high-affinity neurotrophin receptors display high ligand specificity (Rodriguez-Tebar *et al*, 1990). The finding that the survival-promoting effects of NGF and BDNF are observed at concentrations that result in preferential occupancy of their respective high affinity receptors (Greene, 1977; Rodriguez-Tebar and Barde, 1988) suggested that these receptors mediate the response of neurons to neurotrophic factors. The functional high affinity receptors for the NGF family of neurotrophins have been identified as the members of the trk family of tyrosine protein kinases (for review, Barbacid, 1993). The trk gene was first identified as an oncogene in a human colon carcinoma biopsy (Coulier *et al*, 1990). The oncogenic activity of trk resulted from a genomic rearrangement event in which non-muscle tropomyosin sequences were aberrantly fused onto the transmembrane and cytoplasmic domains of trk (hence the name "tropomyosin receptor kinase").

The demonstration that neurotrophic factors promote rapid autophosphorylation of trk tyrosine kinases (Kaplan *et al*, 1991; Klein *et al*, 1991 and Soppet *et al*, 1991) and elicit a response from oocytes and cell lines transfected with trk cDNAs (Nebreda *et al*, 1991; Loeb *et al*, 1991; Cordon

Cardo *et al*, 1991; Squinto *et al*, 1991; Glass *et al*, 1991 and Lamballe *et al*, 1991) shows that these tyrosine kinase receptors play a role in neurotrophic factor signal transduction. Three members of the trk family of receptor tyrosine kinase have been identified in vertebrates: TrkA, TrkB and TrkC (for review see Lamballe *et al*,1991). Expression studies in PC12 cells and fibroblast cell lines have shown that TrkA is a functional receptor for NGF (Klein *et al*, 1991a), TrkB is a functional receptor for BDNF and NT4/5 (Klein *et al*, 1991b), and TrkC is a functional receptor for NT3(Lamballe *et al*,1991) (Fig. 1.1). All three of the Trk receptors are expressed at highest levels in the nervous system (Klein *et al*,1989; Martin-Zanca *et al*, 1990; Lamballe *et al*, 1991).

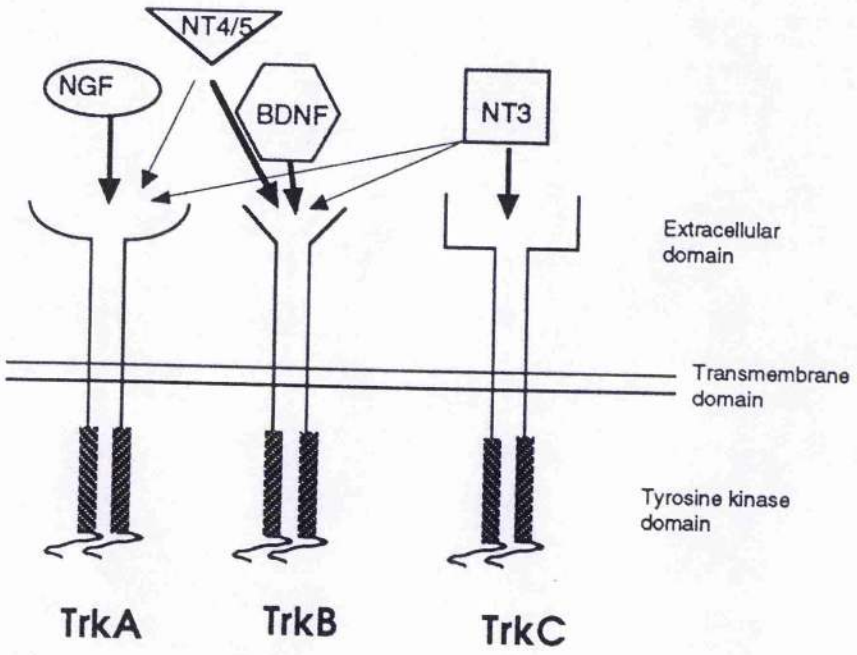


Figure 1.1. Trk receptors and their ligands. The thick arrows denote the preferred ligand. The non-preferred interactions are indicated by the thin arrows.

Low Affinity NGF Receptor (gp75)

There is still much controversy regarding the role that gp75 plays in mediating the neurotrophins effects. The introduction of a recombinant retrovirus containing gp75 cDNA in NGF receptor deficient PC12 cells restores both high and low affinity NGF binding (Hempstead *et al*, 1989). This suggests that the common low affinity neurotrophic factor receptor is a component of the high affinity receptor (Hempstead *et al* 1991). In addition, transfection assays with different amounts of gp75 have shown that it enhances the affinity of NGF binding to TrkA (Benedetti, 1993). However, other studies have shown that TrkA alone can directly bind and mediate responses to neurotrophins in the absence of gp75 (Cordon Cardo *et al*, 1991; Klein *et al*, 1991; Chao, 1991 for review).

gp75 is a single peptide chain of approximately 400 amino acid residues, with a single membrane spanning domain separating a longer extracellular domain from a shorter cytoplasmic domain. gp75 binds to all the known members of the neurotrophin family (reviewed in Davies, 1994). This has led to the hypothesis that gp75 serves as a common subunit for different neurotrophin receptors, the specificity of a receptor for a given ligand being conferred by the second component of the receptor complex (Bothwell, 1991; Thoenen, 1991). However, although all neurotrophins bind to gp75 with similar affinity, their dissociation rates are markedly different, with NGF and NT3 being the fastest and slowest, respectively (Rodriguez-Tebar *et al*, 1992). This difference raises the possibility that gp75 may play a role in discriminating among neurotrophins, thereby tuning the specificity of neurotrophin binding.

gp75 is also expressed during development in a number of tissues not known to respond to NGF or other neurotrophins, including skin mesenchyme, somites and muscle anlage, testis and kidney (Persson *et al*, 1990; Heuer *et al*, 1990; Wyatt *et al*, 1990; Sariola *et al*, 1991). This widespread expression has raised the possibility that the receptor has a more general role in embryonic development (Bothwell, 1995 for review).

Dorsal root sensory and neonatal sympathetic neurons from mice carrying a null mutation in the gp75 locus have a decreased sensitivity to NGF during the period of naturally occurring cell death suggesting that gp75 does play a role in mediating neurotrophin function in some NGF dependent neurons (Lee *et al*, 1994).

TrkA

The TrkA proto-oncogene encodes two tyrosine protein kinase isoforms of 790 or 796 aminoacid residues. These isoforms, designated as TrkA, or $gp140^{TrkA}$, are transmembrane glycoproteins of 140 kDa (Martin Zanca *et al*, 1989; Meakin *et al*, 1992; Barker *et al*, 1993; Horigome *et al*, 1993). Both isoforms have the canonical structure of tyrosine protein kinase cell surface receptors including; a signal peptide, an extracellular domain responsible for the interaction with NGF, a single transmembrane domain and a cytoplasmic catalytic domain. The two isoforms differ in the presence of six amino acid residues (VSFSPV) located in the extracellular domain near the transmembrane region. Whereas the 796 amino acid TrkA molecule is primarily expressed in neuronal cells, the 790 amino acid isoform has been found in cells of non-neuronal origin (Barker *et al*, 1993; Horigome *et al*, 1993). Although no

comparative studies have been carried out so far, it appears that both isoforms have similar biological properties (Klein *et al*, 1991b; Jing *et al*, 1992; Meakin *et al*, 1992).

The extracellular domain of the TrkA receptor exhibits two distinct subsets of cell adhesion-related motifs (Schneider and Schweiger, 1991). The amino-terminal moiety consists of an array of three tandem leucine-rich motifs (LRM) of 24 amino acids, flanked by two distinct cysteine clusters (CCI and CCII). This LRM motif has been found in a diverse superfamily of proteins thought to be involved in specific protein-protein interactions, such as the human platelet von Willebrand factor receptor and several cell adhesion proteins and extracellular matrix components. Next to the second cysteine cluster there are two imunoglobulin-like domains (IgI and IgII) of the C2 type similar to those found in the neural cell adhesion molecules N-CAM and in other tyrosine kinase receptors such as the PDGF and FGF receptor subfamilies (Schneider and Shweiger, 1991). Deletion of 51 amino acid residues encompassing the carboxy-terminal half of the IgII motif (residues 322-372) or replacement of the single Cys^{345} residue in this sequence by Ser results in the constitutive activation of the TrkA receptor (Coulier *et al*, 1990).

The TrkA cytoplasmic domain has a set of structural features unique to this subfamily of receptors (Martin-Zanca *et al*, 1989; Klein *et al* 1989; Lamballe *et al*, 1991). They include; a Thr residue (position 647) instead of the conserved Ala in the highly conserved HRDLAARN kinase motif, a Trp residue (position 722) instead of Tyr in the WEX_7PY sequence and the replacement of the conserved Pro in the CWX_6RP sequence located at the carboxy terminal end of the kinase domain.

A common characteristic of the tyrosine kinase receptors for the neurotrophins of the NGF family is a very short carboxy-terminal tail of 15 amino-acids which includes a conserved Tyr residue (Martin Zanca *et al*, 1989). Outside of this receptor subfamily, the TrkA cytoplasmic region is most closely related to that of several new receptors called orphan receptors. However, these receptors have rather distinct extracellular domains and are likely to recognise ligands unrelated to the NGF neurotrophin family. The deletion of the 15 C-terminal amino-acids, including tyrosine 785 (Y-785), abrogates receptor and substrate phosphorylation activities (Obermeier *et al*, 1993). Therefore, the short C-terminal tail seems to be a crucial structural element of the TrkA cytoplasmic domain.

The identification of gp140 TrkA as an NGF receptor was made after it was noted that its distribution was restricted to targets of NGF action in neural crest derived sensory neurons (Martin Zanca *et al*, 1989). It was later proved to be a receptor whose tyrosine kinase activity and autophosphorylation is stimulated by the binding of NGF (Kaplan *et al*, 1991b). Tyrosine phosphorylation of TrkA by NGF is rapid, specific and occurs with picomolar quantities of factor, indicating that the response is mediated by physiological amounts of NGF (Kaplan *et al*, 1991b).

In addition to the highly effective receptor-ligand interactions previously mentioned, there is evidence for indiscriminate receptor action. Expression studies in fibroblasts have shown that NT-3 is able to elicit a response via TrkA and TrkB, although it is much less potent than the preferred ligands of these receptors (Klein *et al*,1991; Soppet *et al*, 1991;Squinto *et al*, 1991), and NT-4/5 can elicit a response via TrkA in fibroblasts, although it is less potent than NGF acting at this receptor (Berkemeier *et al*, 1991). In neurons, however, there is

some indirect evidence that the neurotrophin receptors have a higher degree of ligand specificity (Ip *et al*, 1993). The neurotrophin specificities of Trks are illustrated in Figure 1.1.

Detection of TrkA mRNA expression in sympathetic ganglia (Schecterson and Bothwell, 1992) and basal forebrain cholinergic neurons (Holtzman *et al*, 1992) has confirmed that TrkA expression is directly associated with classic targets of NGF. For example, cholinergic neurons of the basal forebrain and of the caudate-putamen are sensitive to NGF. Holtzman and collaborators (1992) have shown that TrkA mRNA expression is highly restricted in its distribution in the adult rat forebrain, that it is present in cholinergic neurons and that NGF up-regulates its receptor expression.

A null mutation in the TrkA gene results in mice with a phenotype very similar to that seen after developmental treatment with an activity-blocking NGF antibodies (Smeyne *et al*, 1994). Newborn animals are insensitive to pain and temperature stimuli and few animals survive for more than a month after birth. Ten days after birth, extensive neuronal cell loss was found in trigeminal and dorsal root ganglia. Presumably cells lost were those responsible for detecting pain and temperature (Smeyne *et al*, 1994). In addition, sympathetic neurons of the peripheral nervous system are virtually absent. In contrast TrkA and hence NGF, does not appear to be required for the survival of basal forebrain cholinergic neurons, although the rate-limiting enzyme for neurotransmitter production, choline acetyltransferase, is reduced in the absence of functional TrkA. This is especially interesting as these neurons normally express TrkA and their target fields express NGF during development (Lu *et al*, 1989). In addition, exogenous NGF can prevent the death of many of these cells following injury in the adult (Martinez *et al*, 1985). Curiously, a decrease in choline

acetyltransferase staining is not observed in cholinergic cells of the striatum that normally also expresses TrkA (Mobley *et al*, 1986).

TrkB

TrkB was initially cloned from an adult mouse brain cDNA library using low stringency screening with a TrkA probe (Klein *et al*, 1989). Alignment of the deduced amino acid sequence of the mouse TrkB gene product with that of human TrkA reveals an overall homology of 69%, a 57% homology in their extracellular regions and a 88% homology within their respective tyrosine kinase domains (Klein *et al*, 1989; Martin-Zanca *et al*,1989).

The TrkB locus exhibits a complex transcriptional pattern generating at least eight different transcripts ranging in size from 0.7 to 9 kb. The existence of transcripts that are too small to encode the full length TrkB product led to the search for additional protein products and the isolation of novel cDNAs encoding two truncated receptors in rats (Middlemas *et al*,1991) and one truncated receptor in mice (Klein *et al*,1990). The full length receptor is called $gp145^{TrkB}$ (glycoprotein of 145 KDa) and is referred to as TrkBTK+. The $gp145^{TrkB}$ is a heavily glycosylated molecule of 821 amino acid residues that contains all the canonical motifs of tyrosine kinase receptors. In the extracellular domain most of the residues that are homologous to TrkA are located within the second and third leucine rich motifs and particularly within the second Ig-like domain (Schneider and Schweiger, 1991). The second class of TrkB receptors are referred to as TrkBTK- and have the same extracellular and transmembrane domain as TrkBTK+,. However, they have a very short cytoplasmic domain and lack the entire kinase catalytic region (Klein *et al*, 1990; Middlemas *et al*, 1991).

So far, two isoforms of non-catalytic TrkBTK- receptor isoforms have been identified. $gp95^{TrkB}$ or TrkB.T1 has been isolated from mouse and rat cDNA libraries and has been shown to be expressed in adult mouse brain at levels comparable to those of the signalling TrkBTK+ receptor (Klein *et al*, 1990). The cytoplasmic sequences of TrkB.T1 or $gp95^{TrkB}$ consist of 23 amino acid residues of which the last 11 are unrelated to the other known sequences (Klein *et al*, 1990). The second non-catalytic TrkBTK- receptor, TrkB.T2, has a 21 amino acid-long cytoplasmic domain, of which the last 9 residues are unrelated to those present in TrkB.T1/$gp95^{TrkB}$ (Middlemas *et al*, 1991). All the different receptor forms appear to be the products of the same gene locus and are derived by alternative mRNA splicing. Different variants which contain deletions in the extracellular domain of the TrkB receptor have been recently discovered (Ninkina *et al*, submitted).

Binding studies have shown that TrkB is the preferred receptor for BDNF and NT4/5 (Berkemeier, 1991; Ip *et al*, 1992) although NT3 also binds to TrkB (Soppet *et al*, 1991).

Northern blotting analysis in adult mouse tissues has shown that TrkB transcripts are most abundant in brain and the transcripts of 9.0 and 5.5 kb are specific for the brain. Other smaller transcripts have been found in lung, heart, kidney and muscle, albeit at lower levels (Klein *et al*, 1989). *In situ* hybridisation analysis of 14 and 18 day old embryos indicates that TrkB transcripts are localised in the central and peripheral nervous system including; brain, spinal cord, spinal and cranial ganglia, paravertebral trunk of the sympathetic nervous system and various innervation pathways (Klein *et al*, 1989).

The phenotypic characteristics of TrkB knockout mice include a significant decrease in the number of both cranial and spinal sensory neurons, as well as a loss of some cranial and spinal motoneurons (Klein *et al*, 1993). These animals die soon after birth. Comparisons of the TrkB knockouts with mice lacking either BDNF or NT4 supports the hypothesis that there are at least two endogenous ligands for the TrkB receptor. The reduction in trigeminal sensory neurons in BDNF knockouts (44% fewer than in wild type animals), is much less than that resulting from a null mutation of the TrkB receptor (60% fewer than wild type). More remarkable is the observation that the significant loss of motoneurons observed in the TrkB knockouts does not occur in either the BDNF (Ernfors *et al*, 1994) or NT4 knockouts (Conover *et al*, 1995). In addition, the survival of many motoneurons in the TrkB knockout mice suggests the involvement of still other trophic agents, that can compensate for the loss of signalling through TrkB (for review, see Snider, 1994).

TrkC

TrkC was initially cloned from a porcine brain cDNA library screened with a probe derived from the human TrkA proto-oncogene (Lamballe *et al*, 1991). The TrkC protein has 67% homology with human TrkA and 68% homology with mouse TrkB and has a molecular weight of 145 KDa. The extracellular domains of TrkA and TrkC are 54% homologous and the extracellular domains of TrkC and TrkB are 53% homologous. The catalytic domain is 87% homologous to that of human TrkA kinase and 88% homologous to that of mouse TrkB kinase. The catalytic kinase regions of TrkC exhibit the characteristic features of TrkA and TrkB tyrosine kinases (Klein *et al*, 1989;

Martin-Zanca *et al*, 1989). In addition, TrkC possess the short carboxyl-terminal tail characteristic of TrkA and TrkB (Barbacid *et al*, 1991).

Tyrosine phosphorylation, cross linking, and ligand binding assays indicate that TrkC receptors interact with NT3 and not with the related neurotrophins NGF, BDNF, NT4 or NT5 (Tsoulfas *et al*, 1993).

Screening an adult rat cDNA library has allowed novel forms of TrkC containing kinase inserts to be cloned (Valenzuela *et al*, 1993). The kinase inserts, either 14 amino acids or 39 amino acids, are located at the same position within the tyrosine kinase domain, between subdomains VII and VIII. The 14 amino acid insert is included within the 39 amino acid insert. Tsoulfas *et al* (1993) also report the cloning of four different forms of rat TrkC; a full length one, two different isoforms with either a 14 or 25 amino acid insertion in the tyrosine kinase domain and a truncated receptor that lacks the cytoplasmic catalytic domain.

In transfected NIH3T3 fibroblast, both the full length TrkC isoform and the isoform containing the 14 amino acids insertion are phosphorylated on tyrosine residues upon binding NT3, but not BDNF or NGF. The time course of phosphorylation and the NT3 dose response are similar for both isoforms. Chemical cross-linking experiments show that the full length form of TrkC and the isoform with the 14 amino acids insertion both bind NT3 at physiologically relevant concentrations, but respond differently to NT3 when transfected into PC12 cells. The 14 aminoacid insertion renders the TrkC receptor inactive in neurite outgrowth in PC12 cells even though NT3 can stimulate tyrosine phosphorylation in these cells (Tsoulfas *et al*, 1993). The 39 amino acid form is also unable to transduce a signal leading to neurite outgrowth in PC12 cells

(Valenzuela, 1993). The binding of NT3 to full length TrkC results in proliferation of transfected NIH3T3 fibroblasts. However, neither the 14 or 39 amino acids kinase insert forms of TrkC can mediate fibroblast proliferation in response to NT3 (Valenzuela *et al*, 1993; Tsoulfas *et al*, 1993).

Different isoforms of TrkC have also been found in mouse. Isoforms exist that contain insertions, of either 14 or 25 amino acid residues, in a similar position to those in rat TrkC (Lamballe *et al*, 1993). All three forms become rapidly phosphorylated on tyrosine residues upon interaction with the cognate ligand NT3, but, in the same way as rat TrkC, only the full length isoform without any insertion is able to induce neuronal differentiation in PC12 cells and has mitogenic activity in NIH3T3 cells. These TrkC isoforms may act through different signalling pathways (Lamballe *et al*, 1993).

A full length and a truncated form of chicken TrkC, lacking the tyrosine kinase domain, have also been isolated by screening a cDNA library with a cDNA probe corresponding to the tyrosine kinase domain of porcine TrkC (Okazawa *et al*, 1993). More recently, Garner and Large (1994) have isolated nine different isoforms of chicken TrkC generated by alternative splicing. They have identified TrkC receptors with two different extracellular and four different cytoplasmic domains. The different cytoplasmic domains are; a full length tyrosine kinase motif (FL), a kinase truncation (KT), kinase deletion (KD) and kinase insertion (KI) motif. Like the rat homologue, the avian KD motif replaces the kinase domain of the FL receptor with 39 amino acids, 36 of which are conserved between chicken and rat. In contrast with deletion of the entire kinase domain, a single clone exhibited a KT motif in conjunction with a full length extracellular motif. In contrast with the alternative splicing that deletes regions of the kinase domain, a KI motif adds 25 aminoacid after the double tyrosine

residues (711 and 712) that have been shown to be important regulators of TrkA function (Mitra *et al*, 1991). Only 13 of these aminoacid residues are identical to the 25 amino acid insertion identified in rat (Tsoulfas *et al*, 1993) and mouse TrkC (Lamballe *et al*, 1993).

TrkC is widely expressed in the embryonic and adult central and peripheral nervous system (Lamballe *et al*, 1991; 1993; Ernfors *et al*, 1992; Merlio *et al*, 1992). TrkC is also expressed in certain ganglia of the autonomic enteric nervous system (Lamballe *et al*, 1993). During embryonic development, TrkC expression correlates, both temporally and spatially, with the outgrowth of axons towards their peripheral targets. In the adult mouse, TrkC mRNA is heterogeneously distributed throughout the brain, with highest levels of expression in limbic and diencephalic structures. TrkC mRNA is also expressed in certain non-neuronal tissues such as the mesenchymal cells of arterial walls, the multilocular adipose tissue and the acini of the submaxillary gland (Lamballe *et al*, 1993) *In situ* hybridisation studies have revealed TrkC transcripts in the hippocampus, cerebral cortex and the granular layer of the cerebellum of developing rats (Lamballe *et al*, 1991). TrkC mRNA is highly expressed in the embryonic neural tube in early stages of development (E2) and has its expression in the spinal cord restricted in later stages of development (E4-E5) (Averbuch-Heller *et al*, 1994).

Almost all neural tissues express transcripts encoding for both full-length and truncated TrkC forms. The retina is the only neural region that expresses relatively high levels of transcripts encoding for the full length forms as compared with the truncated forms. The sciatic nerve is unique among neural tissues in that it only expresses transcripts encoding the truncated forms of TrkC. In this same study Valenzuela and colleagues (1993) reported that isolated

neuronal cells from hippocampus express transcripts encoding both full length and truncated forms of TrkC, whereas astrocytes purified from the same region express transcripts only for the truncated forms of TrkC .The two different mouse TrkC isoforms (insertion of 14 or 25 amino acids) have been identified in various structures of the adult murine brain. This suggests that the trophic activities of NT3 might be mediated by different TrkC receptor isoforms (Lamballe, 1993).

When the chicken full length and the kinase insert isoforms of TrkC are transfected into PC12 cells, they are able to stimulate process outgrowth in response to NT3 . However, unlike the full length TrkC isoform, the isoform with the 25 amino acid insertion is unable to support the survival of these cells after 4-5 days in culture.

A TrkC null mutation results in very specific sensory losses and death of the mice within several weeks after birth (Klein *et al*, 1994). Newborn animals exhibit proprioceptive (awareness of limb position) disorders and a significant reduction in spinal sensory cells innervating muscle spindles afferents, but no loss of motoneurons. These animals lack Ia muscle afferent projections to spinal motor neurons and have fewer large myelinated axons in the dorsal root and posterior columns of the spinal cord. This defect may be the cause of the behavioural deficits found in these homozygous mice. TrkC null mutants appear normal at birth but by postnatal day 4 they are reduced in size and most of them die by postnatal day 21(Klein *et al*, 1994). In accordance with the observed deficits in proprioception, they have 19% neuronal loss in dorsal root ganglia that corresponds to the subpopulation of dorsal root ganglia known to express TrkC transcripts (Carrol *et al*, 1992; Mu *et al*, 1993).

1.2.6. Other Neurotrophic Factors

Several proteins that are not structurally related to the neurotrophins are also able to support the survival of particular kinds of neurons *in vitro*. These include ciliary neurotrophic factor (CNTF) (Sendtner *et al*, 1994), basic fibroblast growth factor (bFGF) (Eckenstein, 1994), leukaemia inhibitory factor (LIF) (Murphy *et al*, 1993) and glial cell line derived neurotrophic factor (GDNF) (Lin *et al*, 1993).

CNTF, GPA, IL-6, FGF, LIF

CNTF was initially identified based on its ability to sustain the survival of embryonic chicken ciliary neurons (Adler *et al*, 1979; Manthorpe *et al*, 1985; Manthorpe and Varon, 1985; Barbin *et al*, 1984; Manthorpe *et al*, 1986, Lin *et al*, 1989; Stockli *et al*, 1989). CNTF is an acidic protein with an estimated molecular mass of 20-24 kDa that acts as a survival factor for certain neurons like chicken sympathetic and sensory neurons at E10 (but not at E8) (Manthorpe *et al*, 1986). A physiological role for CNTF in maintaining the function of normal adult motor neurons is supported by recent observations of motor-neuron degeneration in mice where the expression of the CNTF gene was abolished by homologous recombination (Masu *et al*, 1993).

The receptor for CNTF seems to be a heterotrimer of one membrane-linked ligand-binding subunit and two transmembranous signal transducing subunits (Davis *et al*, 1991; Ip *et al*, 1992). Ciliary neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF) and interleukin-6 (IL-6) compose a family of distantly related cytokines (Bazan, 1991; Rose and Bruce 1991) that initiate signalling by inducing either homodimerization of the ß signal transducing

receptor component gp130 (in the case of IL6) or heterodimerization between gp130 and the gp130-related LIFRß (in the case of CNTF, LIF and OSM) (Murakami *et al*, 1993; Davis and Yancopoulos, 1993). CNTF also requires a third receptor component (CNTFR alpha) that is mostly restricted to the nervous system in its expression. In fact, CNTFR alpha is a required receptor component that uniquely characterises CNTF-responding cells (Ip *et al*, 1993)

An additional member of this cytokine group has been isolated. This 20 kDa protein, termed GPA (growth promoting activity) is 50% homologous to mammalian CNTF and has similar biological activities (Leung *et al*, 1992).

Leukaemia inhibitory factor (LIF) is a pleiotropic molecule with a multitude of effects on neurons and non-neuronal cells. It can produce blockage or enhancement of differentiation or proliferation, depending on the responsive cell population (Smith *et al*, 1992). In addition to promoting sensory neuron survival, there is some evidence that LIF is able to influence neurotransmitter and neuropeptide expression in these neurons (Murphy *et al*, 1994).

Another important growth factor is fibroblast growth factor (FGF) which can promote axonal regeneration. FGFs have been characterised by virtue of their mitogenic activities for a variety of cell types of mesodermal and ectodermal origin (Rifkin and Moscatelli, 1989; Vlodavsky *et al*, 1991). Basic FGF (bFGF) enhances differentiation of sympathoadrenal precursor cells and PC12 cells (Ip *et al*, 1994) and is a potent mitogen for certain CNS populations (Gensburger *et al*, 1987; Cattaneo and McKay, 1990; Ray *et al*, 1993; Ray and Gage, 1994; Kilpatrick and Bartlet, 1993). Four high-affinity receptors for the FGF family have been cloned, forming a family of transmembrane proteins with ligand-

activated tyrosine kinase activity (Klagsbrun and Baird, 1991; Yarden and Kelman, 1991).

GDNF, TGFß

Glial cell line derived neurotrophic factor (GDNF) is a newly discovered distantly related member of the transforming growth factor ß superfamily. It exhibits neurotrophic effects on midbrain dopaminergic neurons both *in vivo* and *in vitro*. GDNF was originally isolated from the B49 glial cell line (Lin *et al* 1993). It was found to be expressed in the developing limb bud, suggesting that it has a target- derived role in motor neuron development (Henderson *et al.*, 1994; Oppenheim *et al.*,1995; Yan *et al.*, 1994). It also protects midbrain dopaminergic neurons from MPTP toxicity (Tomac *et al.*, 1994) and axotomy-induced degeneration (Beck *et al.*, 1995). It has also been demonstrated that GDNF is able to support *in vitro* survival of sympathetic, parasympathetic, proprioceptive, enteroceptive and cutaneous sensory neurons of chicken embryos at different stages of their development (Buj-Bello *et al.*, 1995).

Transforming growth factor ß (TGFß) is the prototype of a family of polypeptide growth factors which were originally isolated and purified from several transformed cell lines as well as non-neoplastic tissues. TGFß is a 25 kD homodimer with multiple activities including roles in embryogenesis and in wound-healing responses (reviewed by Roberts and Sporn, 1990). TGFß has been shown to have a neurotrophic effect on sensory neurons *in vitro* and to be synergistic with NGF (Chalazonitis *et al*, 1992). A balanced interaction between neuronal and accessory cells during repair might be maintained via TGF-ß, since

this cytokine promotes Schwann-cell growth and activity (Perry and Brown, 1992).

OBJECTIVES OF THIS STUDY

I) To investigate the responsiveness of various chicken sensory neurons (placode and neural-crest derived) to neurotrophins during embryonic development *in vitro*

II) To analyse cell death in developmental mouse trigeminal neurons in wild type embryos and in embryos that are homozygous for null mutations in the TrkA, TrkB and TrkC genes.

III) To study neurotrophin discrimination among chicken sensory neurons *in vitro*

IV) To study the mRNA expression and regulation of the full length high affinity receptor for NT3 (TrkC) in chicken sensory and sympathetic neurons during development both *in vivo* and *in vitro*

Chapter 2

THE SURVIVAL OF CHICK NEURAL CREST DERIVED BUT NOT PLACODE DERIVED NEURONS IS PROMOTED BY SEVERAL DIFFERENT NEUROTROPHINS EARLY IN THEIR DEVELOPMENT.

2.1. INTRODUCTION

As mentioned in the previous chapter, the peripheral nervous system is derived from precursors in the neural crest and placodes. After precursor cell migration and aggregation to form primitive ganglia, a defined number of neurons is generated by proliferation and differentiation of these precursors. With the exception of factors controlling neuronal death, most of the signals involved in the interactions between developing precursors cells and their surrounding environment are still unknown (Snider, 1994). The differentiated neurons extend processes to their targets fields which are known to produce limited amounts of trophic factors. Only a proportion of the neurons are able to obtain enough of these factors to survive (Davies *et al*, 1994a)

A family of homologous proteins termed neurotrophins, which have been already described and characterised in Chapter 1, are well recognised for promoting the survival of neurons in the developing vertebrate nervous system. The available evidence suggests that each neurotrophin promotes the survival of a particular set of developing neurons. For example, whereas NGF supports

sympathetic neurons (Greene, 1977), neural crest-derived cutaneous sensory neurons (Davies and Lindsay, 1985) and basal forebrain cholinergic neurons (Hatanaka *et al* , 1988), BDNF supports proprioceptive, enteroceptive and placode-derived cutaneous sensory neurons (Davies *et al.*, 1986a, 1986b) and the dopaminergic neurons of the substantia nigra (Hyman *et al.*, 1991). NT3 promotes the survival of proprioceptive neurons (Hohn *et al*, 1990).

Cranial sensory neurons have been especially useful for studying the specificity and time-course of the survival-promoting effects of neurotrophins (Davies, 1987). These neurons can be obtained for experimental studies from the earliest stages of their development. In contrast to the functional heterogeneity of the sensory neurons of DRG, cranial sensory neurons are segregated into functionally distinct populations. Perhaps as a consequence of this functional segregation, different populations of cranial sensory neurons have distinct neurotrophin requirements when cultured during the period of naturally occurring cell death. For example, the small-diameter cutaneous sensory neurons of the jugular ganglion and the dorsomedial part of the trigeminal ganglion (DMTG) are neural crest derived and are supported by NGF (Davies and Lumsden, 1983; 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 placode derived and are supported by BDNF (Davies *et al.*, 1986b) but show little response to NGF (Davies and Lumsden, 1983) or NT-3 (Hohn *et al.*, 1990). The proprioceptive neurons of the trigeminal mesencephalic nucleus (TMN) are neural crest derived and 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 are placode derived and contain a major subpopulation of BDNF-dependent neurons and a minor subpopulation of

NT-3-dependent neurons (Lindsay *et al.*, 1985; Davies *et al.*, 1986b; Hohn *et al.*, 1990) but are unresponsive to NGF (Lindsay and Rohrer, 1985).

Developmental studies have shown that cranial sensory neurons survive independently of neurotrophins when their axons are growing to their targets and acquire neurotrophin dependence close to the time when their axons reach their targets (Davies and Lumsden, 1984; Vogel and Davies, 1991). In the embryonic mouse trigeminal system, where NGF responsive neurons innervate the facial region (Davies and Lumsden, 1984), the onset of NGF production and NGF receptor expression (gp75, Wyatt *et al*, 1990 and TrkA, Wyatt and Davies, 1993) both appeared to be related to the arrival of sensory axons in the target field (Davies, 1987 a). Trigeminal neurons do not express detectable levels of NGF receptors and are not dependent on NGF for survival when peripheral axons are growing to their targets.

However, in a recent *in vitro* study of the survival-promoting effects of different neurotrophins on embryonic mouse trigeminal neurons, a survival response to BDNF and NT-3 switches to responsiveness to NGF during the early stages of target field innervation (Buchman and Davies, 1993). When trigeminal axons come into proximity with their peripheral targets, the neurons become responsive to BDNF and NT-3. Shortly afterwards, the neurons become additionally responsive to NGF. With the onset of cell death in the trigeminal ganglion, the neurons become refractory to BDNF and NT-3 but remain dependent on NGF for survival throughout the phase of naturally occurring cell death (Buchman and Davies, 1993). The present study was undertaken to ascertain if responsiveness to multiple neurotrophins is a universal feature of the early stages of neuronal development and to provide information on how early neurons selectively lose responsiveness to certain neurotrophins by studying the

survival requirements of several different populations of chicken cranial sensory neurons at stages throughout their early development *in vitro*.

2.2. MATERIALS AND METHODS

Chicken Cranial Sensory Neurons

To obtain chicken embryos, fertile White Legorn chicken eggs were incubated in a forced-draft, humidified incubator at 38°C for the required time. To remove embryos from eggs, the egg was held with blunt end uppermost (where the airspace is located). This was swabbed with 70% alcohol and allowed to dry. The shell was cracked in a line around the airspace by tapping it with forceps after which this portion of the shell and the membrane lining the airspace was removed. The embryo together with its adherent membranes was removed with a pair of curved forceps.

The embryos were then washed in a Petri dish containing L15 medium and all membranes were removed with pairs of watchmaker's forceps. The embryos were transferred to a 60 mm Petri dish containing L15 medium. To facilitate visualisation of early ganglia within the partially dissected embryonic head the dissection was carried out on a black background with illumination from above. The contrast was further improved by placing a small volume of liquid between the Petri dish and the underlying black background. All dissections and preparation of neuronal cultures were carried out in the laminar flow hood using

standard sterile procedures. For the final stages of dissection a stereo microscope with zoom lens was used. Using electrolytically sharpened tungsten needles, two transverse cuts were performed to isolate the part of the developing head that contains the trigeminal, geniculate, vestibular, petrosal and jugular ganglia (Fig.2.1). The trigeminal, jugular and nodose ganglia were dissected from chicken embryos of, 5, 6, 8, 10 and 12 days incubation: stages 24, 29, 34, 36 and 38, respectively (Hamburger and Hamilton, 1951). The trigeminal ganglion was sub-dissected (Davies and Lumsden, 1983) into its neural crest-derived dorsomedial pole (DMTG) and placode-derived ventrolateral pole (VLTG) (Fig 2.2) (Davies, 1986). The placode-derived nodose ganglia were dissected from the neck by teasing the tissue apart with two fine forceps where the nodose ganglia were identified by the attached vagus nerve (Fig. 2.3).

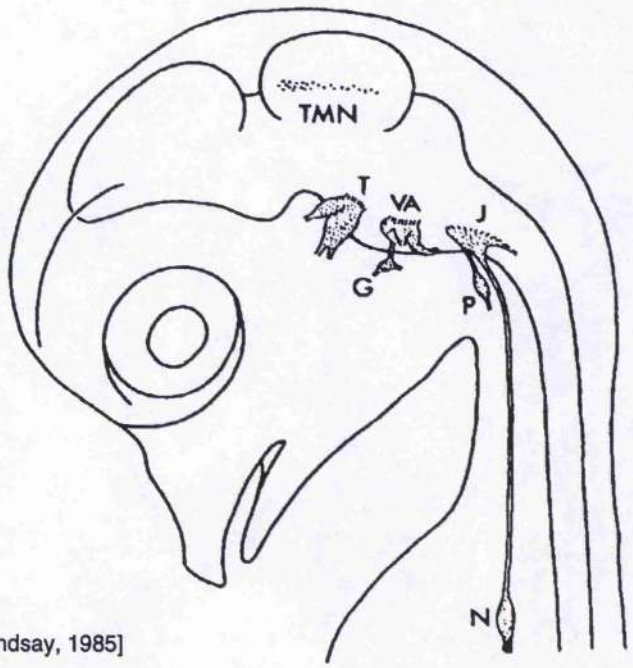


Figure 2.1. Schematic illustration of an E10 chick embryo showing the locations of populations of cranial sensory neurons. TMN: trigeminal mesencephalic nucleus; T: trigeminal ganglion; J: jugular ganglion; N: nodose ganglion; VA: vestibular acoustic ganglion; G: geniculate ganglion; P: petrosal ganglion.

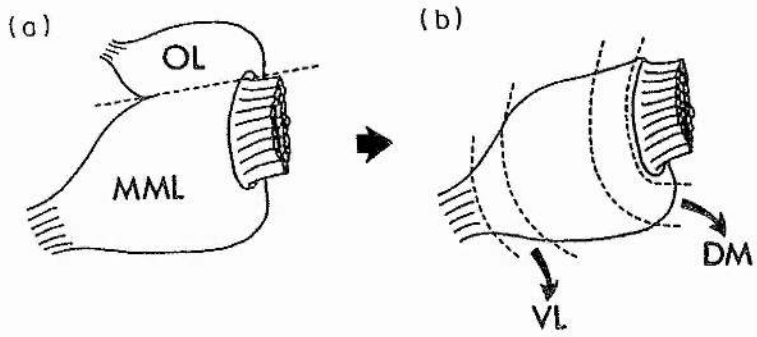


Figure 2.2. Subdissection of the trigeminal ganglion to obtain dorsomedial and ventrolateral neurons. The ophthalmic lobe (OL) is separated from the maxillomandibular lobe (MML) and then the dorsomedial (DM) and ventrolateral (VL) parts are subdissected (From Davies, 1988)

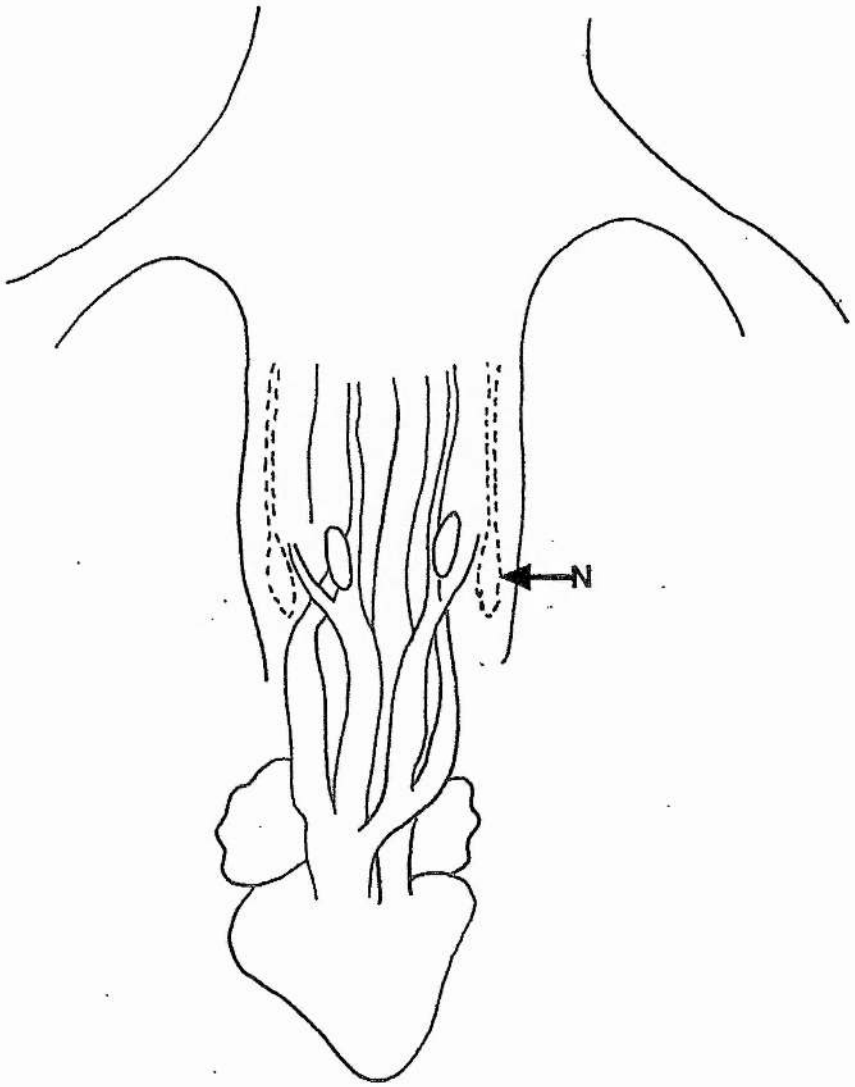


Figure 2.3. Ventral aspect of the thoracic region of an E10 chicken embryo after removal of the skin and exposure of the heart and great vessels. The connective tissue lying either side of the great vessels contains the two nodose ganglia (N) attached to the vagus nerve (Shown by interrupted lines (From Davies, 1995).

Culture Procedure

a) Dissociation of the tissue

The dissected ganglia were incubated in 0.1% trypsin (Worthington) in Ca^{2+}- Mg^{2+} free Hanks balanced salt solution (HBSS) for 10 min for E5 and E6 ganglia, 12 min for E10 ganglia and 20 min for E12 ganglia. After the trypsinization, most of the trypsin solution was removed with a Pasteur pipette and the remaining trypsin was blocked by addition of Ham's F12 medium containing 10% heat-inactivated horse serum (HIHS). The tissue was mechanically dissociated by gentle trituration in a fired-polished Pasteur pipette.

b) Separation of neurons from non-neuronal cells

The neurones were separated from the non neuronal cells by differential sedimentation. This technique depends on differences in sedimentation rates of cells in liquid medium, larger cells sedimenting faster than smaller cells. Thus, neurones sediment more quickly than non-neuronal cells. This technique is applicable to all populations of neurones in the peripheral nervous system and it is very efficient (Davies, 1986). However, it is only effective after stages when there is a clear difference in size between neurons and non-neuronal cells exists. In the present study, this technique was used for E8 and older ganglia. About 60% of the neurones from the starting tissue were recovered uncontaminated by non neuronal cells and the neuronal viability was very high. An autoclaved dropping funnel was filled with 60 ml of Ham's F14 medium plus 10% of HIHS and was placed on an anti-vibration table in a cooling incubator at 2°C overnight

before the dissections were carried out. The dissociated cell suspension (about 2 ml using HBBS or PBS) was layered on the medium inside the dropping funnel. After one hour, fractions of about 5 ml were collected. To determine which fractions contain only neurones, 0.5 ml samples of these fractions were placed in a 24 multi-well plate and were examined with phase contrast microscopy.

c) Culture substrate

The pure neurone samples were plated in 35mm plastic Petri dishes previously coated with poly-ornithine and laminin (Edgard *et al*, 1984). To coat the dishes, 1 ml of poly-ornithine solution (0.5 mg/ml of poly-ornithine in a 0.15 M borate buffer, pH 8.7) was placed in each dish and left overnight at room temperature. The poly-ornithine solution was aspirated the following morning and the dishes were washed three times with sterile water to remove any trace of borate buffer. The dishes were air dried in a laminar flow hood and 120µl of laminin solution (20µg/ml in PBS or Ham's F14) was placed in the centre of each dish and spread over about two-thirds of the dish surface using a plastic pipette tip. The dishes were then placed in a humidified culture incubator at 37°C for at least 4h. After that time, the dishes were removed from the incubator and washed twice with culture medium (Ham's F14 plus 10% HIHS). Finally 1 ml of culture medium was placed in the dishes prior to the addition of 1ml of neuronal suspension. Pure neuronal suspensions from E8 and older ganglia and the dissociated cell suspensions from E5 or E6 ganglia (at which age differential sedimentation is not very effective in removing non-neuronal cells) were plated at very low density in 35 mm tissue culture dishes (250 to 500 per dish) To perform a dose response to neurotrophic factor, a suitable range of concentrations

of this factor was set up in 1ml ofculture medium at double the required final concentrations. Subsequently 1 ml of cell suspension was added to the dishes.

d) Assessing survival

To assess neuronal survival, the number of attached neurons within a standard 12 X 12 mm grid placed under the centre of dishes was counted 6 hours after plating. The number of surviving, process-bearing neurons (the process being defined as an outgrowth three times longer than its cell body) was subsequently counted using the same standard grid 48 hours after plating. By that time almost all neurons had degenerated in control cultures (those containing no neurotrophic factor). The number of neurons surviving after 48 hours was expressed as a percentage of the initial number of neurons counted at 6 hours. In each experiment, control and neurotrophin-supplemented cultures were set up in triplicate. Each experiment was repeated at least twice. The same procedure was used to assess the proportion of neurons surviving in cultures set up in 4-well dishes, except that the entire surface of the wells was examined for neurons.

To ascertain wether the increased number of neurons observed in early ganglion cultures in the presence of neurotrophins was due to a direct survival response of neurons (as opposed to the differentiation of neuron progenitor cells present in these cultures), the fate of individual neurons in low density dissociated cultures was followed (Vogel and Davies, 1991). This was done by recording the location 6 hours after plating of unambiguously identifiable bipolar neurons within a 9 x 9 mm grid scored on the under surface of poly-ornithine/laminin-coated 60 mm culture dishes and monitoring at 12 hourly intervals until 48 hours if each of these neurons was surviving or had

degenerated. To avoid any possible effects of neurotrophins on the size of the initial cohorts, these factors were added after the identification of cohorts at 12 hours.

For experiments in which very high concentrations of neurotrophins were used, the neurons were grown in the 11 mm diameter wells of 4-well dishes (Greiner) that had been pre-coated with polyornithine and laminin. This permitted the use of only 100 µl of culture medium in each well. Purified NGF (gift of W. Mobley) and purified recombinant BDNF and NT-3 (gifts of Gene Burton and John Winslow, Genentech Inc.) were added to these cultures either alone or in combination. In the 4 well dishes, the whole surface of each well was counted. All data are presented as mean ± SEM unless otherwise stated.

2.3. RESULTS

Differences and developmental changes in the response of cranial sensory neurons to neurotrophins

By 48 hours incubation, the great majority and often all the neurons in control cultures of DMTG, jugular, VLTG and nodose neurons had degenerated (Fig.2.4). This permitted the survival response of these neurons to NGF, BDNF and NT-3 to be clearly observed at this time. The graphs in figure 2.5. show the magnitude and age-related changes in the survival-promoting effects of neurotrophins on these neurons from E6 to E12.

The bar charts in figure 2.6 compare the survival of neurons grown with each neurotrophin alone with the survival of neurons grown in the presence of different combinations of neurotrophins. Where two neurotrophins promote the survival of neurons in a given population, comparison of the numbers of neurons surviving in the presence of each neurotrophin alone with the number surviving in the presence of both neurotrophins will reveal whether the neurotrophins support the same or different subsets of neurons in the population.

(i) DMTG neurons

Figures 2.5 and 2.6 shows that the survival of the majority of DMTG neurons was promoted by NGF throughout the E6 to E12 age range. In addition, at E6, almost as many neurons were supported by either BDNF or NT-3. This

early response to BDNF and NT-3 was, however, short-lived. There was a marked fall in the number of neurons surviving in the presence of BDNF or NT-3 between E6 and E8, and by E12 less than 5% of the neurons were supported by these factors, compared with over 60% supported by NGF.

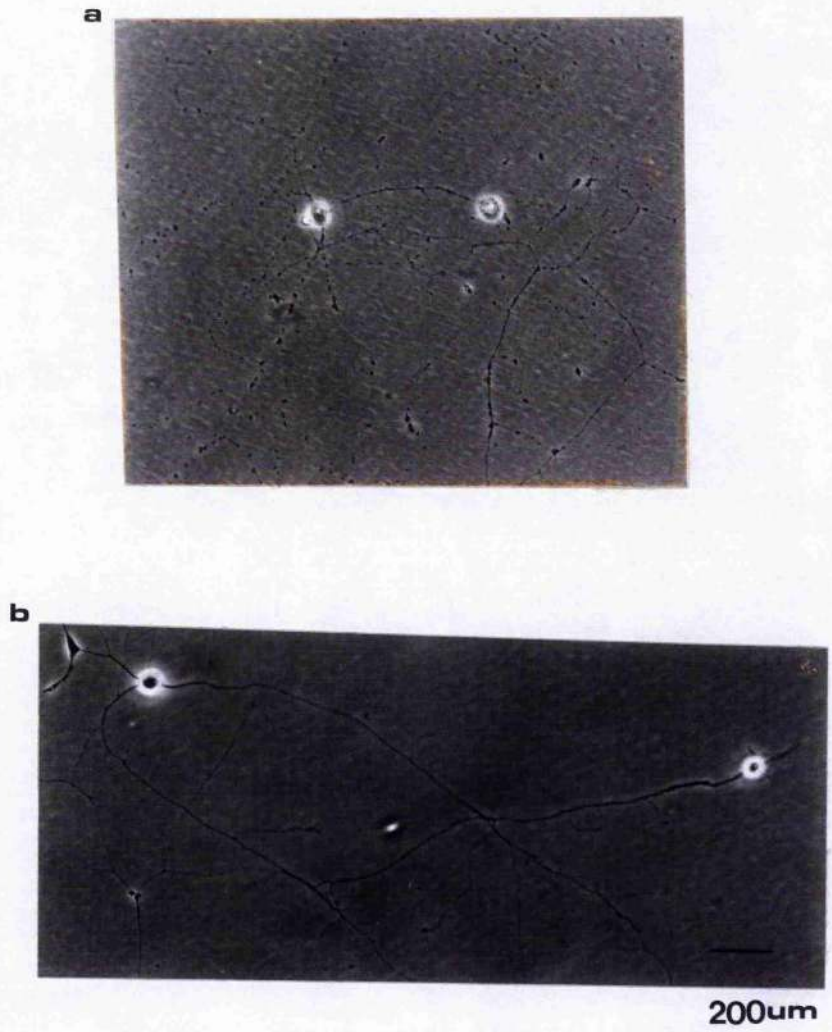


Figure 2.4. Phase contrast micrograph of E10 DMTG neurons after 48h in control culture (a) or in media supplemented with NGF (2ng/ml) (b).

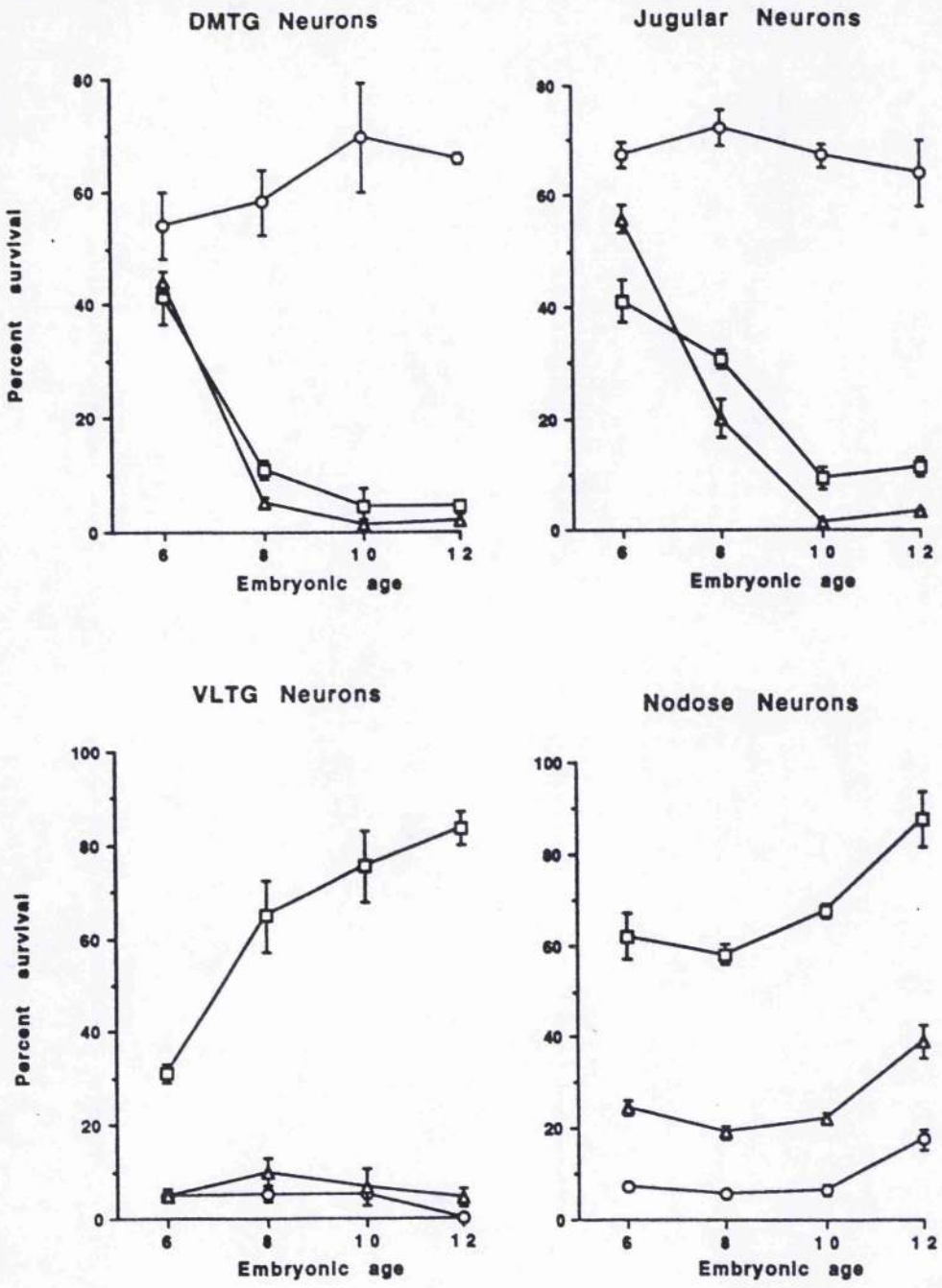


Figure 2.5. Age-related changes in the survival responses of cranial sensory neurons to neurotrophins. Graphs showing the percent survival of DMTG, Jugular, VLTG and Nodose neurons from E6 to E12 embryos after 48 hours incubation with 2ng/ml of NGF (circles), BDNF (squares) or NT3 (triangles). The mean +/- the standard error of the mean are shown (n=3). Stardard error of the mean is the standard deviation divided by the square root of n.
Control data for each type of neurons are presented in Figures 2.6-2.10.

Additionally if E6 DMTG neurons were grown in the presence of NGF plus BDNF or NGF plus NT-3, there were very few additional surviving neurons compared with cultures containing NGF alone. (Fig 2.6) Likewise, there was negligible additional neuronal survival in cultures containing BDNF plus NT-3 compared with cultures containing either of these neurotrophins alone. These results indicate that there are not separate subpopulations of neurons that respond each neurotrophin at E6, rather, the majority of E6 neurons respond to all three neurotrophins.

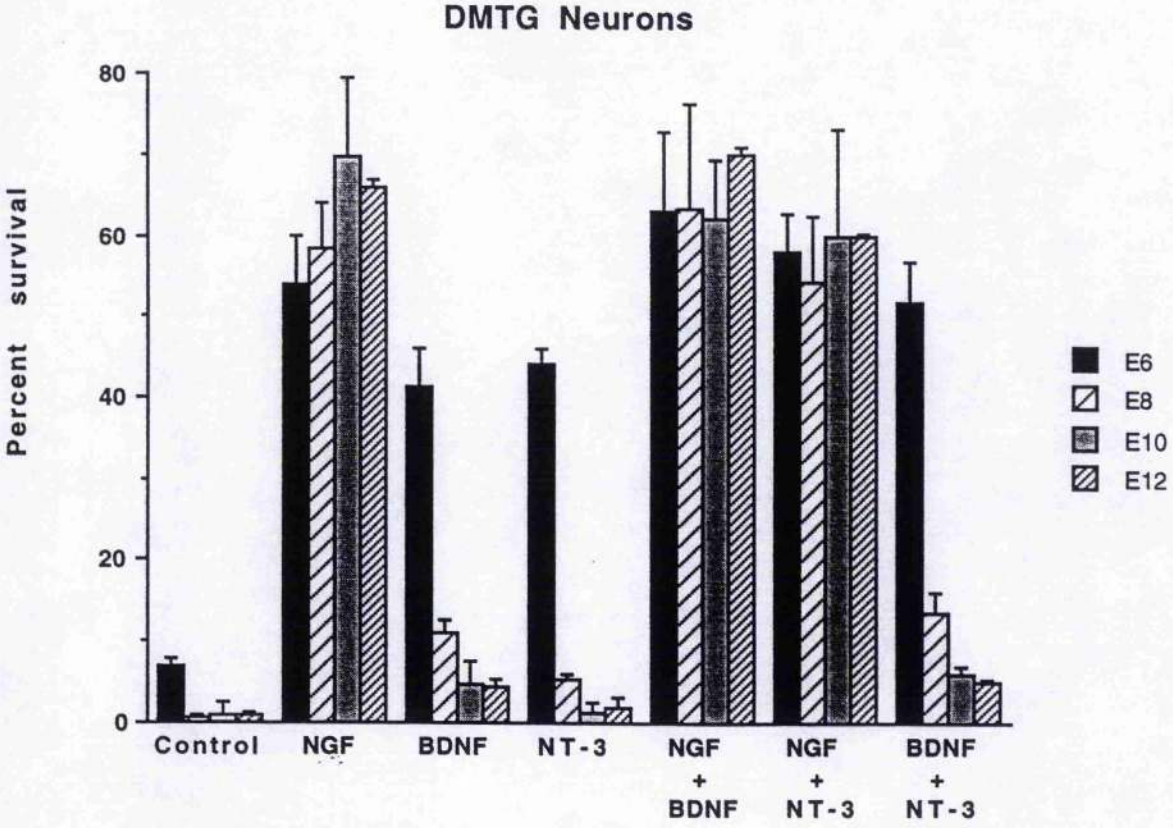


Figure 2.6. Survival responses of DMTG neurons to neurotrophins alone and in combination. Bar chart showing the percent survival of DMTG neurons from E6 (black bars), E8 (light hatched bars), E10 (stippled bars) and E12 (dark hatched bars) embryos after 48h incubation with 2ng/ml of NGF, BDNF, NT3, NGF+BDNF, NGF+NT3 or BDNF+NT3. The mean ± the standard error are shown (n=3).

(ii) Jugular neurons

As with DMTG neurons, the survival of the majority of jugular neurons was promoted by NGF at all ages. Jugular neurons also showed a marked, transitory survival response to BDNF and NT-3 at E6. The number of neurons surviving in the presence of BDNF or NT-3 decreased markedly at later ages, reaching 10% or less by E10 (Fig. 2.7).

There was negligible additional neuronal survival in E6 cultures containing NGF plus BDNF or NGF plus NT-3 compared with cultures containing NGF alone, indicating that at this early stage the majority of neurons supported by NGF also respond to BDNF and NT-3. Likewise, there was negligible additional neuronal survival in E6 cultures containing BDNF plus NT-3 compared with cultures containing either neurotrophin alone (Fig. 2.7).

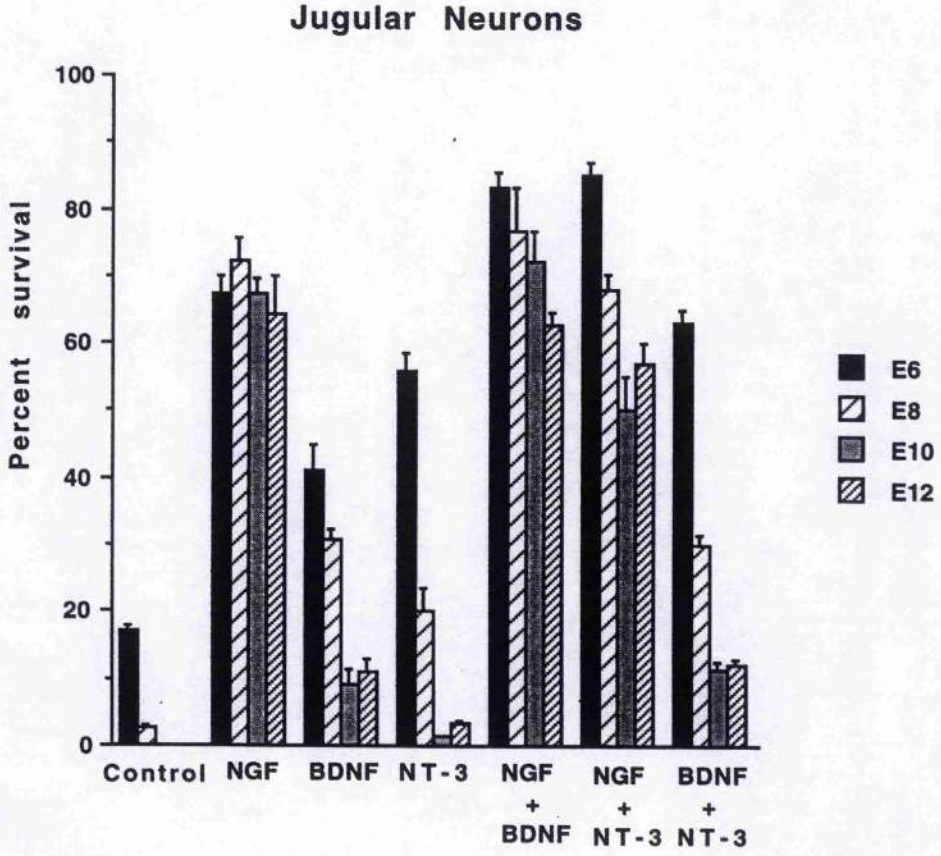


Figure 2.7. Survival responses of chicken Jugular neurons to neurotrophins alone and in combination. Bar chart showing the percent of survival of Jugular neurons from E6 (stippled bars) and E12 (dark hatched bars) embryos after 48 hours of incubation with 2ng/ml of NGF, BDNF, NT3, NGF+BDNF or BDNF+NT3. The mean ± the standard errors are shown (n=3).

(iii) VLTG neurons

At all ages, the survival of VLTG neurons was supported by BDNF. This response to BDNF increased from E6 to E8, after which it remained in the region of 70 to 80% survival. In marked contrast to the early response of DMTG and jugular neurons to all three neurotrophins, only a small percentage of VLTG neurons were supported by NGF or NT-3 (less than 10% at any age) and there were no age-related changes in this response (Fig.2.5). The percentage survival of VLTG neurons in the presence of BDNF was not significantly changed by the addition of either NGF or NT-3 to the culture medium (Fig. 2.8).

Because many VLTG neurons are generated before DMTG neurons (D'Amico-Martel and Noden, 1980), it is possible that VLTG neurons may have passed through a period of responsiveness to multiple neurotrophins before E6, the age at which DMTG neurons respond to NGF, BDNF and NT-3. To exclude this possibility, VLTG neurons were cultured with different neurotrophins at E5 (Fig. 2.9). After 48 hours incubation $9.0 \pm 0.8\%$ of the neurons were surviving in control cultures and there were no significant differences in the number of neurons surviving in the presence of either NGF ($8.5 \pm 0\%$) or NT-3 ($13.0 \pm 5.9\%$). There was, however, a clear survival response to BDNF ($63.4 \pm 2.5\%$). Thus, in contrast to early DMTG neurons, VLTG neurons at an equivalent early stage in their development do not exhibit responsiveness to multiple neurotrophins.

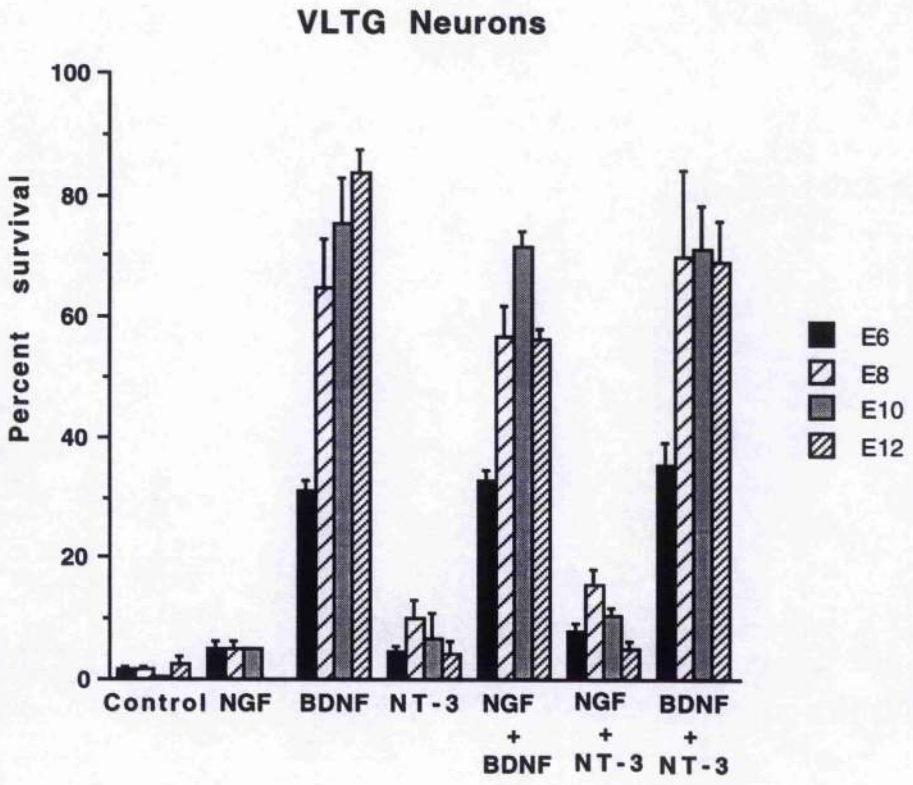


Figure 2.8. Survival responses of VLTG neurons to neurotrophins alone and in combination. Bar chart showing the percent survival of VLTG neurons from E6 (black bars), E8 (light hatched bars), E10 (stippled bars) and E12 (dark hatched bars) embryos after 48 hours incubation with 2ng/ml of NGF, BDNF, NT3, NGF+BDNF, NGF+NT3 or BDNF+NT3. The mean ± the standard error are shown (n=3)

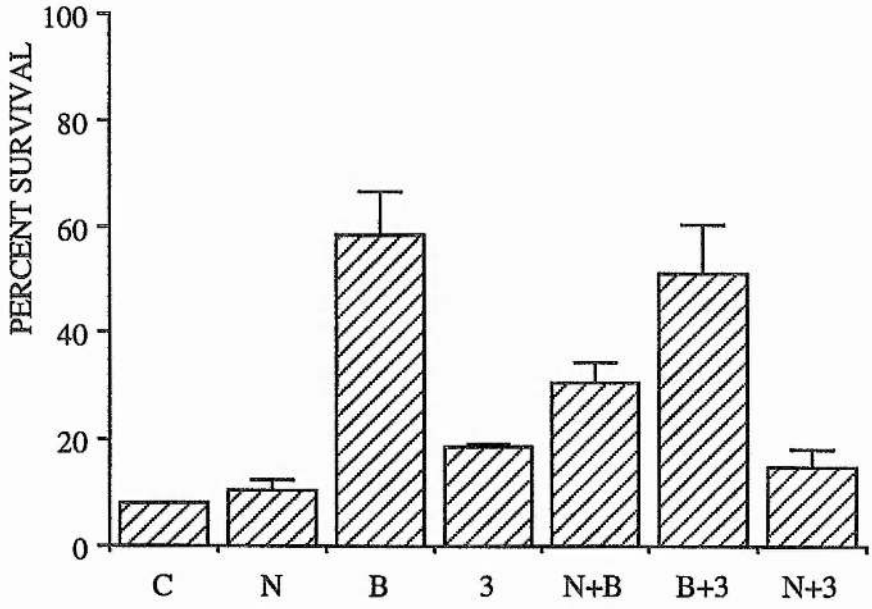


Figure 2.9. Survival responses of E5 VLTG neurons to neurotrophins alone and in combination. Bar chart showing the percent survival after 48h in culture. C: control; N: NGF 2ng/ml; B: BDNF 2ng/ml; 3: NT3 2ng/ml; N+B: NGF+BDNF; B+3: BDNF+NT3; N+3: NGF+NT3. The mean ± standard error are shown (n=3).

(iv) Nodose neurons

Throughout the period studied, the survival of nodose neurons was supported by BDNF and NT-3. Of the two, BDNF was more effective (60% to over 80% of the neurons surviving with this factor compared with 20 to 40% with NT-3). At all ages, the numbers of neurons surviving in the presence of NGF was not significantly greater than the numbers of neurons in control cultures (Fig.2.10). Almost all neurons survived in the presence of both BDNF and NT-3 (Fig. 2.10), indicating that the nodose ganglion comprises two partially overlapping populations of BDNF-responsive and NT-3-responsive neurons. These neurons are responsive to BDNF and NT3 even earlier in development, E5 (Fig 2.11). The survival response to NT3 decreases from E5 to E6.

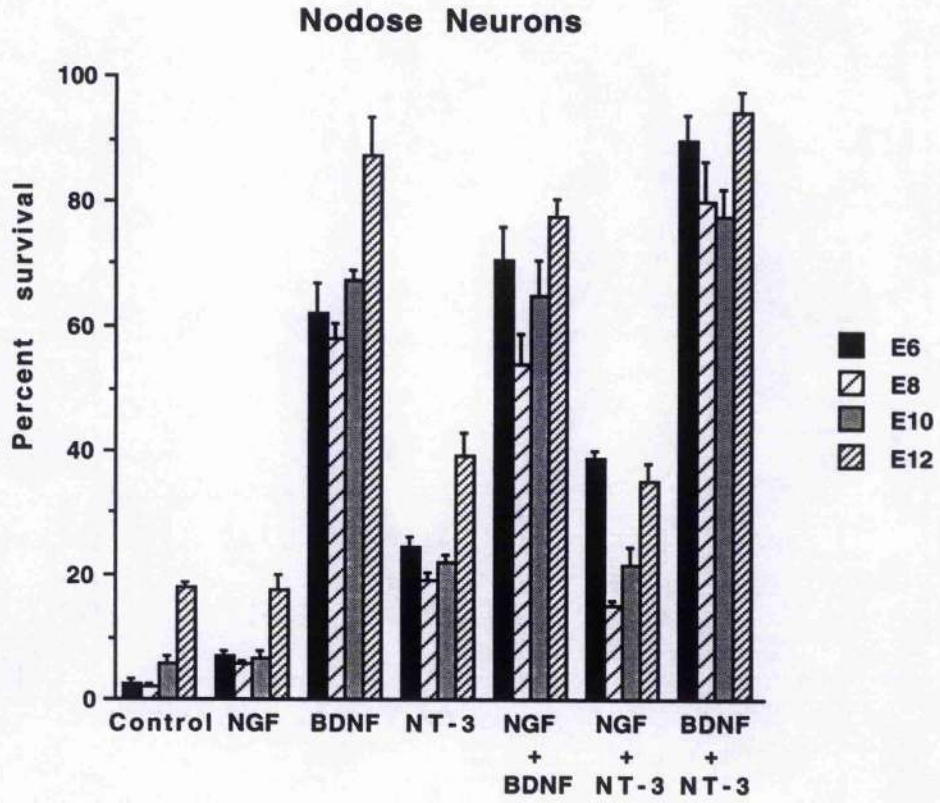


Figure 2.10. Survival responses of nodose neurons to neurotrophins alone and in combination. Bar charts showing the percent survival of Nodose neurons from E6 (black bars), E8 (light hatched bars), E10 (stippled bars) and E12 (dark hatched bars) embryos after 48 hours incubation with 2 ng/ml of NGF, BDNF, NT3, NGF+BDNF, NGF+NT3 or BDNF+NT3. The mean ± the standard error are shown (n=3).

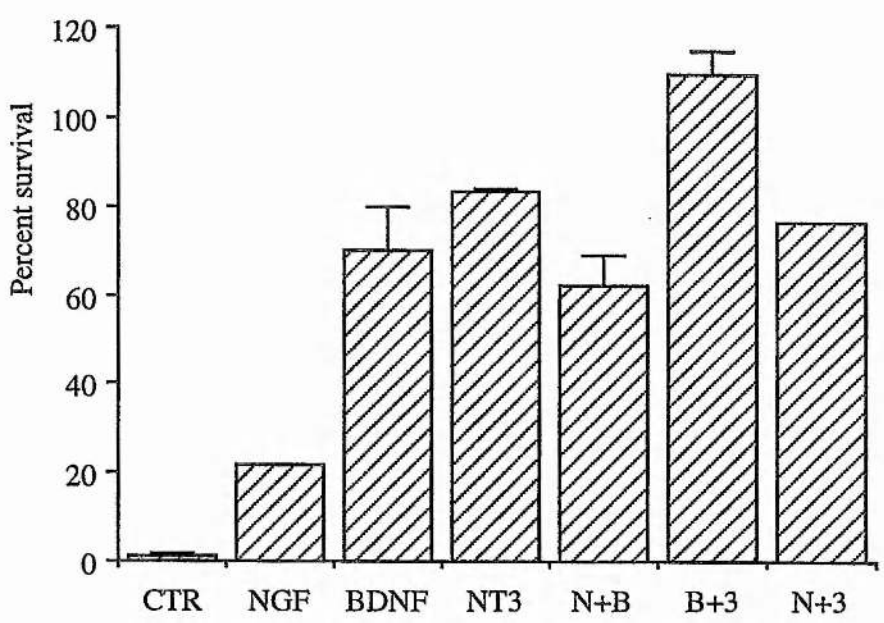


Figure 2.11. Survival responses of E5 Nodose Neurons to neurotrophins alone (at 2ng/ml) and in combination. Bar chart showing the percent survival in culture after 48h. N+B: NGF + BDNF; B+3: BDNF + NT3; N+3: NGF + NT3. The mean ± standard errors are shown (n=3).

BDNF and NT-3 exert a direct survival response from early DMTG and jugular neurons

Because the neural crest-derived DMTG and jugular neurons are born between E4 and E6 *in vivo* (D'Amico-Martel, 1982), cell cultures set up at E6 may contain neuron progenitor cells that are capable of differentiating into neurons *in vitro* (Rohrer *et al.*, 1985). Thus, it was important to ascertain if the increase in the number of neurons in these cultures in the presence of BDNF or NT-3 was due to a survival response of differentiated neurons or was due to enhanced proliferation or differentiation of progenitor cells. This is especially pertinent because BDNF may direct pluripotent neural crest cells to differentiate along the sensory neuron lineage (Sieber-Blum, 1991) and NT-3 increases the proliferation of neural crest cells (Kalcheim *et al.*, 1992) and enhances the proliferation or differentiation of dorsal root ganglion neuron progenitor cells (Wright *et al.*, 1992).

Figure 2.12. shows the percent survival after 48 hours incubation in cohorts of E6 DMTG neurons identified 6 hours after plating. The majority of neurons in these cohorts were still surviving in the presence of either BDNF or NT-3, whereas only 10% were alive in control cultures. Moreover, the percentage survival in control and neurotrophin-supplemented neuronal cohorts was very similar to the overall percentage survival in E6 DMTG cultures in which the fate of individual neurons was not serially monitored (Fig.2.6). These results demonstrate that the effect of BDNF and NT-3 on the number of neurons in E6 DMTG cultures is due mainly, if not exclusively, to enhanced survival. A

smaller number of cohort experiments carried out with E6 jugular neurons gave similar results (data not shown).

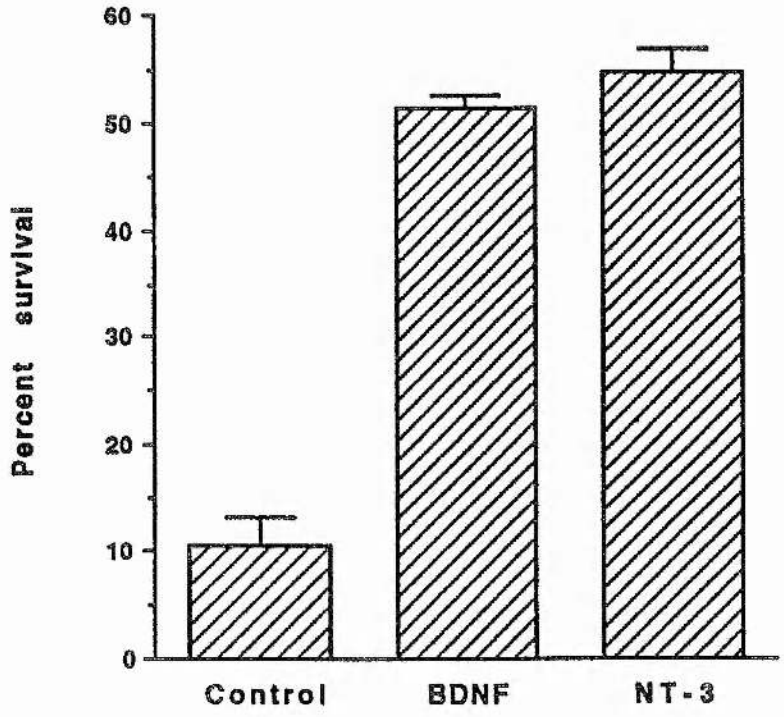


Figure 2.12. BDNF and NT3 exert a direct survival-promoting effect on E6 DMTG neurons. Bar chart of the percent survival after 48 hours incubation of cohorts of E6 DMTG neurons identified 6 hours after plating. Cohorts were grown either without neurotrophins (control) or were supplemented with 2 ng/ml of BDNF or NT3 at 6 hours. The mean +/- the standard error are shown (n=3).

Dose responses of DMTG neurons to neurotrophins shift to higher concentrations with age

The loss of the early survival response of DMTG and jugular neurons to BDNF and NT-3 could be due either to increasing numbers of the neurons becoming completely unresponsive to these neurotrophins with age or to a marked shift in the dose response of neurons in the population as a whole. To distinguish between these possibilities, E6 to E12 DMTG neurons were grown with BDNF or NT-3 over a broad range of concentrations up to 5 μg/ml (which is over 4 orders of magnitude higher than the saturating concentration of these neurotrophins for nodose and VLTG neurons, data not shown). For comparison, dose responses of DMTG neurons to NGF were also carried out at these ages.

Figure 2.13. shows the dose responses of E6, E8, E10 and E12 DMTG neurons to NGF, BDNF and NT-3. At E6, the majority of DMTG neurons were supported by saturating concentrations of each neurotrophin, and the concentrations of each neurotrophin that promoted half-maximal survival were similar (3.8, 4.7 and 12.7 pg/ml for NGF, BDNF and NT-3, respectively). By E8, however, the BDNF and NT-3 dose-responses showed a marked shift to higher concentrations. Although the majority of neurons were still promoted by the highest concentration of BDNF and NT-3 used (5 μg/ml), the concentrations of BDNF and NT-3 that promoted half-maximal survival had increased by 4 orders of magnitude by E8 (to 98 and 124 ng/ml for BDNF and NT-3, respectively). There appeared to be further shifts in the BDNF and NT-3 dose responses to higher concentrations at E10 and E12 (seen most clearly in the case

of NT-3). However, because maximal neuronal survival was not reached at the highest concentration used (5 μg/ml), the half-maximally effective concentrations cannot be reliably calculated at these ages.

There was also a shift in the NGF dose response to higher concentrations between E6 and E8. However, in contrast to the very large shifts in the BDNF and NT-3 dose responses during this period, the half-maximally effective NGF concentration increased by only one order of magnitude from 3.8 to 28 pg/ml. In contrast to the continued shifts in the BDNF and NT-3 dose responses at later ages, the NGF dose responses were similar at E8, E10 and E12 and there was no significant difference in the half-maximally effective NGF concentrations at these ages.

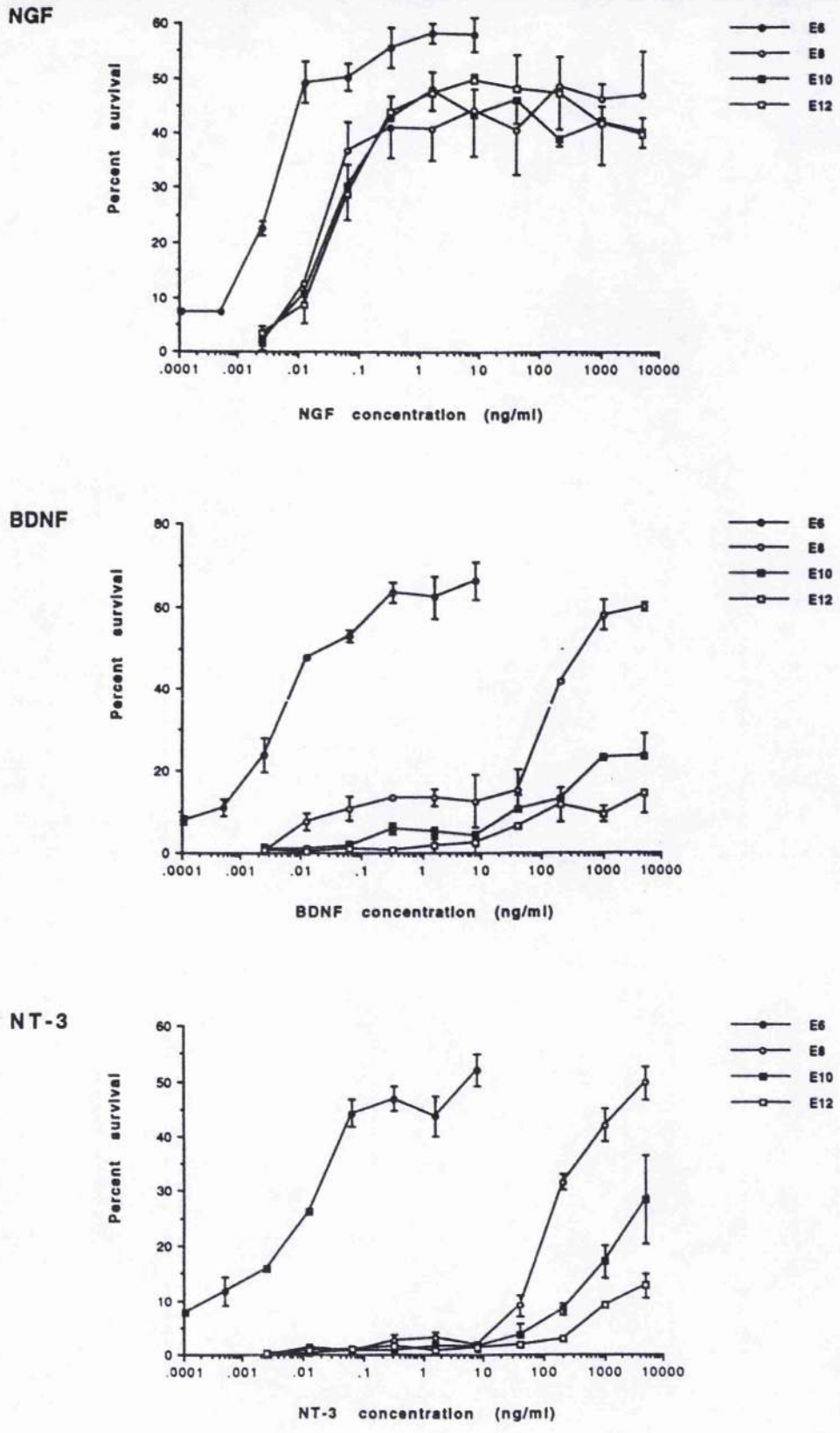


Figure 2.13. Dose responses of DMTG neurons to neurotrophins.
Graphs showing the percent survival of DMTG neurons from E6 (filled circles), E8 (open circles), E10 (filled squares), and E12 (open squares) embryos after 48 hours incubation with NGF, BDNF or NT3 at concentrations ranging from 0.1pg/ml to 5μg/ml. The mean +/- the standard error from typical experiments are shown.

2.4. DISCUSSION

The experiments outlined in this chapter suggest that NGF promotes the survival of DMTG and jugular neurons and BDNF promotes the survival of VLTG and nodose neurons grown in low-density cultures that are virtually free of non-neuronal cells. These data are in agreement with results of previous *in vitro* studies of the effects of NGF and BDNF on the survival of mid-embryonic cranial sensory neurons (Davies and Lindsay, 1985; Lindsay and Rohrer, 1985; Lindsay *et al.*, 1985; Davies *et al.*, 1986b; Davies *et al.*, 1986a), In addition, by studying neurons at earlier developmental stages, it was shown that the survival of NGF-dependent DMTG and jugular neurons is transiently supported by BDNF and NT-3. Because there was negligible additional survival in E6 DMTG and jugular cultures grown with combinations of NGF, BDNF and NT-3, most of these neurons responded to all three factors during this early stage of their development. By monitoring the survival of individual E6 DMTG neurons between 6 and 48 hours *in vitro*, it was shown that BDNF and NT-3 exert a direct, through transient, survival-promoting action on NGF-dependent sensory neurons at an early stage in their development.

Previous work on the NGF-dependent trigeminal ganglion neurons of the mouse embryo has shown that the transient survival-promoting effects of BDNF and NT-3 on these neurons is largely over by the time the number of neurons in the trigeminal ganglion begins to decline as a result of naturally occurring neuronal death (Buchman and Davies, 1993). Although there are no available data on the timing of neuronal death in the chicken trigeminal ganglion, a developmental study of neuronal death in the sensory ganglia of cranial nerves

IX and X in chicken embryos (Hiscock and Straznicky, 1986) has shown that the total number of neurons in the jugular ganglion peaks at E8 and decreases by half over the next five days of development. Thus, for at least chicken jugular ganglion neurons, the timing of the early survival response to BDNF and NT-3 appears to be similar to that observed for mouse trigeminal ganglion neurons in that it is largely over by the time neurons numbers begin to fall as a result of cell death.

In vitro studies of developing dorsal root ganglion (DRG) neurons have shown that NGF and BDNF supported the survival of distinct subsets of neurons at E12, whereas at E6 more than half of the neurons were supported by either factor alone (Lindsay *et al.*, 1985; Acheson *et al.*, 1987), indicating that there is some overlap between NGF-responsive and BDNF-responsive neurons during the early stages of DRG development. In a detailed *in vitro* study of the survival requirements of DRG neurons from the earliest stages of their development (Ernsberger and Rohrer, 1988), it was shown that after an initial phase of neurotrophin independence, the survival of all neurons was promoted by either NGF or BDNF and that a proportion of the neurons subsequently lost responsiveness to BDNF. The current observations of early DMTG and jugular neurons are consistent with the early response of NGF-dependent neurons to BDNF and the subsequent loss of BDNF responsiveness. In addition, it was shown that NGF-dependent neurons are also transiently supported by NT-3 early in their development. Furthermore, by studying several different populations of cranial sensory neurons, it was demonstrated that the broad neurotrophin responsiveness of NGF-dependent neurons at early developmental stages does not extend to other populations of developing sensory neurons. VLTG neurons are supported by BDNF throughout their early development, but show virtually no response to either NGF or NT-3. Nodose neurons respond to BDNF and NT-

3 from an early developmental stage, but show negligible response to NGF at any stage in their development. Thus, responsiveness to multiple neurotrophins is not a ubiquitous feature of the early stages of neuronal development, but among sensory neurons appears to be restricted to those that depend on NGF for survival during the phase of naturally occurring cell death.

In accordance with these observations, it has been shown that sympathetic neurons depend on NGF for survival by the time they innervate their targets (Cohen, 1960; Levi-Montalcini and Booker, 1960), but not earlier during development (Coughlin and Collins, 1985; Ernsberger *et al*, 1989; Birren *et al*, 1993). These neurons, however, respond transiently to NT3 (Birren *et al*, 1993 ; DiCicco-Bloom *et al*, 1993, Barde , 1995) produced within the ganglion (Schechtersson and Bothwell, 1992) before the neurons become dependent on target-derived NGF.

The current studies show that the loss of the response of the NGF-dependent DMTG neurons to BDNF and NT-3 during development is associated with marked shifts in the BDNF and NT-3 dose responses to higher concentrations. Between E6 and E8 there is a shift of over four orders of magnitude in the BDNF and NT-3 dose responses and further increases are apparent at later stages. The marked shifts in the BDNF and NT-3 dose responses may be due to reduced levels of expression of TrkB and TrkC in DMTG neurons with development or to the increasing expression of truncated TrkB and TrkC isoforms that may act as dominant-negative receptors (Jing *et al.*, 1992). Interestingly, there is a smaller shift of one order of magnitude in the NGF dose response of DMTG neurons to higher NGF concentrations between E6 and E8. A similar shift in the NGF dose response is observed in mouse trigeminal neurons during a roughly equivalent stage of development between

E12 and E15 (Buchman and Davies, 1993). Curiously, the mean level of TrkA mRNA increases in trigeminal neurons over this period of development (Wyatt and Davies, 1993) and there is no evidence for the existence of a TrkA isoform lacking the tyrosine kinase domain in developing trigeminal neurons (Rosenthal and Davies, personal communication). This developmental shift in the NGF dose response is also not dependent on the expression gp75 because it is still observed in developing trigeminal neurons obtained from mouse embryos that have a null mutation of the gp75 gene (Davies *et al.*, 1993).

Chapter 3

TIMING OF NEURONAL DEATH IN *TrkA*, *TrkB* and *TrkC* MUTANT MOUSE EMBRYOS DEMONSTRATES NEUROTROPHIN SWITCHING IN DEVELOPING TRIGEMINAL NEURONS .

3.1. INTRODUCTION

Developing sensory neurons survive in culture independently of neurotrophins during the stage when their axons are growing to their targets (Davies and Lumsden, 1984; Ernsberger and Rohrer, 1988; Vogel and Davies, 1991). The *in vitro* survival of many populations of cranial sensory neurons is initially promoted by BDNF or NT3 (Vogel and Davies, 1991; Buchman and Davies, 1993). Whereas several populations of cranial sensory neurons retain dependence on these neurotrophins in culture throughout the phase of naturally occurring neuronal death (Chapter 2 of this thesis; Davies *et al.*, 1986a, 1986b; Hohn *et al.*, 1990), other populations switch dependence to NGF during the early stages of target field innervation (Chapter 2 of this thesis; Buchman and Davies, 1993)

The *in vitro* switch from BDNF/NT3 dependence to NGF dependence has been studied most extensively in the trigeminal ganglion neurons of the mouse embryo (Buchman and Davies, 1993; Paul and Davies, 1995), but also occurs in cultured jugular ganglion neurons (Davies *et al.*, 1993a). When grown at low density in defined medium, E10 mouse trigeminal neurons, which have

not yet innervated their peripheral targets, die between 24 and 48 hours in the absence of neurotrophins. The death of these early neurons is prevented by BDNF or NT3 but not by NGF. NGF promotes the survival of a small proportion of E11 neurons and virtually all E12 neurons for 48 hours in culture. Concomitant with the acquisition of the NGF survival response, the survival responses to BDNF and NT3 are lost. In E13 cultures, less than 10% of the neurons are supported for 48 hours by BDNF, and the survival response to NT3 falls to a similar level in E14 cultures. It is not known, however, if this apparent switch in the survival response of developing trigeminal neurons from BDNF/NT3 to NGF observed in culture takes place *in vivo* during normal development. Moreover, because the *in vitro* assessment of the effects of neurotrophins on developing neurons involves incubation periods of 48 hours or more, it is difficult to relate the timing of *in vitro* switching to the developmental stage of the neurons *in vivo*.

Analyses of mice that have targeted null mutations in the neurotrophin genes (Crowley *et al.*, 1994; Ernfors *et al.*, 1994a, 1994b; Farinas *et al.*, 1994; Jones *et al.*, 1994; Conover *et al.*, 1995; Liu *et al.*, 1995) and trk genes (Klein *et al.*, 1993, 1994; Smeyne *et al.*, 1994) have been extremely useful in assessing the physiological relevance of earlier *in vitro* studies of the effects of neurotrophins on neuronal survival and in extending our understanding of the functions of neurotrophins in neuronal development (See Table 3.1)

Mutation	Phenotype		
	Behavioural	**PNS (Neuron Loss)**	**CNS (Neuron Loss)**
gp75	viable and fertile	Sympathetic ganglia, no loss; smaller DRG	
NGF	viable by birth; reduced responses to painful stimuli; death within a month	SCG, 99% DRG, 70-80% Trigeminal ganglion, 70% Nodose, no loss of neurons	Motor neurons, no loss Basal forebrain, cholinergic neurons; normal differentiation and reduced ChAT immunoreactivity Reduced axonal branching.
TrkA	viable at birth, death within a month; no orientation in response to vibrissal stimulation. Basic motor functions normal	Trigeminal ganglia, 70-90%; DRG, 70-90% SCG severe loss.	Decrease in AChE in cholinergic fibbers
BDNF	Some survival for few weeks. Head turning, spinning in week 2	SCG, no loss DRG, 30-50% Vestibular, >80% Nodose, 45-65% Trigeminal ganglion, 25-55%	Motor neurons, no loss

NT4	long-lived; no obvious neurological defects	Trigeminal ganglion, no loss Vestibular ganglion, no loss Nodose' some loss Petrosal, some loss	Motor neurons, no loss
TrkB	Absence of feeding activity, most die in 48h after birth	Trigeminal ganglia, DRG Nodose, substantial loss,	Motor neurons, 35%
NT3	Strikingly abnormal movements and postures; loss of proprioception, most die shortly after birth.	SCG, 55% DRG, 55% Nodose, 50% Trigeminal ganglion, 65% Cochlear, 85%	Motoneurons, no loss Loss of spinal proprioceptive afferents and their peripheral sense organs (muscle spindles and Golgi tendon organs)
TrkC	Abnormal movements and posture; death within a month	DRG, 20%	Motoneurons, 30%

Table 3.1. Phenotypes of Neurotrophin and Trks (-/-) Mutant Mice.Adaptede from Snider, 1994.

Several studies of mutant mice suggest that BDNF and NT3 play a role in trigeminal ganglion development. By birth and the postnatal period there are marked reductions in the neuronal complement of the trigeminal ganglia of *BDNF*$^{-/-}$ (Ernfors *et al.*, 1994a; Jones *et al.*, 1994), *TrkB*$^{-/-}$ (Klein *et al.*, 1993) and *NT3*$^{-/-}$ mice (Ernfors *et al.*, 1994b). However, because trigeminal ganglia were not studied at intervals throughout embryonic development, it is not known at which stage of development BDNF and NT3 are required for neuron survival or whether they influence the neuronal complement of the trigeminal ganglion by promoting precursor cell proliferation and/or differentiation. The latter possibilities are raised by the finding that BDNF directs pluripotent neural crest cells to differentiate *in vitro* along the sensory neuron lineage (Sieber-Blum, 1991) and that NT3 is a mitogen for cultured neural crest cells (Kalcheim *et al.*, 1992) and enhances the proliferation or differentiation of sensory neuron progenitor cells in culture (Wright *et al.*, 1992). Although many foetal trigeminal neurons are supported by NGF *in vitro* (Buchman and Davies, 1993), the dependence of trigeminal neurons *in vivo* has not been formally demonstrated and the relationship between the stage at which the neurons are critically dependent on NGF *in vivo* and the timing of naturally occurring neuronal death in the ganglion is not known.

To resolve the above issues, I have quantified the extent of neuronal death in the trigeminal ganglia of *trk*A$^{-/-}$, *TrkB*$^{-/-}$, *TrkC*$^{-/-}$ and wild type mice at closely staged intervals throughout embryonic development. My results demonstrate that neurotrophin switching takes place during embryogenesis and have clarified the role of Trk receptor tyrosine kinases in sensory neuronal

development by providing precise data on the timing of neuronal dependence on Trk signalling *in vivo*.

3.2. MATERIALS AND METHODS

Animals

Embryos were obtained from overnight matings of (i) $TrkA^{+/-}$ mice, (ii) $TrkB^{+/-}$ and (iii) $TrkC^{+/-}$ mice. Pregnant females were killed by cervical dislocation at the required stage of gestation and the precise stage of development of the embryos was determined by the criteria of Theiler (Theiler, 1972). The genotypes of the embryos were determined by a PCR-based technique using DNA isolated from embryonic tissues (Schimmang *et al.*, 1995). DNA extracted from a small sample of the embryo tissue was amplified by PCR using specific primers.

Quantification of numbers of pyknotic nuclei in the trigeminal ganglion

Embryos were fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer pH 7.3. The brain was removed from E14 and older embryos to allow proper fixation of the trigeminal ganglia. After fixation for at least a week, the embryos were dehydrated and embedded in paraffin wax. E15 and older embryos were first decalcified with 1M EDTA. Coronal sections of the head were cut at 8 μm. These were mounted on gelatinised slides and were stained with cresyl fast violet.

To evaluate the extent of cell death, all pyknotic nuclei were counted at 400X magnification in every fourth section along the entire rostrocaudal extent of the trigeminal ganglia of E11 to E19 embryos. Estimates of the total number of pyknotic nuclei in each ganglion were obtained by multiplying the sum of these counts by four. At each age, between four and ten ganglia of the following genotypes were studied: *trkA*$^{-/-}$ mice, *TrkB*$^{-/-}$, *TrkC*$^{-/-}$ and wild type. In all cases, the sections were coded prior to counting to avoid any observer bias.

Quantification of total numbers of neurons in the trigeminal ganglion

To estimate the total number of neurons in the trigeminal ganglia of selected embryos, the profiles of every 10th coronal section of the ganglion were made using a drawing tube at 100X magnification. The cross-sectional area of each counted section was measured using an Image Processing and Analysis Program (NIH Image). The average density of neurons was quantified in each of these sections at 1,000X magnification using a 0.01mm^2 grid. Neurons were identified by virtue of the Nissl substance and their large, round, pale-stained nuclei (Konigsmark, 1970). The total number of neurons in each section was calculated from the section area and average neuron density in each section. The total number of neurons in the ganglion was estimated by adding these numbers and multiplying by 10. Correction for split nucleoli was not made as these do not appreciably affect the neuronal estimate (Jones, 1937). The estimates of the total number of neurons in the trigeminal ganglia of normal embryos in the present study are very similar to those previously obtained (Davies and Lumsden, 1984) by counting neuronal nuclei and correcting for split nucleoli by the method of Abercrombie (Abercrombie, 1946).

Immunohistochemistry

To identify the proportion of neurons undergoing cell death in the early trigeminal ganglia of wild type and *TrkB*$^{-/-}$ embryos, 8μm frozen sections were doubled labelled for neuron-specific markers (peripherin or 160 kDa neurofilament protein) and 3' DNA ends generated by DNA fragmentation in apoptotic cells. Sections were incubated with either rabbit anti-peripherin antibody (Chemicon) diluted 1:300 in phosphate-buffered saline containing 0.1% Triton and 1% goat serum for 12 hours or mouse anti-160 kDa monoclonal antibody (Sigma) diluted 1:200 in phosphate-buffered saline containing 0.3% Triton and 1% horse serum for 48 hours. These primary antibodies were localised by an immunoperoxidase technique using a biotinylated secondary antibody and an avidin/biotinylated horse radish peroxidase macromolecular complex (Vectastain ABC Kit, Vector Laboratories). This was followed by *in-situ* DNA end-labelling with fluorescein-nucleotides (ApopTag Direct Kit, Oncor). The sections were examined and photographed using a Axioskop microscope. Cells undergoing apoptosis were recognised by an intensely fluorescent nucleus. Apoptotic cells were classified as neurons if a peroxidase-labelled rim of cytoplasm was clearly visible.

3.3. RESULTS

Developmental changes in the number of pyknotic nuclei

Pyknotic nuclei in developing trigeminal ganglia were recognised as one or more very darkly stained spherical structures contained within a clearly visible membrane (Fig.3.1). The great majority of these pyknotic nuclei were observed in large degenerating cells, suggesting that these were neurons (Oppenheim, 1991). In late foetal ganglia, a very small number of pyknotic nuclei were also observed in very small cells that were of a similar size to satellite cells, suggesting that a minority of the supporting cells undergo cell death in the ganglion. These latter pyknotic cells were not counted.

(i). Wild type embryos

In wild type embryos, pyknotic nuclei were observed as early as E11. The number increased steadily by five-fold to reach a peak at E14 after which there was a decrease to a negligible number by the end of foetal development at E19 (Fig 3. 1).

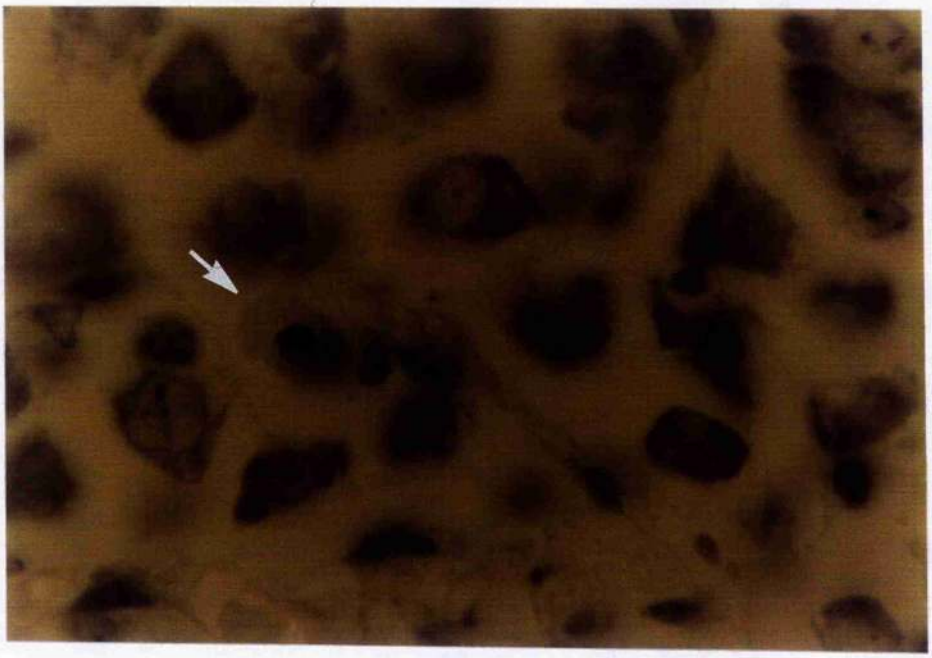


Figure 3.1. Bright field view of a wild type E17 trigeminal ganglion showing the typical appearance of a pyknotic nucleus. A darkly stained spherical structure (arrow) is seen enclosed within a membrane. Magnification = X 1,200

(ii). *TrkA* $^{-/-}$ embryos

The number of pyknotic nuclei in the trigeminal ganglion of *trkA*$^{-/-}$ embryos was very similar to that of wild type embryos at E11 and E12 (Fig. 3.2). In contrast to wild type embryos, there was a marked increase in the number of pyknotic nuclei between E12 and E13. At E13 and E14 the number of pyknotic nuclei in the trigeminal ganglia of *trkA*$^{-/-}$ embryos was approximately three-fold greater than in ganglia of wild type embryos. After E14 there was a marked fall in the number of pyknotic nuclei to less than in wild type ganglia at E15. The number of pyknotic nuclei fell further to the same negligible level by just before birth (E19) as in wild type embryos. There was no obvious difference in the appearance of individual pyknotic nuclei in the trigeminal ganglia of *trkA*$^{-/-}$ embryos and wild type embryos at equivalent stages of development.

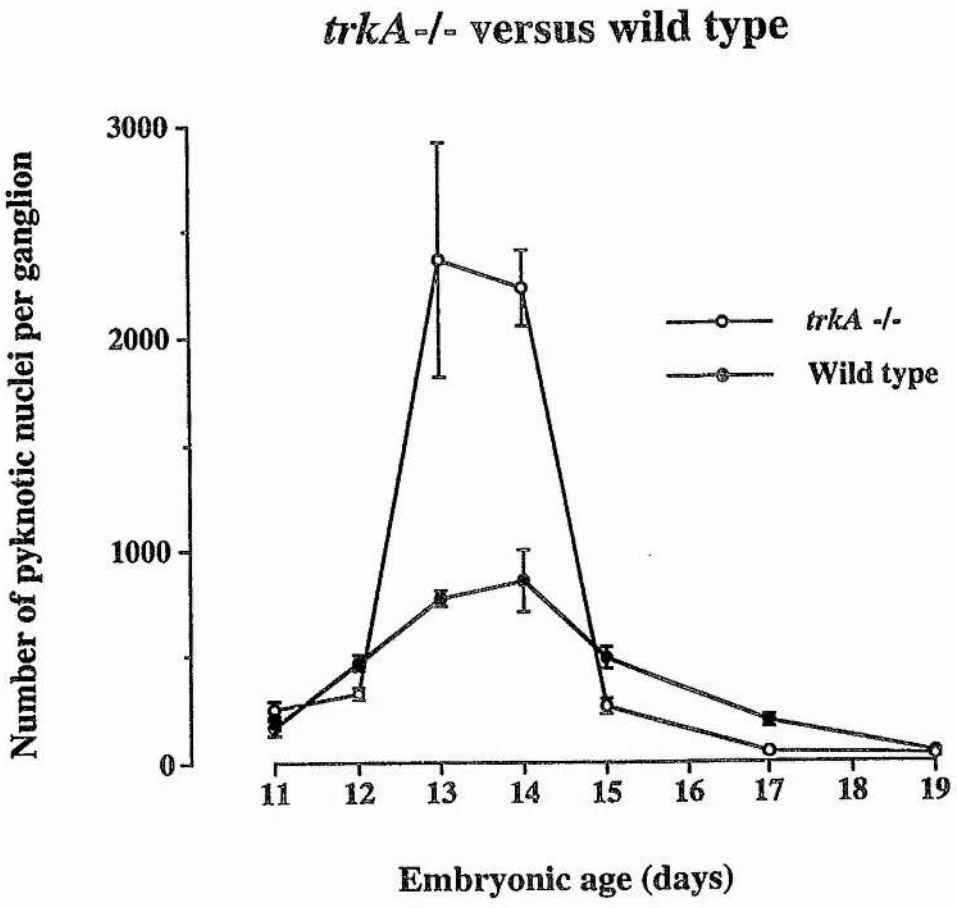


Figure 3.2. Graph comparing the number pyknotic nuclei in the trigeminal ganglia of wild type and *trkA*$^{-/-}$ embryos from E11 to E19. The data were obtained from 4 to 10 ganglia of wild type embryos and from 4 to 6 ganglia of *trkA*$^{-/-}$ embryos at each age. The means and standard errors are shown.

(iii). *TrkB*$^{-/-}$ embryos

The number of pyknotic nuclei in the trigeminal ganglion of *TrkB*$^{-/-}$ embryos was substantially higher than that of wild type embryos at E11 and E12 (Fig.3.3). There were over four-fold more pyknotic nuclei at E11 and over three-fold more pyknotic nuclei at E12. From a clear peak in the number of pyknotic nuclei in *TrkB*$^{-/-}$ ganglia at E12 there was a marked fall to less than in wild type ganglia at E13. At E15 and later ages, the number of pyknotic nuclei in *TrkB*$^{-/-}$ ganglia was similar to that in wild type ganglia. There was no obvious difference in the appearance of pyknotic nuclei in the trigeminal ganglia of *TrkB*$^{-/-}$ embryos and wild type embryos at equivalent stages of development.

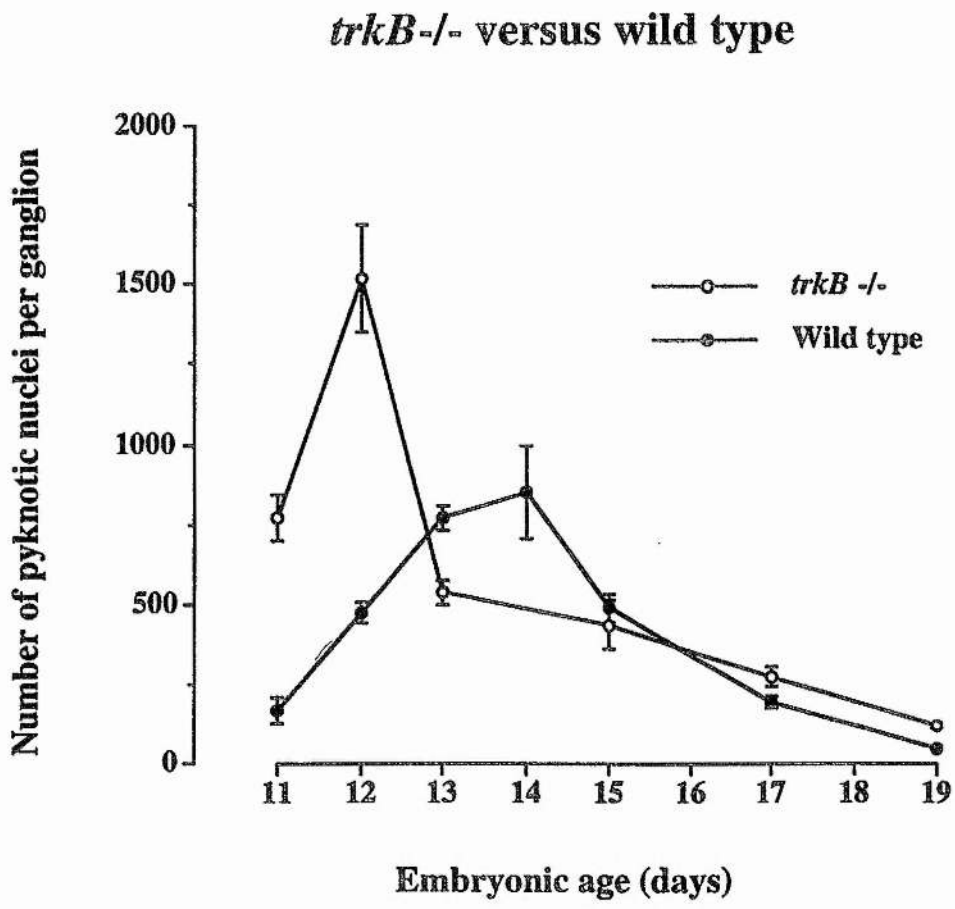


Figure 3.3. Graph comparing the number pyknotic nuclei in the trigeminal ganglia of wild type and *TrkB*$^{-/-}$ embryos from E11 to E19. The data were obtained from 4 to 10 ganglia of wild type embryos and from 4 to 6 ganglia of *TrkB*$^{-/-}$ embryos at each age. The means and standard errors are shown.

(iv). *TrkC*$^{-/-}$ embryos

There was a small increase in the number of pyknotic nuclei in the early trigeminal ganglia of *TrkC*$^{-/-}$ embryos (Fig.3.4). The number of pyknotic neurons was just over a two-fold greater than in wild type ganglia at E11, and was 1.5-fold greater at E12. Although these increases in pyknotic nuclei in *TrkC*$^{-/-}$ ganglia were far less than in *TrkB*$^{-/-}$ ganglia, they were nonetheless statistically significant (t-tests: E11, $p < 0.001$; E12, $p < 0.02$). At E13 and later ages there were similar numbers of pyknotic nuclei in *TrkC*$^{-/-}$ and wild type ganglia. As in *trkA*$^{-/-}$ and *TrkB*$^{-/-}$ ganglia, there were no obvious differences in the appearance of pyknotic nuclei in the trigeminal ganglia of *TrkC*$^{-/-}$ embryos and wild type embryos at equivalent stages of development.

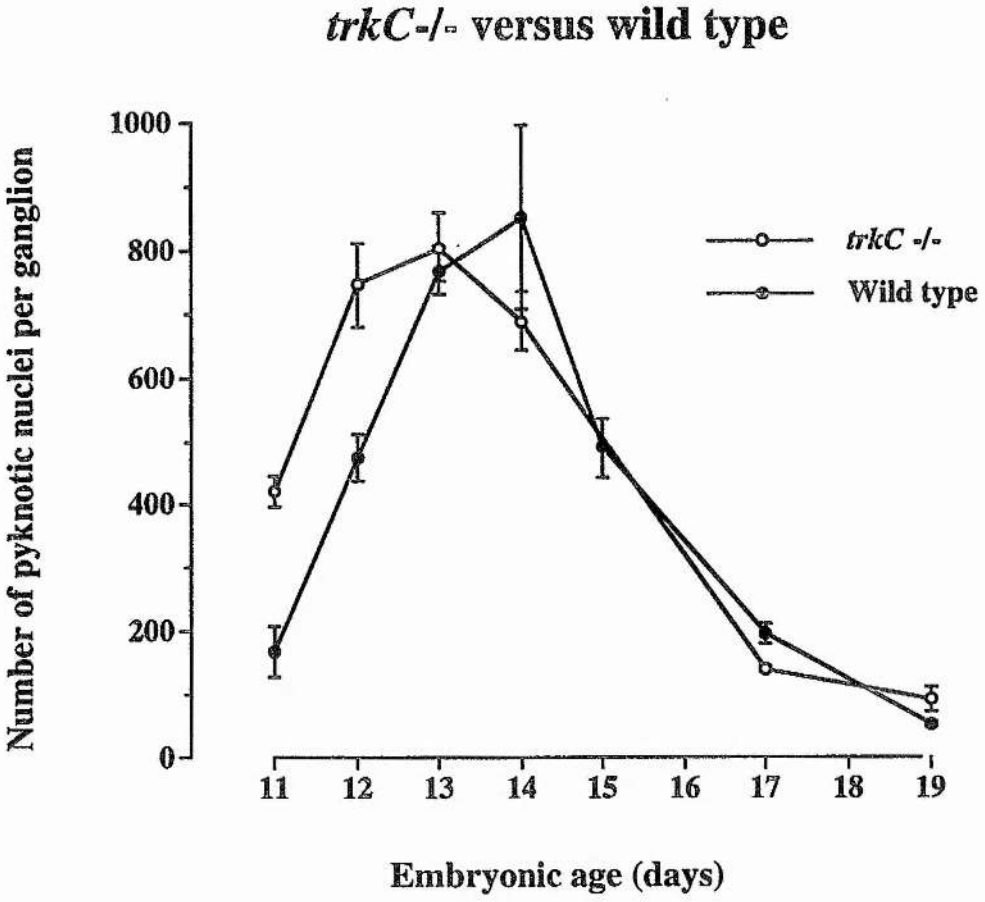


Figure 3.4. Graph comparing the number pyknotic nuclei in the trigeminal ganglia of wild type and *TrkC*$^{-/-}$ embryos from E11 to E19. The data were obtained from 4 to 10 ganglia of wild type embryos and from 4 to 8 ganglia of *TrkC*$^{-/-}$ embryos at each age except for E18 where 2 *TrkC*$^{-/-}$ ganglia were studied . The means and standard errors are shown.

Differences in the neuronal complement of the trigeminal ganglion of wild type and mutant embryos at key stages of development

To ascertain the consequences of the differences in the number of dying cells in the trigeminal ganglia of wild type and mutant embryos at different stages of development, the total number of neurons in the ganglion was estimated at several key stages (Fig.3.5). At E13, when the total number of neurons in the trigeminal ganglion reaches a peak during normal development (Davies and Lumsden, 1984), there were no statistically significant differences in the total numbers of neurons in the trigeminal ganglia of wild type, *trkA*$^{-/-}$ and *TrkC*$^{-/-}$ embryos (t-tests: $p > 0.1$). In contrast, there was a 42%, statistically significant reduction in the number of neurons in the ganglia of *TrkB*$^{-/-}$ embryos at this stage of development (t-test: $p < 0.001$). At E15, shortly after the number of neurons in the trigeminal ganglion begins to decline in normal development (Davies and Lumsden, 1984), there was a marked, 64%, statistically significant reduction in the number of neurons in *trkA*$^{-/-}$ ganglia compared with wild type ganglia (t-test: $p < 0.001$). The number of neurons in E15 *TrkB*$^{-/-}$ ganglia was significantly reduced by 33% compared with wild type ganglia (t-test, $p < 0.01$). By E17, close to the end of the phase of naturally occurring neuronal death, the reduction in the number of neurons in *trkA*$^{-/-}$ ganglia had further increased to 69% compared with wild type ganglia. The number of neurons in E17 *TrkB*$^{-/-}$ ganglia was still significantly reduced by 33% compared with wild type ganglia (t-test, $p < 0.0005$). By E17, however, there was a 22% reduction in the number of neurons in the ganglia of *TrkC*$^{-/-}$ embryos compared with wild type embryos that was just within the limits of significance (t-test, $p < 0.05$).

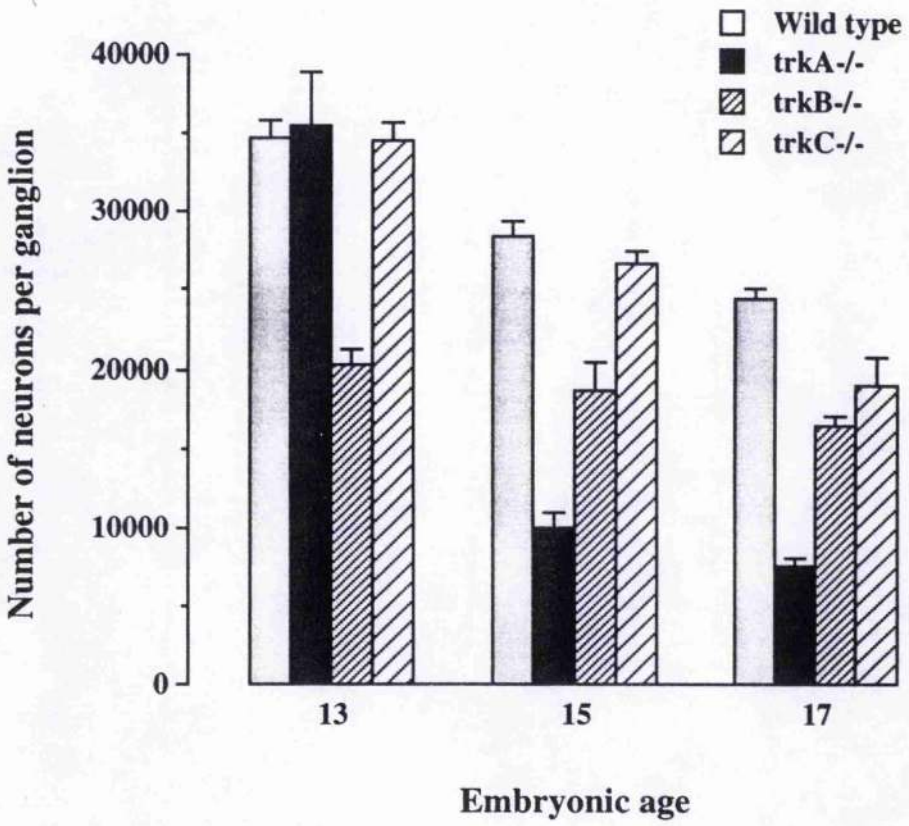


Figure 3.5. Bar chart of the total numbers of neurons in the trigeminal ganglia of wild type, *trkA*$^{-/-}$, *TrkB*$^{-/-}$, and *TrkC*$^{-/-}$ embryos at E13, E15 and E17. Four ganglia of each kind were studied at each age except at E15 where *TrkC*$^{-/-}$ ganglia were not studied. The means and standard errors are shown.

The majority of cells dying in early *TrkB*$^{-/-}$ ganglia are neurons

To determine if the increased cell death in early *TrkB*$^{-/-}$ trigeminal ganglia was due to increased loss of neurons, sections of *TrkB*$^{-/-}$ and wild type embryos were double labelled for neuron markers and apoptotic nuclei at the peak of cell death in *TrkB*$^{-/-}$ trigeminal ganglia at E12. Apoptotic cells were recognised by intense nuclear fluorescence following *in-situ* DNA end-labelling with fluorescein-nucleotides. Neurons were positively identified by cytoplasmic staining for either 160 kDa neurofilament protein or peripherin using an immunoperoxidase technique. All of the apoptotic cells in several sections of each ganglion were scored as either neurons or other cells. An apoptotic cell was scored as a neuron if the fluorescent nuclear staining was clearly and unambiguously contained within a distinct rim of peroxidase-labelled cytoplasm (Fig.3.6). There was no significant difference between the proportion of apoptotic cells positively identified as neurons by neurofilament staining in E12 wild type embryos (67.3 ± 3.6%, n = 3 ganglia from different embryos) and E12 *TrkB*$^{-/-}$ embryos (69.0 ± 1.9%, n = 3). Likewise, there was no significant difference between the proportion of apoptotic cells positively identified as neurons by peripherin staining in E12 wild type embryos (70.0 ± 3.7%, n = 3) and E12 *TrkB*$^{-/-}$ embryos (71.6 ± 5.6%, n = 3). These results suggest that the majority of dying cells in early wild type and *TrkB*$^{-/-}$ trigeminal ganglia are neurons. However, because of the strict criteria for classifying apoptotic cells as neurons, the actual proportion of dying cells that are neurons is likely to be even greater than our estimates suggest. Figures 3.7a and 3.7b illustrate the large

difference in the numbers of apoptotic cells in wild type and *TrkB*$^{-/-}$ ganglia at E12.

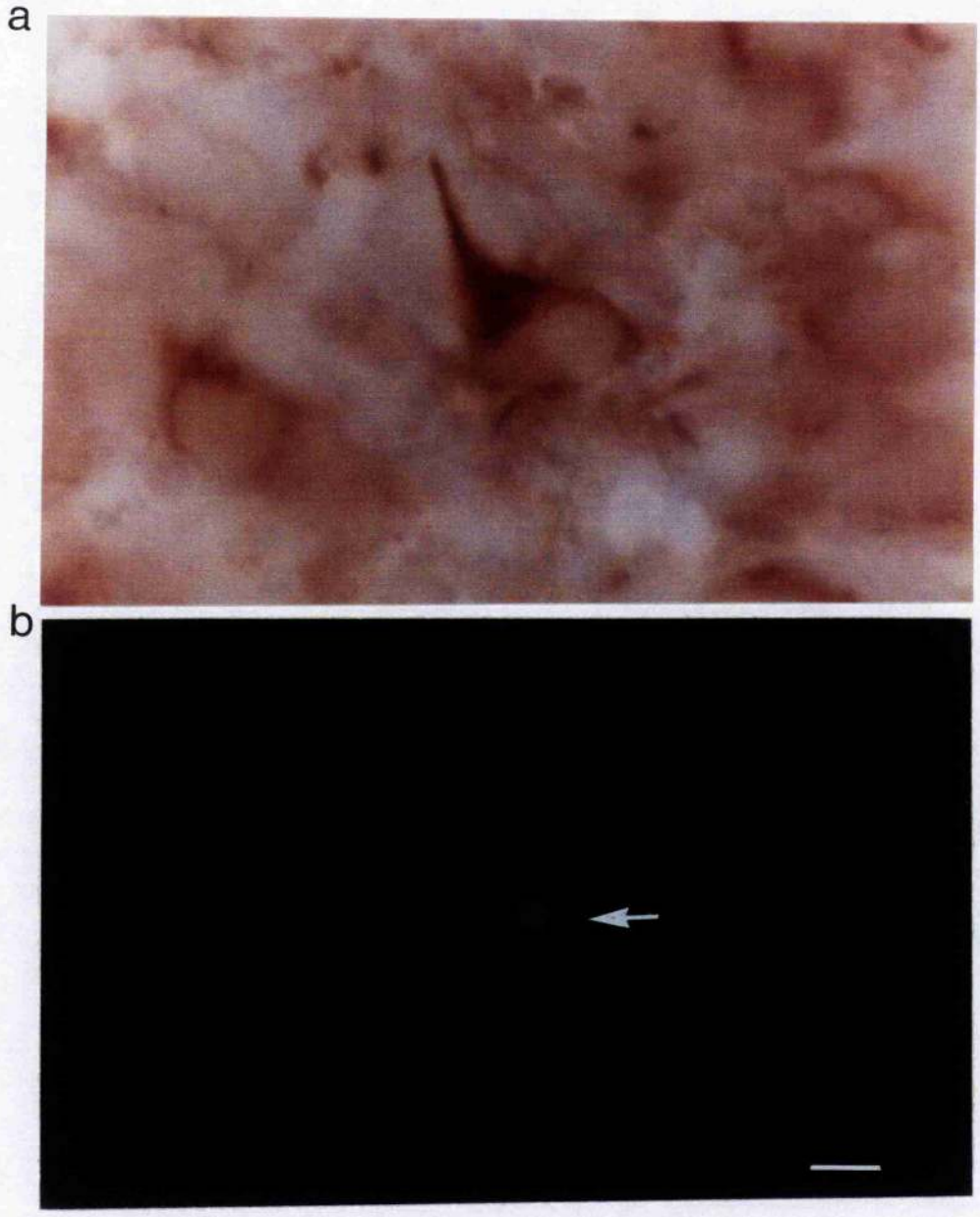


Figure 3.6. Matching bright field (a) and fluorescence (b) photomicrographs of a section through the trigeminal ganglion of an E12 *TrkB*$^{-/-}$ embryo. In the plane of focus, two neurons are clearly identified by cytoplasmic staining for peripherin (a). One of these has fluorescent staining for 3' DNA ends in the nucleus characteristic of cells in the early stages of apoptosis (arrow, b). A nearby circle of fluorescent staining is present in a cell that was not stained with peripherin. Scale bar = 20μm.

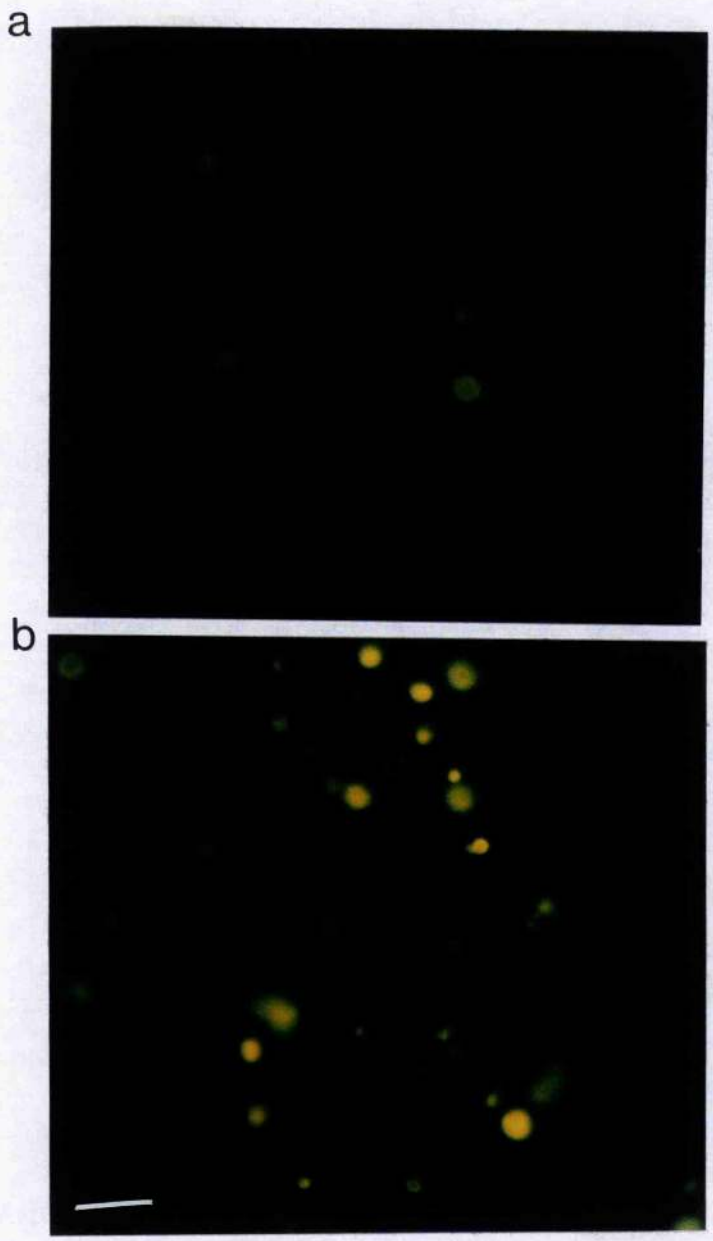


Figure 3.7. Photomicrographs of sections of the equivalent regions of E12 trigeminal ganglia of wild type (a) and *TrkB*$^{-/-}$ (b) embryos labelled with fluorescent nucleotides for 3' DNA ends generated by DNA fragmentation in apoptotic cells. An increased number of labelled cells is clearly evident in b. Scale bar = 100μm.

3.4. DISCUSSION

Several of my findings suggest that many trigeminal neurons require functional TrkB receptors for survival at an early stage in their development. First, the number of pyknotic nuclei in E11 and E12 ganglia was substantially greater in *TrkB*$^{-/-}$ embryos compared with wild type embryos. Second, the majority of cells dying in early *TrkB*$^{-/-}$ ganglia were positively identified as neurons. Third, the total number of neurons in the trigeminal ganglia of *TrkB*$^{-/-}$ embryos was substantially reduced at E13 compared with wild type neurons. TrkB is the preferred receptor for two neurotrophins, BDNF (Glass *et al.*, 1991; Klein *et al.*, 1991b; Soppet *et al.*, 1991; Squinto *et al.*, 1991) and NT4 (Berkemeier *et al.*, 1991; Ip *et al.*, 1992; Klein *et al.*, 1992). Previous *in vitro* studies have shown that both BDNF and NT4 promote the survival of the majority of embryonic mouse trigeminal ganglion neurons at an early stage in their development and that the majority of neurons lose responsiveness to these factors by E13 (Buchman and Davies, 1993; Davies *et al.*, 1993a; Paul and Davies, 1995). The finding that postnatal *BDNF*$^{-/-}$ mice (Ernfors *et al.*, 1994a; Jones *et al.*, 1994), but not *NT4*$^{-/-}$ mice (Conover *et al.*, 1995; Liu *et al.*, 1995), have marked reductions of the numbers of neurons in the trigeminal ganglion, suggests that BDNF, not NT4, is the physiologically important ligand for developing trigeminal ganglion neurons. However, because NT3 can also promote the survival of embryonic trigeminal neurons by signalling via TrkB (Davies *et al*, 1995), I cannot exclude the possibility that NT3 acting via TrkB is also important for the early survival of trigeminal neurons *in vivo*.

Although studies of postnatal *BDNF*$^{-/-}$ mice (Ernfors *et al.*, 1994a; Jones *et al.*, 1994) and *TrkB*$^{-/-}$ mice (Klein *et al.*, 1993) have shown that BDNF is important for the development of a proportion of trigeminal ganglion neurons, because the trigeminal ganglia of these animals were not studied throughout embryogenesis, it could not be ascertained at which stage of development BDNF is crucial. I have shown that BDNF is required at a very early stage of neuronal development and provide formal genetic proof for the proposal based on *in vitro* studies (Buchman and Davies, 1993; Paul and Davies, 1995) that many trigeminal neurons depend on BDNF or NT4 for survival at this stage.

Whereas the peak of cell death in the trigeminal ganglia of *TrkB*$^{-/-}$ embryos occurs earlier than in wild type embryos, Schimmang and colleagues (1995) have shown that the increased loss of neurons that occurs in the vestibular ganglia of *TrkB*$^{-/-}$ embryos occurs during the same time frame as in wild type embryos. However, unlike trigeminal neurons which lose dependence on BDNF early in their development, vestibular neurons remain dependent on BDNF for survival *in vitro* throughout the peak period of naturally occurring neuronal death (Davies *et al.*, 1986b) and do not acquire survival dependence on NGF (Davies and Lindsay, 1985). Thus, it is not unexpected that the accelerated death of TrkB-deficient vestibular neurons should occur over the same period of development as these neurons normally die when competing for a limiting supply of BDNF *in vivo*.

This study has shown that trigeminal neurons depend on functional TrkA receptors for survival at a later stage of development than their dependence on TrkB signalling. At E11 and E12, when there are substantially more dying cells in the trigeminal ganglia of *TrkB*$^{-/-}$ embryos than wild type embryos, there are

normal numbers of dying cells in the trigeminal ganglia of *trkA*$^{-/-}$ embryos. Accordingly, at E13, there is no significant difference between the total number of neurons in the trigeminal ganglia of *trkA*$^{-/-}$ and wild type embryos, whereas there is a marked reduction in the neuronal complement in the ganglion of *TrkB*$^{-/-}$ embryos by this stage. At E13 and E14, there are substantially higher numbers of dying neurons in the trigeminal ganglion of *trkA*$^{-/-}$ embryos than wild type embryos, and by E15 there is a marked reduction in the neuronal complement of the *trkA*$^{-/-}$ ganglion. The later onset of survival dependence of trigeminal neurons on TrkA signalling *in vivo* is consistent with the later acquisition of the survival response of cultured trigeminal neurons to NGF (Buchman and Davies, 1993), the preferred TrkA ligand (Hempstead *et al.*, 1991; Kaplan *et al.*, 1991; Klein *et al.*, 1991a). My findings therefore provide genetic evidence for the switch in survival dependence of early trigeminal ganglion neurons from TrkB to TrkA ligands..

The switch in survival dependence of early trigeminal ganglion neurons from BDNF to NGF is consistent with the timing and sequence of expression of the respective neurotrophin and trk genes. *BDNF* mRNA is expressed in the presumptive maxillary territory of the trigeminal ganglion as early as E9.5, before the arrival of the earliest trigeminal axons, and reaches a peak of expression at E12 (Buchman and Davies, 1993). *BDNF* mRNA is also expressed in the hindbrain from as early as E9.5, although its level remains low and there is no peak in the expression early in development (Buchman and Davies, 1993). *NGF* mRNA and protein are detected in the maxillary process with the arrival of the earliest axons at E11 (Davies *et al.*, 1987) and peaks at E13 (Buchman *et al.*, 1994). Transcripts encoding a TrkB protein that possesses a catalytic kinase domain are detected in embryonic mouse trigeminal ganglia as early as E10 and fall after E12 when transcripts for TrkB variants that lack a

kinase domain predominate (Ninkina *et al.*, 1996). The levels of transcripts for TrkA and gp75, a neurotrophin receptor that enhances the survival response of trigeminal neurons to NGF (Davies *et al.*, 1993b), increase markedly in trigeminal neurons after E12 (Wyatt and Davies, 1993) coincident with the acquisition of NGF dependence of trigeminal neurons *in vivo*.

Estimates of the number of neurons in the trigeminal ganglion towards the end of the phase of naturally occurring neuronal death at E17 indicate that during embryonic development TrkA signalling is required for the survival of more trigeminal neurons than TrkB signalling. This in turn suggests that not all trigeminal neurons depend on BDNF for survival before becoming NGF dependent. Because my data suggest that TrkB signalling is important for the survival of many neurons during the early stages of trigeminal ganglion development (at E11 and E12), it is possible that only early-born trigeminal neurons are dependent sequentially on BDNF and NGF for survival. Although detailed information on the time-course of neuronal differentiation in the mouse trigeminal ganglion is not available, the demonstration that the number of trigeminal neurons increases in the ganglion to E13 (Davies and Lumsden, 1984) and that new axons are recruited to the trigeminal nerve up to this time (Davies, 1987) suggests that trigeminal neurons are born until at least E13. The transient dependence of early-born trigeminal neurons on BDNF has been proposed to sustain the survival of many of these neurons until the majority of later-born neurons have extended axons to their targets so that most neurons would compete for target-derived NGF during the same period of development (Davies, 1994b).

Although I have observed a small, statistically significant increase in the number of pyknotic nuclei in the trigeminal ganglia of TrkC-/- embryos at E11 and E12, there was no significant reduction in the total number of neurons in the

trigeminal ganglia of TrkC$^{/-}$ embryos at E13. This is surprising given the early survival response of cultured trigeminal neurons to NT3 (Buchman and Davies, 1993; Paul and Davies, 1995) and the expression of TrkC mRNA by many neurons in the early trigeminal ganglia of the rat embryo (Ernfors *et al.*, 1992). Furthermore, there is a 61 to 64% reduction in the number of neurons in the trigeminal ganglia of postnatal *NT3*$^{-/-}$ mice (Ernfors *et al.*, 1994b; Farinas *et al.*, 1994), and recent analysis of the trigeminal ganglion of *NT3*$^{-/-}$ embryos suggests that NT3 acts early in trigeminal ganglion development to promote the survival of proliferating trigeminal neuron precursor cells (ElShamy and Ernfors, 1996b). These findings suggest that whereas NT3 plays an important role in the early development of the trigeminal ganglion, TrkC tyrosine kinase receptor does not play a major role in mediating the effects of NT3 at this stage.

Although there was no significant reduction in the neuronal complement of the *TrkC*$^{-/-}$ ganglion at E13, I observed a small, statistically significant reduction in the number of neurons in the trigeminal ganglion of E17 TrkC$^{-/-}$ embryos compared with wild type, suggesting that a small proportion of foetal trigeminal neurons depends on TrkC signalling for survival. It is known that a small proportion of neurons are supported in cultures of late foetal trigeminal cultures by low concentrations of NT3 and that these neurons are larger than those that are supported by NGF (Davies *et al.*, 1993b; Davies *et al.*, 1995). Thus, it is possible that this small, distinctive subset of neurons is lost in the *TrkC*$^{-/-}$ ganglion.

Previous studies have also revealed differences between the phenotypes of *TrkC*$^{-/-}$ and *NT3*$^{-/-}$ mice. There is a 55 to 78% reduction in the number of neurons in the lumbar DRG of *NT3*$^{-/-}$ neonates (Ernfors *et al.*, 1994b; Farinas *et al.*, 1994) compared with only a 17 to 19% reduction in the lumbar DRG of *trk*C^{-}

$^{-/-}$ neonates (Klein *et al.*, 1994; Minichiello *et al.*, 1995). Likewise, the neuronal complement of the spiral ganglion of *NT3*$^{-/-}$ neonates is reduced by 85 to 87% (Farinas *et al.*, 1994; Ernfors *et al.*, 1995) compared with a 51% reduction in *trk*C$^{-/-}$ neonates (Schimmang *et al.*, 1995). These differences are likely to be due to the ability of NT3 to signal via TrkA and TrkB in embryonic sensory neurons (Davies *et al.*, 1995). NT3 is able to promote the *in vitro* survival of the majority of trigeminal and nodose neurons obtained from E14 *trk*C$^{-/-}$ embryos but not from embryos that also lack functional TrkA or TrkB receptors. Interestingly, the ability of NT3 to signal via TrkA and TrkB in sensory neurons decreases during development, becoming negligible by E18 (Davies *et al.*, 1995). Thus, it is possible that NT3 promotes the survival of trigeminal neuron progenitor cells by acting at least in part via non-preferred trk receptor tyrosine kinases.

Chapter 4

HIGH SPECIFICITY OF NEUROTROPHINS IN THE EMBRYONIC CHICKEN TRIGEMINAL SYSTEM

4.1. INTRODUCTION

Equilibrium binding studies have shown that neurotrophin-responsive neurons possess two classes of receptors: a common low-affinity receptor that binds NGF, BDNF and NT3 with similar Kd in the range of 0.8 x 10^{-9}M to 1.7 x 10^{-9}M and specific high-affinity receptors have Kds in the region of 2 x 10^{-11}M (Sutter *et al.*, 1979; Rodriguez-Tebar and Barde, 1988; Rodriguez-Tebar *et al.*, 1990, 1992). Two kinds of cell surface receptors have been described: the common-low affinity receptor gp75 (Chao, 1994) and members of the Trk family of receptor tyrosine kinases which bind neurotrophins with higher affinity (Barbacid, 1994; Klein, 1994).

In vitro studies of neurons obtained from mice with a null mutation in the gp75 gene (Lee *et al.*, 1992) have demonstrated that gp75 is not essential for neurotrophin signal transduction but that it enhances the sensitivity of embryonic sensory and postnatal sympathetic neurons to NGF (Davies *et al.*, 1993; Lee *et al.*, 1994). Trk receptor tyrosine kinases are essential for neurotrophin signal transduction (Barbacid, 1994; Klein, 1994) and initiate a cascade of protein phosphorylations in cells following ligand binding (Kaplan and Stephens, 1994).

Expression studies in cell lines have shown that TrkA is the receptor for NGF (Kaplan *et al.*, 1991; Klein *et al.*, 1991), TrkB is the receptor for BDNF and NT4 (Berkemeier *et al.*, 1991; Glass *et al.*, 1991; Klein *et al.*, 1991, 1992; Soppet *et al.*, 1991; Squinto *et al.*, 1991; Ip *et al.*, 1992) and TrkC is the receptor for NT3 (Lamballe *et al.*, 1991). In addition to these preferred receptor/ligand interactions, studies of Trk receptor tyrosine kinases expressed in fibroblasts have shown that NT3 is also able to signal through TrkA and TrkB (Lamballe *et al.*, 1991; Soppet *et al.*, 1991; Squinto *et al.*, 1991) and that NT4/5 is able to signal through TrkA (Berkemeier *et al.*, 1991). Likewise, NT3 can signal via TrkA and TrkB in developing sensory neurons (Davies et al, 1995).

In contrast to the cross-activation of high-affinity neurotrophin receptors by non-preferred ligands in fibroblasts, binding and cross-activation are only observed with much higher concentrations of non-preferred ligands in neuronal cell lines and neurons. For example, whereas the BDNF and NT4/5 dose-responses of TrkB-expressing PC12 cells and TrkB-expressing fibroblasts are similar, 100-fold higher concentrations of NT3 are required to elicit responses from TrkB-expressing PC12 cells compared with TrkB-expressing fibroblasts (Ip *et al.*, 1993). Likewise, a 10-fold higher concentration of NT3 is required to prevent BDNF binding to TrkB-expressing sensory neurons than to a TrkB-expressing kidney cell line (Dechant *et al.*, 1993a). Despite the higher ligand specificity of Trk receptor tyrosine kinases in neuronal cell lines and neurons, neurotrophins can be displaced from their respective high-affinity receptors on neurons by very high concentrations of heterologous neurotrophin ligands. The binding of NGF and BDNF to their respective high-affinity receptors on sympathetic and dorsal root ganglion (DRG) neurons is reduced to 50% by 13-fold and 36-fold molar excesses of NT3, respectively (Dechant *et al.*, 1993a, 1993b). 1,000-fold excesses of NGF and BDNF are required to cause a 50%

reduction in the binding of each other to their respective high-affinity receptors on DRG neurons (Rodriguez-Tebar *et al.*, 1990). The binding of NT3 to its high-affinity receptor on sympathetic and dorsal root ganglion (DRG) neurons is reduced to 50% by 1,300-fold and 2,100-fold molar excesses of NGF and BDNF, respectively (Rodriguez-Tebar *et al.*, 1992). Very high concentrations of neurotrophins can also exert functional responses from neurons expressing heterologous high-affinity neurotrophin receptors. For example, NGF at a concentration of 5 μg/ml is able to promote the survival of the BDNF-dependent nodose ganglion neurons of the E6 chicken embryo (Rodriguez-Tebar *et al.*, 1990). At concentrations of NT3 sufficient to completely saturate high-affinity BDNF receptors on E8 nodose ganglion neurons (20 μg/ml), the survival-promoting effects of saturating concentrations of BDNF were completely blocked (Dechant *et al.*, 1993a). These studies show that very high concentrations of neurotrophins can exert either agonist or antagonist effects on heterologous high-affinity neurotrophin receptors.

Although the concentrations of neurotrophins used in the above studies are greater than the anticipated physiological range, these studies provide clear illustrations of the differing specificities of neurotrophin receptors in different cellular environments and give an indication of the capacity of different neurons to distinguish between neurotrophins. The aim of the current study was to extend this experimental analysis to the well-characterised neuronal populations of chicken embryo trigeminal system which have different and distinctive neurotrophin survival requirements during the mid-embryonic period of development. In particular, I wished to ascertain whether the agonist and antagonist actions of high concentrations of neurotrophins observed for previously studied neurons can be generalised to other populations of neurons. Previous work has shown that the proprioceptive neurons of the trigeminal

mesencephalic nucleus (TMN) are supported by physiological concentrations of BDNF (Davies *et al.*, 1986a) and NT3 (Hohn *et al.*, 1990) but not by NGF (Davies *et al.*, 1987). The small-diameter neurons of the dorsomedial pole of the trigeminal ganglion (DMTG) consist predominantly of NGF-dependent neurons and very few BDNF-dependent neurons, whereas the large-diameter cutaneous sensory neurons of the ventrolateral pole of the trigeminal ganglion (VLTG) consist predominantly of BDNF-dependent neurons and very few NGF-dependent neurons (Davies *et al.*, 1986b). My studies of these neurons have revealed no significant agonist or antagonist effects of very high concentrations of neurotrophins acting on heterologous neurotrophin receptors, demonstrating that the specificity of neurotrophins for these neurons is far higher than shown previously for other neurons.

4.2. METHODS

The tissue culture procedures used in these experiments were exactly the same as the ones described in Chapter 2 . The neurons used in this study were from the ventrolateral portion of the trigeminal ganglia (VLTG), the dorsomedial portion of the trigeminal ganglia (DMTG), which were previously described in details in Chapter 2 and the trigeminal mesencephalic nucleus (TMN). The TMN neurons are primary sensory neurons derived from the neural crest and are located in the midbrain (Fig.4.1).

Non neuronal cells were removed from the dissociated suspension of cells by differential sedimentation (as described in Chapter 2). The column fractions containing the neurons were centrifuged at 2000g for 5 min and the neurons were plated in the 11mm diameter wells of four-well culture dishes that had been coated with polyornithine and laminin. The cells were cultured in 100 µl of Ham's F14 medium supplemented with 10% HIHS, penicillin (60 mg/l), streptomycin (100 mg/l) and 24 mM $NaHCO_3$ with or without neurotrophins. The pure neuronal suspension was plated in 4 well dishes to make feasible the use of high concentrations of neurotrophins. In the case of treatment with two neurotrophins, they were added at the same time; at the plating time.

The survival was assayed as described in Chapter 2.

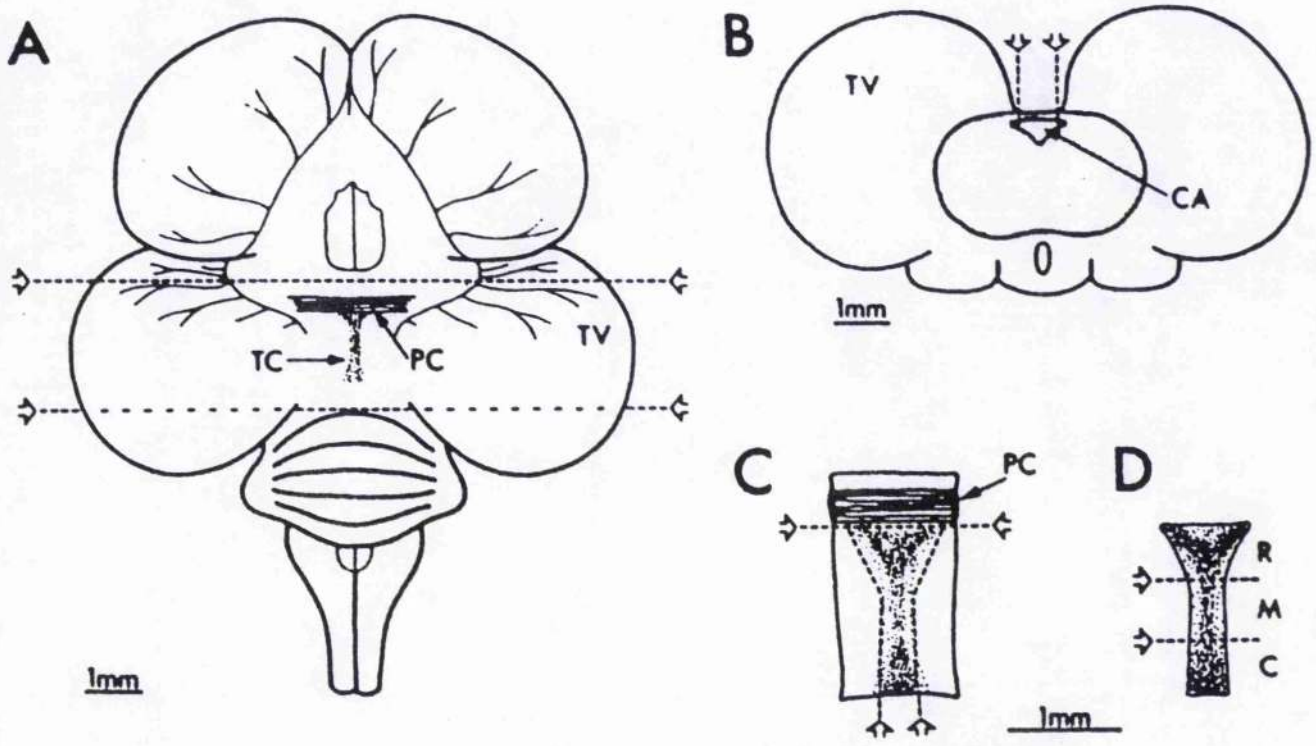


Figure 4.1. Drawings of the dissection of a chicken TMN from an E12 embryo. (A) Dorsal aspect of the brain showing the location of the two coronal incisions (interrupted lines) for isolating the midbrain. (B) caudal aspect of the isolated midbrain showing the location of the two parasagital incisions for removing the roof of cerebral aqueduct after careful striping of the overlaying pia matter. (C) Dorsal aspect of the cerebral aqueduct showing the location of the incisions for cutting out the median part of the TMN. Tectal vesicle (TV), tectal comissure (TC), posterior comissure (PC), cerebral aqueduct (CA); (D) Rostral (R), Medial (M) and Caudal (C) TMN. From Davies, 1988.

4.3. RESULTS

TMN neurons

All TMN neurons died in control cultures by 48 hours incubation. At this time, the survival of almost 80% of the neurons was promoted by saturating levels of BDNF (2 ng/ml) and very high concentrations of BDNF (5 μg/ml) promoted the survival of similar numbers of neurons (Fig. 4.2). In contrast, neither 2 ng/ml nor 5 μg/ml of NGF, which is over 6 orders of magnitude greater than the half-maximally effective concentration (EC_{50}) of NGF acting on E6 DMTG neurons (Chapter 2 of this thesis), promoted the survival of TMN neurons. NGF at 25 μg/ml also failed to promote the survival of TMN neurons. These results indicate that NGF at very high concentrations does not act as an agonist at the BDNF receptor expressed in TMN neurons.

To investigate whether NGF at very high concentrations could act as an antagonist at the BDNF receptor in TMN neurons, these neurons were grown with saturating and subsaturating levels of BDNF plus 5 μg/ml of NGF. Figure 4.3 shows that the dose responses of TMN neurons to BDNF were very similar in the presence and absence of NGF. These results indicate that very high concentrations of NGF do not antagonise the action of BDNF on TMN neurons.

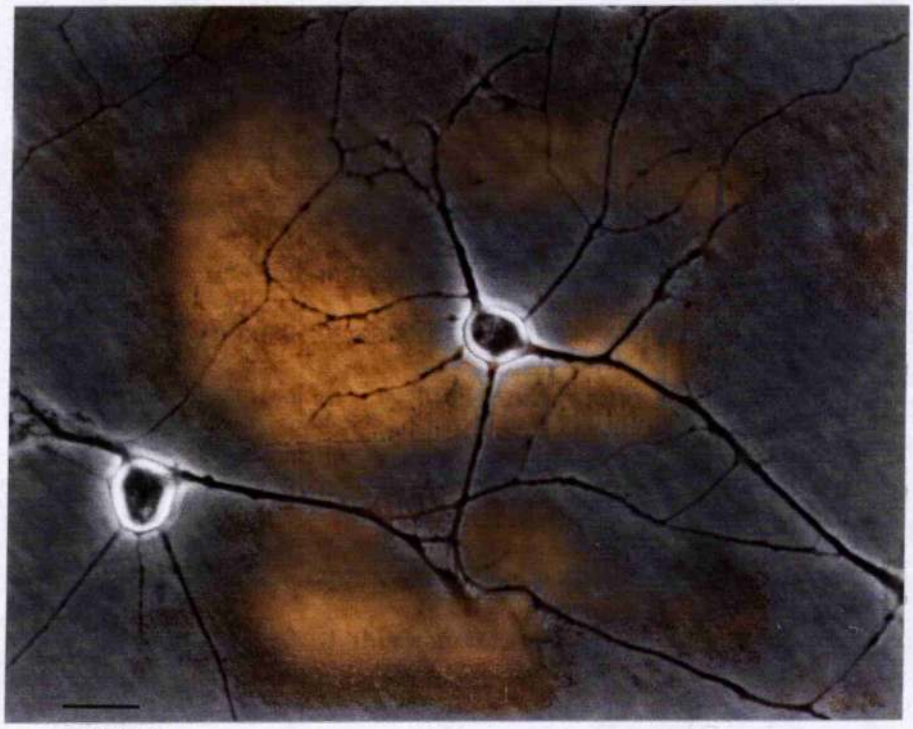


Figure 4.2. Phase contrast micrograph of two E10 trigeminal mesencephalic neurons after 48 h in culture in medium supplemented with BDNF (5µg/ml)

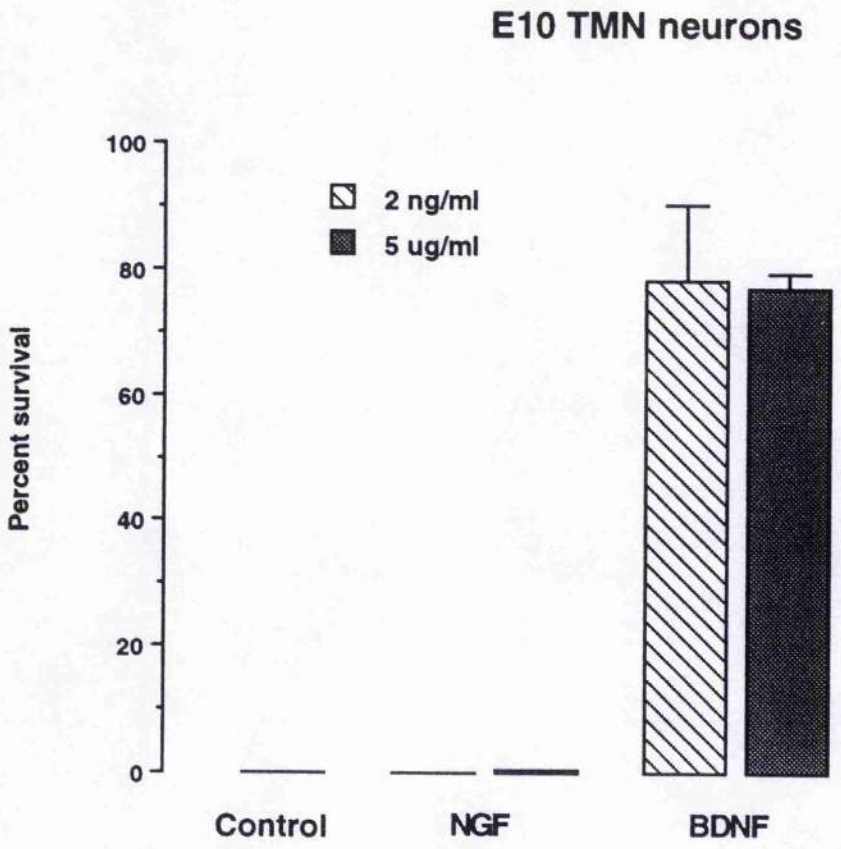


Figure 4.3. Bar chart of the percent survival of E10 TMN neurons in control cultures and cultures containing either NGF or BDNF at concentrations of 2 ng/ml and 5 μg/ml. The mean and standard error are shown.

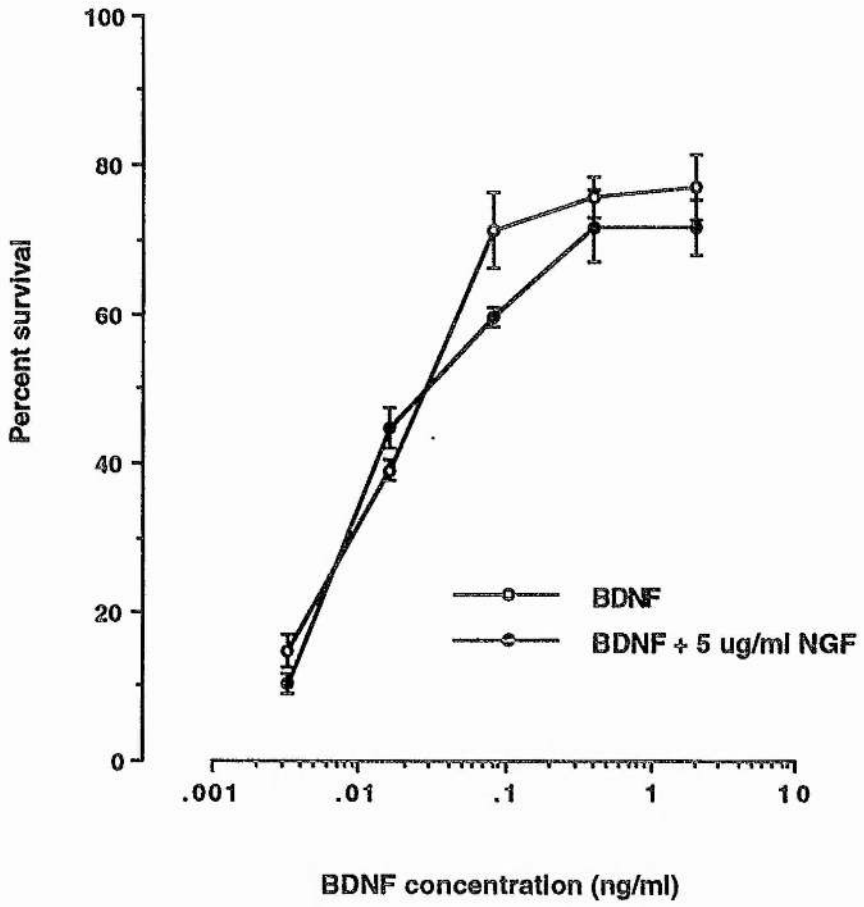


Figure 4.4. Graph of the percent survival of E10 TMN neurons grown with BDNF at concentrations ranging from 3.2 pg/ml to 2 ng/ml either alone or with NGF at a concentration of 5 μg/ml at each BDNF concentration. The mean and standard error are shown.

VLTG neurons

Like TMN neurons, all VLTG neurons died in control cultures by 48 hours incubation and almost 80% were supported by BDNF (Fig.4.5). Previous work has shown that VLTG neurons include a minor subset of NGF-responsive neurons in addition to BDNF-responsive neurons (Davies *et al.*, 1986b). In the current study, 5% of the VLTG neurons were supported by 2 ng/ml NGF, and increasing the NGF concentration to 5 μg/ml caused only a further 10% increase in the number of surviving neurons. Eight percent of VLTG neurons were supported by 2 ng/ml of NT3. Increasing the NT3 concentration to 5 μg/ml caused only a small additional increase (to 14%) in the number of surviving neurons. These data indicate that neither NGF nor NT3 at very high concentrations act as an agonists at the BDNF receptor expressed on the majority of VLTG neurons.

To investigate whether NT3 at very high concentrations could act as an antagonist at the BDNF receptor in VLTG neurons, these neurons were grown with saturating and subsaturating levels of BDNF plus 5 μg/ml of NT3. Figure 4.6 shows that the dose responses of VLTG neurons to BDNF were very similar in the presence and absence of NT3. These results indicate that very high concentrations of NT3 do not antagonise the action of BDNF on the great majority of VLTG neurons.

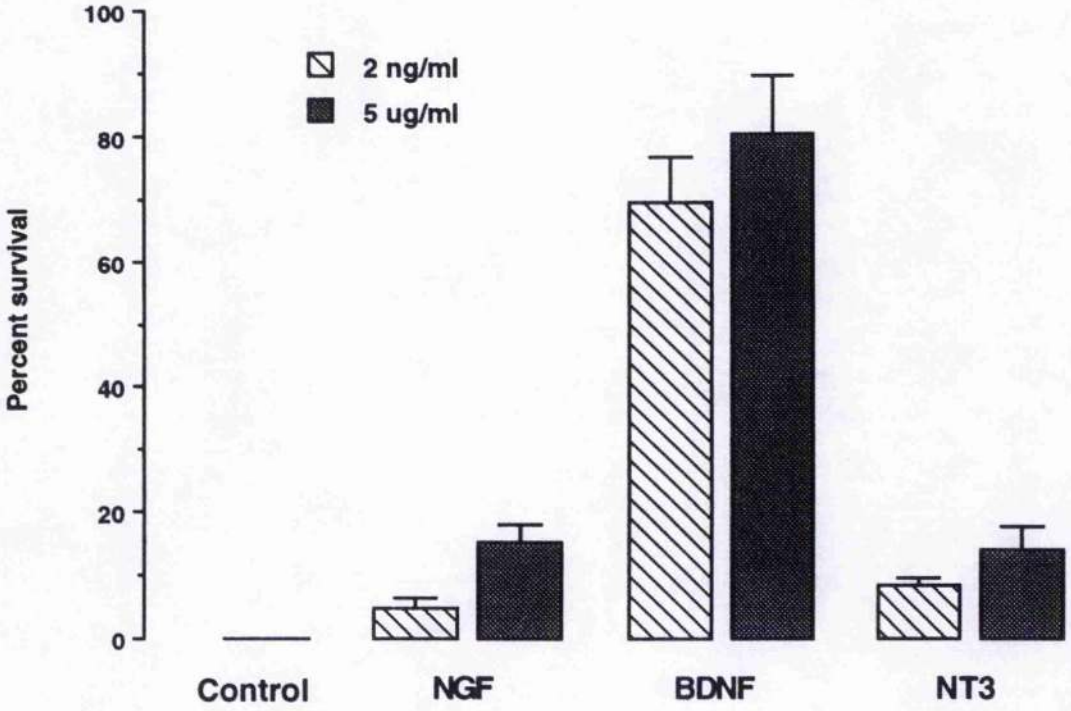


Figure 4.5. Bar chart of the percent survival of E10 VLTG neurons in control cultures and cultures containing NGF, BDNF or NT3 at concentrations of 2 ng/ml and 5 μg/ml. The mean and standard error are shown.

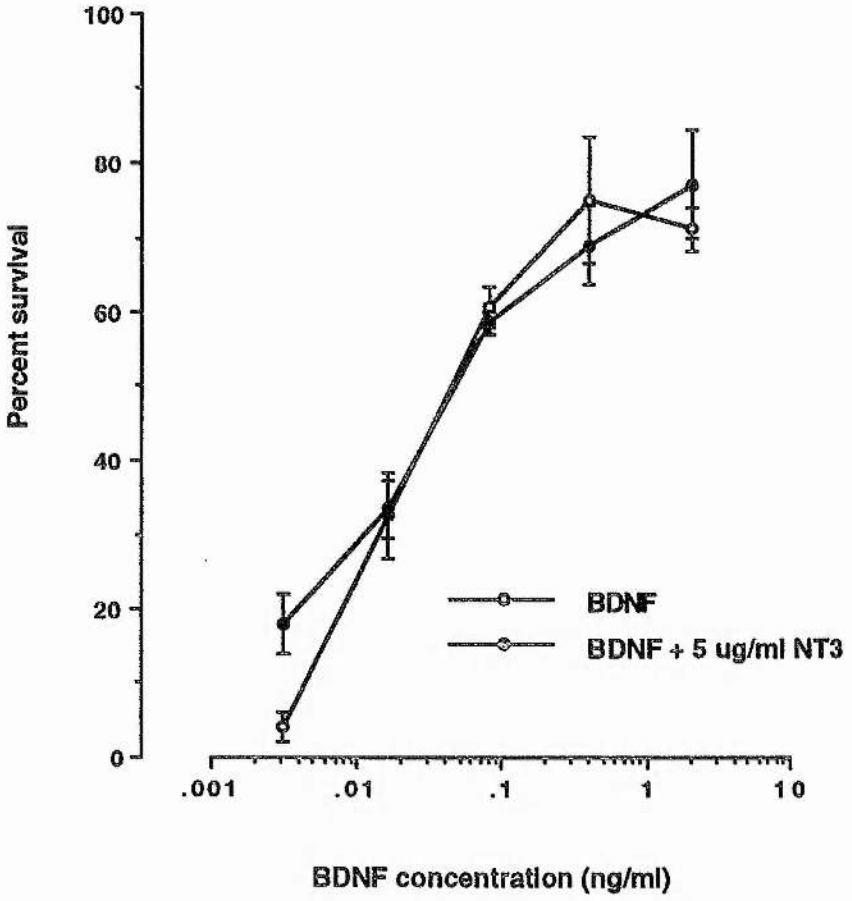


Figure 4.6. Graph of the percent survival of E10 VLTG neurons grown with BDNF at concentrations ranging from 3.2 pg/ml to 2 ng/ml either alone or with NT3 at a concentration of 5 μg/ml at each BDNF concentration. The mean and standard error are shown.

DMTG neurons

The great majority of DMTG neurons died in control cultures by 48 hours incubation and 75% of the neurons were supported by 2 ng/ml of NGF (Fig. 4.7). Previous work has shown that DMTG neurons include a minor subset of BDNF-responsive neurons in addition to the major set of NGF-responsive neurons (Davies *et al.*, 1986b). Figure 4.7 shows that almost 15% of the DMTG neurons were supported by 2 ng/ml BDNF. Increasing the BDNF concentration to 5 μg/ml did not further enhance the survival of DMTG neurons but caused a small decrease in the number of surviving neurons. These data clearly indicate that BDNF at very high concentrations does not act as an agonist at the NGF receptor expressed on the majority of DMTG neurons. NT3 at a concentration of 2 ng/ml promoted the survival of 7% of the DMTG neurons, but increasing its concentration caused a greater than 4-fold increase in the number of surviving neurons to over 30%. Thus, it is possible that very high concentrations of NT3 could promote neuronal survival by acting on the NGF receptor in an appreciable proportion of DMTG neurons.

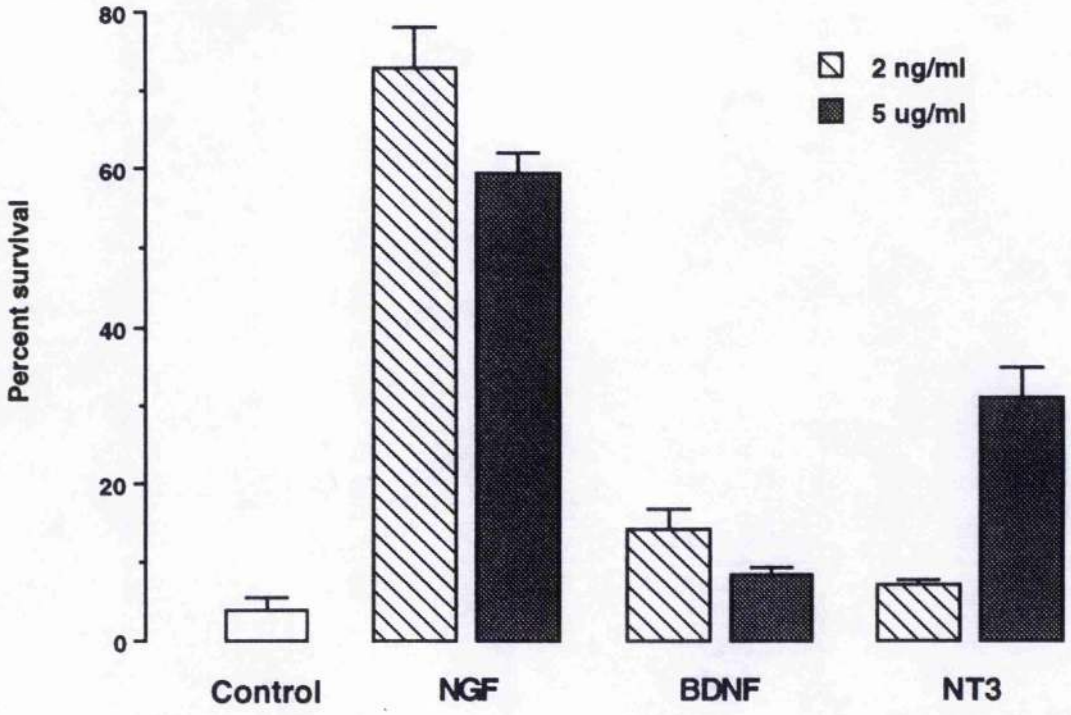


Figure 4.7. Bar chart of the percent survival of E10 DMTG neurons in control cultures and cultures containing NGF, BDNF or NT3 at concentrations of 2 ng/ml and 5 µg/ml. The mean and standard error are shown (n=3).

4.4. DISCUSSION

I have shown that neurotrophins exhibit a very high degree of specificity in the embryonic chicken trigeminal system. Whereas previous work has shown that a high concentration of NGF (5 μg/ml) can promote the survival of the BDNF-dependent neurons of the E6 nodose ganglion (Rodriguez-Tebar *et al.*, 1990), I have demonstrated that the same high concentration of NGF has no effect on the survival of BDNF-dependent E10 TMN neurons. Moreover, 5 μg/ml of NGF has no effect on the dose response of TMN neurons to BDNF even at BDNF concentrations that are over 1.5 million-fold lower (3.2 pg/ml). Likewise, 5 μg/ml of NGF has negligible effect on the survival of the BDNF-dependent VLTG neurons. In the converse experiment, I have shown that a high concentration of BDNF (5 μg/ml) has no effect on the survival of NGF-dependent E10 DMTG neurons. Thus, in the trigeminal system I find no evidence for agonist effects of NGF and BDNF acting at heterologous receptors at concentrations that are 6 orders of magnitude (Chapter 2 of this Thesis) greater than the EC_{50} for the preferred neurotrophin.

In contrast to the antagonist effects of high concentrations of NT3 on the response of E8 nodose neurons to saturating levels of BDNF (Dechant *et al.*, 1993a), I did not observe any antagonistic action of high concentrations of NT3 on the response of E10 VLTG neurons to saturating or subsaturating levels of BDNF. The EC_{50} for BDNF acting on these neurons was unaffected by the presence of high concentrations of NT3. Taken together, these findings clearly demonstrate that some populations of neurons are able to discriminate between

neurotrophins to a far greater extent than was previously appreciated. The only exception in the avian trigeminal system is the response of DMTG neurons to NT3, which is negligible with 2 ng/ml NT3 but increases to almost half of the number of neurons surviving with NGF with 5 μg/ml NT3. Previous *in vitro* studies of embryonic chicken DMTG neurons have shown that these neurons respond to low concentrations of NT3 at E6 and that the NT3 dose response shifts to higher NT3 concentrations with age (Chapter 2 of this Thesis). Thus, the response of DMTG neurons to NT3 at E12 essentially represents the tail-end of this response. Whether this response is mediated by very low, residual levels of TrkC expression at this stage or whether it is mediated via TrkA is not known.

What is the explanation of the differing abilities of neurons to discriminate between neurotrophins? The demonstration that cross-activation of Trk receptor tyrosine kinases by heterologous neurotrophins is more restricted in neuronal cell lines than in fibroblast cell lines (Ip *et al.*, 1993), suggests that the molecular environment of the cell in which these receptors is expressed affects their ability to discriminate between neurotrophins. With regard to NT3 discrimination, there is some evidence to suggest that gp75 may be involved. gp75 is expressed in PC12 cells but not fibroblasts (Ip *et al.*, 1993) and expression of a truncated gp75 receptor in PC12 cells results in decreased expression of wild type gp75 and increased neurite outgrowth in response to NT-3 (Benedetti *et al.*, 1993). Function-perturbing antibodies to gp75 also potentiate the response of PC12 to NT3 (Clary and Reichardt, 1994). Moreover, sympathetic neurons from postnatal mice with a null mutation in the gp75 gene are more sensitive to NT3 than sympathetic neurons from wild type animals (Lee *et al.*, 1994). Thus, direct or indirect interactions between Trk receptor tyrosine kinases and other co-expressed molecules may enhance the ability of Trk receptor tyrosine kinases to discriminate between neurotrophins. The differing

abilities of various kinds of neurons to discriminate between neurotrophins could perhaps be due to differences levels of expression of these putative molecules. Alternatively, there could be structural variations in the ligand binding domains of Trk receptor tyrosine kinases expressed in different neurons that are responsible for differences in ligand discrimination. For example, TrkA and TrkC isoforms have been described that differ in their extracellular domains (Barker *et al.*, 1993; Garner and Large, 1994) and a TrkA isoform that contains an 18 bp exon in the extracellular domain shows significantly higher activation by NT3 in PC12 cells compared with the isoform that lacks this exon (Clary and Reichardt, 1994).

Why do neurons differ in their capacity to discriminate between neurotrophins? The concentrations of neurotrophins used in this and other studies (Rodriguez-Tebar *et al.*, 1990; Dechant *et al.*, 1993a; Dechant *et al.*, 1993b) to test the ability of neurons to discriminate between neurotrophins are likely to be much greater than those normally encountered by neurons *in vivo*. Yet, quite clear differences were observed in the ability of neurons to discriminate between neurotrophins. Although these differences are clear-cut at very high neurotrophin concentrations, lower concentrations of the heterologous neurotrophin might have important *in vivo* effects on neurons whose discrimination between neurotrophins is not as stringent as others. Perhaps some degree of overlapping neurotrophin responses is important for some developing neurons whereas absolute neurotrophin specificity is essential for others.

Chapter 5

TRKC mRNA EXPRESSION IN CHICKEN CRANIAL SENSORY AND SYMPATHETIC GANGLIA DURING DEVELOPMENT.

5.1. INTRODUCTION

The data presented in Chapter 2 demonstrates that chick neural-crest derived neurons show a marked decrease in the response to NT3 and BDNF during development, whereas NGF responsiveness remains unchanged. Curiously, neuronal populations that are placode-derived and BDNF dependent such as nodose and VLTG, do not appear to be additionally NT3 or NGF responsive at earlier developmental stages.

Mouse trigeminal neurons growing at low density *in vitro* also display a transient survival response to NT3 and BDNF during the earliest stages of target field innervation, before the onset of natural neuronal death and NGF dependence. The responsiveness to BDNF and NT3 is predominantly lost later in development as these neurons become dependent on NGF, however, a small subset of trigeminal neurons remain responsive to NT3 *in vitro* (Buchman and Davies, 1993). The data presented in chapter 3 backs up the results of these *in vitro* studies. A null mutation of the TrkB gene results in an early peak of cell death, compared with wild-type animals, coinciding with the stage when trigeminal neurons are responsive to BDNF *in vitro*. In the absence of TrkA,

mouse trigeminal neurons undergo increased cell death between E13 and E14, a time when they are responsive to NGF *in vitro* (Chapter 3 of this thesis; Piñón *et al*, 1996).

Evidence is beginning to accumulate to suggest that the ability of a neuronal population to respond to neurotrophins, at least in the case of BDNF and NGF, is regulated at the level of neurotrophin receptor expression. The acquisition of NGF dependence in mouse trigeminal neurons is accompanied by a marked up-regulation in the expression of TrkA mRNA (Wyatt and Davies, 1993). Trigeminal neurons express very low levels of TrkB transcripts during the stage of neurotrophin independence. Between E10 and E12, when trigeminal neurons respond to BDNF, there is high level expression of a 9Kb transcript encoding the full length catalytic isoform of TrkB. The level of this transcript decreases after E12 as trigeminal neurons lose responsiveness to BDNF. In addition, there is a concomitant increase in the expression of 8.2Kb and 2.5 Kb transcripts that code for TrkB variants lacking the tyrosine kinase domain (Ninkina *et al*, 1996). It has been proposed that truncated forms of TrkB may act in a dominant negative manner to quench TrkB signalling and BDNF responsiveness. The expression of mRNA encoding the full length TrkB isoform also parallels the acquisition of BDNF responsiveness in chick vestibular and nodose neurons (Robinson and Davies, 1996).

Because Trk receptors (Klein *et al*, 1991; Radeke and Feinstein, 1991; Holtzman *et al*, 1992) and gp75 (DiStefano *et al*,, 1992; Chao, 1994) can each mediate biological activities independently, it is not clear whether their expression is temporally correlated in neurotrophin responsive cells. In motoneurons there is a correlation between the onset of TrkB, but not of the gp75, expression and BDNF sensitivity (McKay *et al*, 1996). While this does not

rule out a role for gp75 in mediating the effects of BDNF, it does indicate that TrkB alone may be capable of mediating the survival effects of BDNF (Weskamp and Reichardt, 1991). This may be also true among sensory neurons since high levels of TrkB are expressed by a sub-population of DRG neurons, (presumably by cells responsive to BDNF), yet these cells express very little gp75 (Ernfors *et al*, 1988). In mouse trigeminal and superior cervical ganglia, gp75 and TrkA mRNAs are co-expressed from the earliest ages in development (Wyatt and Davies, 1993), whereas in developing chick vestibular and nodose ganglia gp75 mRNA expression precedes that of TrkB mRNA (Robinson et al, 1996). While gp75 may increase the affinity of Trk proteins for neurotrophins, the role of gp75 in the context of neurons *in vivo* remains unclear. One possible explanation to account for the observed switch of neurotrophin responsiveness in developing neurons is that variations in the ratios of the high and low affinity receptors could affect the ability of neurons to discriminate between neurotrophins. The low affinity neurotrophin receptor, gp75, has been shown to increase the affinity of TrkA expressing cell lines for NGF (Benedetti *et al*, 1993).

Determining the role that changing neurotrophin receptor expression plays in regulating the transient NT-3 survival response in neural-crest derived neuronal populations is potentially problematic. Unlike NGF and BDNF which show good receptor specificity, NT-3 can mediate biological effects via TrkA and TrkB in addition to its preferred receptor, TrkC (Lamballe *et al*, 1991; Davies *et al*, 1995). Any, or all of these receptors may therefore play a role in mediating the transient NT-3 survival response in developing neural crest derived neurons. Rat sympathetic neurons, like neural crest derived sensory neurons, also show a brief early NT-3 survival response in culture before they become NGF dependent (Di Cicco-Bloom *et al*, 1993). The sequence of TrkC and TrkA

receptor gene expression detected in sympathetic neurons *in vivo* parallels the progression from NT-3 to NGF responsiveness. TrkC mRNA is highly expressed in mouse E15.5 SCG neurons, but decreases more than ten-fold by post-natal day 1. There is a reciprocal pattern of expression for TrkA mRNA whereby initial low levels of expression are increased 40-fold by birth (Birren *et al*, 1993; DiCicco-Bloom *et al*, 1993;Verdi and Anderson, 1994). Thus, in the case of sympathetic neurons, stage-specific responsiveness to NT-3 may reflect developmentally regulated expression of TrkC. In accordance with the lack of response of sympathetic neurons to BDNF, TrkB transcripts are not detectable in sympathetic neurons at any developmental stage (Fagan *et al*, 1996).

In chick embryos, TrkC mRNA is present in neuron progenitor cells in the nascent, trigeminal ganglion at stage 13 (Williams *et al*, 1995). TrkB mRNA expression does not appear until stage 19, peaks at E9 and then falls sharply to much lower levels by E18. TrkA mRNA is the last receptor mRNA to appear at stage 21/22 (embryonic day 3.5). TrkC mRNA has been detected as early as E3 in the avian neural tube and DRG (Pinco *et al*, 1993; Kahane and Kalcheim, 1994; Zhang *et al*, 1994). In the early stages of development, TrkC transcripts are only present in neural crest cell populations that have neurogenic potential and subsequently by a sub-population of differentiated neurons (Henion *et al*, 1995). A detailed analysis of early quail DRGs indicates that TrkC expression is limited to neuroblasts, and not to other cell types (Zhang *et al*, 1994; Henion et al, 1995). The very early onset of TrkC mRNA expression, prior to the appearance of an NT-3 survival response in neural crest-derived sensory neurons, is consistent with the multiple roles that NT3 appears to play in neurogenesis. Experimental evidence suggests that NT-3 can act as a differentiation factor, promote the survival of proliferating sympathetic and sensory neuroblasts and induce terminal mitosis in sympathetic and sensory precursors (Kalcheim *et al*,

1992; Write *et al*, 1992; DiCicco-Bloom *et al*, 1993; Gaese *et al*, 1994; ElShamy and Ernfors, 1996 a, b; El Shamy et al, 1996; Verdi and Anderson, 1994; Ockel *et al.*, 1996).

In an attempt to determine the role that TrkC plays in mediating the early transient NT-3 survival response of sympathetic and sensory neurons, I have assayed the levels of TrkC mRNA in developing chick sensory and sympathetic ganglia. TrkC mRNA levels were assayed using both a sensitive quantitative RT-PCR technique (Wyatt and Davies, 1993) and a less precise semi-quantitative RT-PCR assay. The results show that TrkC mRNA is highly expressed early in development in these ganglia, and that TrkC mRNA levels fall at later stages of development. I have also investigated the regulation of TrkC mRNA expression in low density cultures of E6 DMTG neurons which were cultured for 24 or 48h in the presence of different neurotrophins. Using this approach I have found that NT3 upregulates TrkC mRNA expression *in vitro*.

5.2. METHODS

DISSECTION AND CELL CULTURE TECHNIQUES

The neurons used in this study were from the DMTG and the lumbosacral paravertebral sympathetic chain. All procedures were carried out using standard sterile technique in a laminar flow hood. The DMTG dissection has already been described in Chapter 2. The separation of the neurons by differential sedimentation and the culture procedures were also described in Chapter 2.

The sympathetic chain

The sympathetic chain was removed using a pair of watchmaker's forceps after removing the thoracic and abdominal viscera .The chain was removed from mid-thoracic to sacral regions and separated from connective tissue using sharpened tungsten needles (Fig 5.1). The dissociation procedure was the same as that described for the other neurons in the Methods Section of Chapter 2. The neurons from the sympathetic chain ganglia are quite small early in development therefore it was only possible to separate neuronal cells from non-neuronal cells by differential sedimentation from E10 onwards.

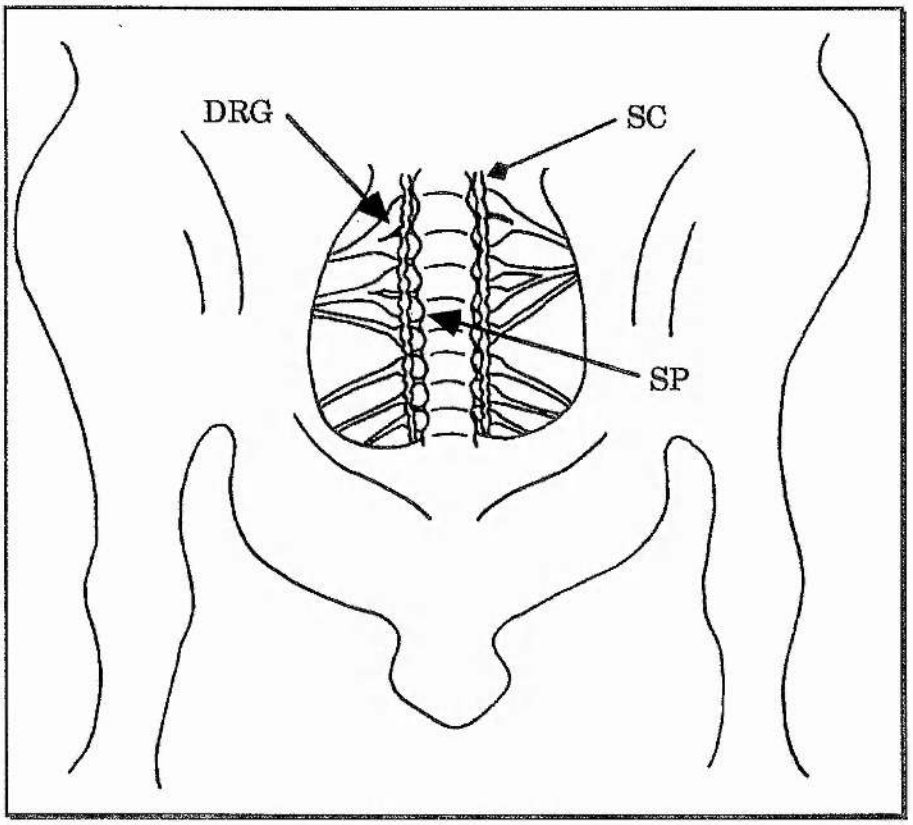


Figure 5.1. Camera lucida drawing of the ventral aspect of the lumbosacral region of an E10 chick embryo after evisceration showing the location of the sympathetic ganglia (Adapted from Davies, 1988).DRG: dorsal root ganglia; SC: sympathetic chain; SP: spinal cord.

MOLECULAR BIOLOGY TECHNIQUES

1. RNA Isolation and Purification

The acid guanidinium thiocyanate RNA extraction method described by Chomczynski and Sacchi, (1987) provides a pure preparation of undegraded RNA in high yield. It is particularly useful for processing large numbers of samples and for isolation of RNA from minute quantities of cells or tissue samples. Immediately after removal from the animal, tissue was minced on ice and homogenised at room temperature with 500 µl of solution D (4M guanidiniun thiocyanate, 25mM sodium citrate, pH 7.0, 0.5% sarcosyl and 0.1M 2-mercapto-ethanol) in an Eppendorf tube. The following were added sequentially to the homogenate; 1/10 volume of 2M sodium acetate, pH 4, the same volume of water saturated phenol and 2/10 volumes of chloroform-isoamyl alcohol mixture (24:1). The reagents were mixed thoroughly by inversion after each addition . The final suspension was shaken vigorously for 10 sec, cooled on ice for 15 min and microfuged at 14,000 rpm for 15 min at 4°C. Following removal of the upper aqueous phase to a fresh Eppendorf, RNA was precipitated by the addition of two volumes of absolute ethanol or 0.6 volumes of isopropanol. The precipitate was allowed to form at -20°C overnight. RNA was then recovered by microfuging at 14,000 rpm for 20 min and the resulting pellet was washed thoroughly with 70% ethanol before being allowed to dry. After resuspending the RNA pellet in 50µl of a buffer containing; 10mM Tris, 10 mM $MgCl_2$ and 20mM vanadyl ribonucleoside, contaminating DNA was removed by incubation with 20-30 units of RNA-free DNaseI, for 1 hour, at 37°C. After this treatment the RNA tubes were removed to a 'DNA free' clean room that was

reserved for setting up PCR reactions. The RNA was further purified using the RNAID kit from BIO 101. In brief, three volumes of 3M NaClO3 were added to the RNA followed by 5-20 µl of RNA binding matrix (the exact volume was dependent on the amount of starting tissue or cells). After thoroughly mixing, the slurry was incubated at room temperature for 10 min to allow the RNA to bind to the matrix. The matrix was sedimented by brief centrifugation and washed with 500 µl of RNAID wash solution. This wash procedure was repeated twice followed by a final sedimentation of the matrix and complete removal of the wash solution. The RNA was then eluted from the matrix material by resuspension in 10- 50 µl of pure water (the exact amount depending of the amount of starting tissue or cells), followed by heating at 60°C for 5 min. Following centrifugation for 1 min at full speed, the RNA solution was removed to a fresh Eppendorf tube and stored at -80°C until required.

2. Cloning the cDNA competitor templates by PCR

In order to mate the cRNA control or competitor species used to calibrate the quantitative RT-PCR assay, it was first necessary to isolate a fragment of TrkC cDNA encoding part of the tyrosine kinase domain.

2.a) Primer selection and PCR reaction

TrkC cDNAs were isolated by PCR using first strand cDNA from E8 chick brain. The cDNA synthesis reaction consisted of:

20 µl of Gibco Superscript buffer
10 µl of 0.1 M DTT
10 µl 5µM dNTPs
1µl 100 µM Randon Hexanucleotides

2.5 µl RNA guard
1µl of RNA from E8 chicken brain
55 µl H_2O

After incubating this mixture for a few minutes at 37°C, 1µl of Superscript (Gibco) was added and incubation was continued for further 30 minutes.

To clone a cDNA fragment corresponding to the chicken TrkC tyrosine kinase domain, primers in the tyrosine kinase domain were designed based on alignment studies between the three chicken Trks (Fig 5.2). The regions chosen for primers contained the most mismatches between TrkC and the other Trks. The primers proved to be very specific, since the amplification using these primers produced only one single band (Fig 5.3). These primers were:

sense: 5' TCCGACAAGGTCACAACTG 3'

antisense: 5' TTGGCAGCCCGCAAGGAT 3'

PCR cycling conditions were; 40 cycles consisting of 94°C for 45 seconds, followed by 57°C for 30 seconds followed by 72°C for 30 seconds.

The PCR reaction mixture consisted of:

10µl of first strand chicken cDNA from whole brain at E8.
1µl of dNTP 5mM
4µl 10X Taq Buffer
1µl of $MgCl_2$
1µl Taq polymerase
5µl of each primer at 15nM
13µl H2O

The PCR products were run out on a 1.5% low melting agarose mini gel and the DNA band of interest was removed from the gel with a sharp razor blade. The agarose slice containing TrkC DNA was melted in three volumes of STE and

subjected to phenol chloroform extraction. Basically, the same volume of phenol was added and the aqueous phase and organic phases were thoroughly mixed. The two phases were separated by centrifuging for 10 min at 13000 rpm and the supernatant was transferred to a fresh Eppendorf tube. This process was repeated twice and was followed by an extraction with a mixture containing equal volumes of phenol and chloroform. The DNA present in the supernatant was precipitated by the addition of 2 volumes of ethanol and 1/10 vol of 3M NaAc. The precipitation was carried out overnight. The following morning, DNA was recovered by centrifuging for 5 min at 13000 rpm and the resulting DNA pellet was washed twice with 70% ethanol. The dried DNA was resuspended in 30-50 µl of H_2O. The purified DNA was then ligated into pGEM-T vector (Promega) according the manufacturers recommendations. The ligation mixture was used directly to transform highly competent *E. coli* Xl 1 cells (Maniatis, 1989).

2.b) Introducing Plasmids into E. Coli

Competent cells (E. Coli XL1 Blue MRF') were kept at -70°C. They were removed from the freezer and defrosted on ice. The cells were kept on ice throughout the whole of this procedure. 10µl of the ligation mixture was added to 100µl of competent cells in an Eppendorf and the mixture was incubated on ice for 40 minutes.

The tube was then transferred to a water bath at 42°C for 2 minutes (heat shock). The cells were returned to ice and 10 volumes of LB-medium, without antibiotic, were added. The cells were incubated at 37°C for 40 minutes before they were plated onto petri dishes containing LB agar and 100mg/ml Ampicillin. This media was selective for cells containing the plasmid as the vector carried an

ampicillin resistance gene. In some cases X-Gal and IPTG were also used as selectors for positive colonies. After 16 - 20 hours at 37°C, colonies were picked and used to inoculate 3ml of LB medium containing ampicillin. These cultures were grown for 10 - 16 hours at 37°C for mini-prep plasmid DNA extraction.

2.c) Plasmid DNA isolation by alkaline lysis

Isolation of plasmid DNA was performed by alkaline lysis (Birnboim and Doly,1979 and Birnboim, 1983). 3ml overnight cultures of E. coli were used for mini preparations of DNA. 1.5 ml Eppendorf tubes were filled with the E. coli cell suspension and gently centrifuged (6000rpm, 2 - 4 °C) for 3 minutes. The supernatant was poured off and the tubes were drained on a tissue. The pellet was resuspended in 200µl of solution number I (50mM glucose, 25mM Tris/HCl pH 8.0, 10mM EDTA and 10mg/ml of lysosyme). The pellet was resuspended in the solution using a rotamix and then put on ice for 10 minutes, 400µl of solution number II (0.2M NaOH and 1% SDS) was added and the solution was mixed gently. Subsequently, 300µl of solution number III (5M potassium acetate pH 4.8) was quickly added, the solution was mixed gently and then put on ice for 30 - 60 mins. The tubes were removed from the ice and the cell debris was pelleted by centrifuging at maximum speed (13,000 rpm) for 10 minutes. After transferring the supernatant to another set of tubes, 500µl of isopropanol was added and the solution was mixed and left at -20°C for 30 minutes. The samples were then centrifuged at maximum speed (13,000 rpm) for 10 minutes to pellet plasmid DNA. After this, the supernatant was tipped off and the pellet was washed with 70% ethanol, followed by centrifugation at 13,000 rpm for 5 minutes. The ethanol was emptied off and the pellet was dried under vacuum for 20 minutes. After resuspending the pellet in 50µl of water, 100µl of 7.5M

ammonium acetate was added and the mixture was left at -20°C for 30 minutes. Following centrifugation at 13,000 rpm for 10 minutes at 0°C, the supernatant was removed to a new tube and 100µl of isopropanol was added. The solution was then left at -20°C for 10 minutes before being centrifuged at 13,000 rpm for 10 minutes. Finally, the supernatant was tipped off, and the pellet was washed with 70% ethanol before being dissolved in 20µl of deionised water. Plasmid DNA was stored at -20°C. This method produced a purified sample of plasmid DNA suitable for restriction endonuclease digestion and sequencing.

When it was necessary the DNA was further purified by ammonium acetate and polyethylene glycol (PEG) precipitation. Briefly, two volumes of 7.5 M ammonium acetate were added to the DNA solution. After 10 min at -20°C, the precipitated RNA, proteins and polysaccharides were removed by centrifugation for 10 min at 4° C (13 000 rpm). The DNA was precipitated from the supernatant with 0.6 volumes of isopropanol and following centrifugation, dissolved in water. An equal volume of PEG-NaCl solution (5.6g of PEG 6000; 7ml of $NaCl_2$ 5M; H_2O up to 50ml) was added to the DNA solution. After 30 min on ice, precipitated linear DNA was removed by centrifugation. Another volume of PEG-NaCl solution was added to the supernatant and the mixture was left for another 30 min on ice. Supercoiled plasmid DNA precipitates at this concentration of PEG and can be obtained as a pellet after centrifugation for 10 min at 4°C (13 000 rpm). After a few washes with 70% ETOH the pellet was dried and resuspended in an appropriate volume of water. This pure DNA solution can be stored at -20°C for a few months. The DNA prepared by this method is as pure as that obtained by caesium chloride ($CsCl_2$) gradient. DNA purification by the $CsCl_2$ method starts by pelleting cells from a large volume of culture (1 litre or 500 ml), at 10 000 rpm, in a Sorvall GSA rotor . The pellet was resuspended in 6ml of solution I and the tubes placed on ice. 2 volumes (12 ml)

of solution II, were added followed by gently mixing. Immediately, 9ml of solution III were added and the lysate was incubated on ice for 10 min. The lysate was cleared by spinning at 10000rpm for 10 min in a Sorvall SS-34 rotor or equivalent. 0.6 volumes of isopropanol were added to the supernatant and the plasmid DNA was pelleted by spinning at 10 000 rpm for 10 min. The pellets were then well drained and resuspended in 6 ml of H_2O in which 7g of caesium chloride was dissolved. To facilitate the separation of plasmid and chromosomal DNA bands, 100µl of 10mg/ml ethidium bromide was added. The tubes were then topped up with liquid paraffin, carefully balanced and spun in a Beckman 70Ti rotor, at 48 000 rpm, for at least 16h. The plasmid DNA was harvested through the wall of the tube using a syringe and needle. Ethidium bromide was extracted from the DNA by mixing with CsCl-isopropanol several times, allowing the phases to separate, and removing the upper non-aqueous layer. The clear final aqueous layer was diluted 2-fold with H_2O and the DNA was precipitated with 0.6 volumes of isopropanol. The DNA pellet was washed with 70% ethanol and air dried before resuspending in H_2O. The concentration of the purified DNA was measured using a spectophotometer.

2.d) Restriction Analysis

Restriction endonucleases cleave double stranded DNA at specific sites within or adjacent to specific recognition sequences. Restriction endonucleases cleave DNA into discrete fragments in a sequence dependent manner. Such cleavage is used to generate fragments of DNA that can be subcloned in appropriate vectors, to confirm the presence of ligated inserts, or to generate fragments to be used as labelled probes in Northern or Southern blotting. Restriction endonuclease cleavage is accomplished simply by incubating the enzyme(s) with the DNA under appropriate reaction conditions. The amounts of

enzyme and DNA, the buffer and ionic concentrations, and the temperature and duration of reaction will vary depending upon the specific application. In principle 1U of restriction endonuclease completely digests 1µg of purified DNA in 60 min using the producer's recommended assay conditions. However, crude DNA preparations often require more enzyme and/or more time for complete digestion. The volume of restriction endonuclease added should be less than 1/10 the volume of the final reaction mixture, because glycerol in the enzyme storage buffer may interfere with the reaction.

2.e) Electrophoresis of DNA

Electrophoresis of DNA was usually carried out in a 1% agarose gel. 300ml of agarose-water mixture was heated for 4 minutes in the microwave to dissolve the agarose. When the solution was clear, 6ml of 50X TAE buffer (1 x TAE is 0.04M Tris-acetate, 1mM EDTA, pH 7.5) and 15µl of 10mg/ml Ethidium bromide were added. The gel solution was left to cool before pouring it into a gel tray whose ends had been sealed with masking tape. Any air bubbles that were present were flamed with a Bunsen burner before gently adding the comb. When the gel had set, a small amount of TAE buffer was poured around the comb and the comb was removed. The electrophoresis tank was filled with 1X TAE buffer until it just covered the gel. 15µl of marker ladder solution (BRL) was then added to the 1st and to the last slot on the gel and the DNA digests were then loaded into the intervening wells (the whole tube volume was loaded). Electrophoresis was started slowly (100v) but when the samples had run into the gel the voltage was turned up to 200v. DNA fragment bands were observed under U.V. light.

2.f) Subcloning fragments of DNA

The pGEM T vector into which the PCR isolated TrkC cDNA was initially cloned did not contain an appropriate restriction site to allow linearization of plasmid DNA prior to the generation of sense cRNA transcript. For this reason the TrkC cDNA was subcloned into the Bluescript vector. The TrkC cDNA was excised from the pGEM T vector with the restriction enzymes SacI and SacII and ligated into SacI and SacII digested Bluescript vector.

0.1ng of vector DNA, 0.5ng of TrkC cDNA fragment (isolated from a LMP agarose gel) and 2µl of ligation buffer (0.5M Tris pH 7.4, 0.1M $MgCl_2$, 0.1M dithiothreitol, 10mM spermidine, 10mM ATP and 1mg/ml BSA) were added to an Eppendorf tube and the final volume was made up to 10 µl with water. 1µl (5 units) of T4 DNA ligase was added and the mixture was left in a water bath at 15°C for 3 hours for the ligation reaction to proceed.

2.g) Sequencing DNA

DNA sequencing was used to check the identity of the TrkC cDNAs cloned by PCR and the accuracy of the "filling" in reaction used to construct the TrkC cDNA control. The sequencing method used was the chain-termination method (Sanger *et al*, 1977). The chain-termination method involves the synthesis of a DNA strand by a DNA polymerase *in vitro* using a single-stranded DNA or a denatured double-stranded DNA as template. DNA synthesis is primed by a synthetic oligonucleotide and is carried out in two steps. The first is the labelling step and the second is the chain-termination step using dideoxynucleosides. In the first step, the primer is extended using limiting

concentrations of the deoxynucleoside triphosphates, including radioactively labelled dATP. This step continues until almost complete incorporation of labelled nucleotide into DNA chains which are distributed randomly in length from several nucleotides to hundreds of nucleotides. In the second step, the concentration of all the deoxynucleoside triphosphates is increased and a dideoxynucleoside triphosphate is added. Processive DNA synthesis occurs until all growing chains are terminated by a dideoxynucleoside. During this step, the chains are extended on average by only several dozen nucleotides. The reactions are terminated by the addition of EDTA and formamide. The products of the reaction are denatured by heating and resolved by electrophoresis.

I) Denaturation of plasmid DNA:

4 - 5 μg of plasmid DNA was denatured with 0.2M NaOH and 2mM EDTA in a final volume of 20μl over 10 minutes. The solution was then neutralised by adding 6μl of 7.5M ammonium acetate. The denatured DNA was precipitated by adding 100μl of ethanol and placing at -20°C for at least 1 hour.

II) The sequencing reactions.

The DNA sample was removed from -20°C and centrifuged at maximum speed (13,000 rpm) for 10 minutes. The pellet was washed with 70% Ethanol and then centrifuged for a further 2 minutes. Following this, the 70% ethanol was poured off and the pellet was dried under vacuum.

The labelling mixture was made up for 12 DNA templates as follows:

24.6µl of nucleotide mixture (7.5mM dGTP, 7.5mM dATP and 7.5mM dTTP);12.3µl of DTT 0.1M;3.5ml of a ^{32}P dCTP (10mCi/ml; 3000Ci/mmol) and 2.5µl of water,

The solution was vigorously mixed prior to use.

The twelve 0.5ml Eppendorf tubes containing dry pellets of denatured DNA templates were prepared for the annealing reaction as follows. 2µl of sequenase reaction buffer (USB kit) was added to the side of each tube. Next, 2µl of the appropriate primer was added to the side of the tube near the reaction buffer and 6µl of water was then added to the base of the tube. The buffer and the primer were tapped down and the whole mixture was vortexed followed by a brief spin in a microfuge. The mixture was left in a water bath at 37°C for 15 minutes before being left on the bench for 2 minutes to cool down prior to the labelling reaction. Next, forty eight 0.5ml Eppendorf tubes were prepared for the termination reaction. The lids were cut off the tubes and 2.5µl of the appropriate termination solution (USB kit) was added to the side of the tube, i.e. 4 rows of 12 tubes with each row containing one of the four dNTPs. The solutions were tapped down and the tubes were then placed in a 37°C water bath.

2.5µl of Sequenase 2.0 enzyme (USB kit) was diluted with 22.5µl of enzyme dilution buffer (USB kit), then the stock was immediately returned to -20°C.

The next step in the sequencing protocol was the labelling reaction. 3.5µl of the labelling mixture were added to the side of each of the tubes containing the annealed primer/template mixtures. 2µl of the diluted enzyme was then added to the side of each tube then both labelling and enzyme mixtures were tapped down to the base of the tubes. After 2 minutes at room temperature 3.5 µl of each of the products of the labelling reactions were quickly added to each of the termination

solutions. All of this should be done at speed. The termination reactions were then placed in a water bath (37°C) for 5 minutes. After 5 minutes, 3µl of stop solution was added (USB kit) to stop the reactions This was added to the side of the tubes then it was tapped down. The tubes were then placed in a water bath (90°C) for 20 minutes to concentrate the mixtures and denature the DNA. Finally, the tubes were removed and put on ice prior to electrophoresis.

III) Preparing the glass plates for electrophoresis of sequencing products.

One plate was silanized using dimethyldichlorosilane solution. This was liberally spread over the surface of the plate and vigorously rubbed in. The plate was then washed down with industrial methylated spirits and left to dry.

5ml of ethanol, 15µl of acetic acid and 15µl of Bind silane were mixed vigorously, and a second glass plate was coated with this mixture. The plate was washed with industrial methylated spirits and left to dry.

IV) Pouring the acrylamide gel .

29.4g of urea was added to 4.2g of an acrylamide/bis mixture (19:1). This mixture was made up to a final volume of 63ml using deionised water. When the reagents were completely dissolved, a small amount of deionising resin (AG50X8 (BioRad)) was added and the solution was stirred for another 10 minutes. 7ml of the 10X electrophoresis buffer (108g of Tris base (890mM), 55g of Boric acid (890mM), 40 ml of 0.5 M EDTA pH8 (20mM) and made up to an end volume of 1l, optimum pH of 8.3) was added to the solution which was subsequently filtered. 0.055g of ammonium persulphate was added and the solution was put under vacuum for ten minutes. The solution was removed from

vacuum and 45µl of TEMED was added to initiate polymerisation. The gel solution was rapidly poured between the two glass plates that had been previously prepared as described above. Care was taken to avoid producing air bubbles when pouring the gel. A sharktooth comb (48 slots) was positioned upside down between the two glass plates to produce an 0.3cm deep grove and the glass plates were clipped tightly together using bulldog clips. After polymerisation was completed, the bulldog clips were replaced with two slide-on clips and the system was transferred into the electrophoresis tank taking care to avoid air bubbles between the bottom of the plates, the presence of such bubbles can interrupt the flow of current through the gel. A thermometer was attached to the outside of one glass plate to ensure that the electrophoresis ran at the optimum temperature of 55^{o}c. The comb was removed and the groove was washed with buffer using a syringe and needle. The gel was pre-run until the temperature reached the optimum of 55°C, after which the current was turned off and the comb was used to pierce the surface of the groove by about 1mm to produce 48 slots for sample loading. The gel was then loaded.

V) Loading the gel.

The slots were rinsed using a syringe of buffer to remove any unpolymerised material and urea that had diffused out of the gel. 1.5µl of each termination reaction was added to each slot. Each template was loaded in the order T,C,G,A for ease of reading after developing the autoradiograph. 12 samples could be loaded at once and then the tank had to heat up to the optimum temperature before any more samples were loaded. The electrophoresis was run at 50W constant power for either 3 - 4 hours for a long run or 2 hours 15 minutes for a short run.

VI) Fixing the gel to the glass plate.

After electrophoresis had finished and the glass gel plates were separated, the gel stuck to the glass plate that had been pre-treated with Bind Silane. This glass plate was soaked in 10% Acetic acid until the marker dye was no longer visible. The glass plate was then baked in an oven (80^{o}C) for 20 minutes. The glass plate was removed from the oven and allowed to cool down.

VII) Loading the film.

The glass plate was taken to the dark room where a Kodak X-ray film was placed over it. Another glass plate was placed over the film and the two plates were clamped together using bulldog clips. The plates were wrapped in black plastic and a cloth, to keep the light out, and stored in a cupboard overnight. The film was then developed and read from the base upwards and the sequence noted.

3. Quantitative and Semi-Quantitative RT-PCR assay

Traditionally, levels of individual mRNAs have been analysed by procedures such as Northern blots, RNA dot/slot blots, nuclease protection and *in situ* hybridisation. Application of the polymerase chain reaction (PCR) technique provides another method of mRNA analysis. The PCR-based method has been variously termed; RNA-PCR, RT-PCR, RNA phenotyping and Message Amplification Phenotyping (MAPPing). The term adopted by this study is RT-

PCR. RT-PCR has been shown to be thousands of times more sensitive than the traditional RNA blotting techniques. This exquisite sensitivity gives RT-PCR the ability to detect extremely rare mRNAs, in addition to mRNAs in small numbers of cells and cell specific mRNAs expressed in mixed-cell populations.

While RT-PCR is an extremely sensitive method of mRNA analysis, obtaining quantitative information with this technique can be very difficult. This is due primarily to the fact that there are two sequential enzymatic steps involved: the synthesis of DNA from the RNA template and the polymerase chain reaction. In practise, the exponential nature of the polymerase chain reaction and the practical aspects of performing PCR pose the most serious obstacles to obtaining quantitative information. With some adaptations, however, RT-PCR can yield accurate quantitative results.

Apart from the quantitative RT-PCR assay, I also performed semi-quantitative RT-PCR analysis in part of my studies. This approach is not so accurate and precise but gives an idea of the relative levels of expression of a target molecule in different samples.

The goal of quantitative PCR is to deduce, from the final amount of PCR product, either the initial number of target molecules (No) or the relative starting levels of target molecules among several samples. Several methods are commonly used to quantify PCR products. The most straight forward approach is to measure the incorporation of labelled nucleotides or primers into PCR products resolved by gel electrophoresis. Although direct, the use of labelled nucleotides in PCR can be problematic. For this reason, I decided to use 5' end-labelled PCR primers rather than labelled nucleotides.

In this study a quantitative and a semi-quantitative reverse transcription/polymerase chain reaction (RT/PCR) technique has been employed to determine the developmental expression of TrkC mRNA in peripheral neurons. The quantitative RT-PCR technique is based on the co-reverse transcription and co-amplification of TrkC mRNA and a slightly larger, *in vitro* transcribed, TrkC RNA control (4bp were inserted between the PCR primer sites). The PCR products of the control and native cDNA templates were resolved on 7% non-denaturing polyacrylamide gels that were subsequently dried and autoradiographed. Reactions were set up such that the autoradiographic signals from the PCR products of the native and control cDNA templates were approximately equal. The autoradiographs were scanned with a Molecular Dynamics Personal Laser Densitometer and the intensity of the respective signals was ascertained using ImageQuant software (Molecular Dynamics). These values enabled the levels of TrkC mRNA in the initial total RNA to be calculated.

Total RNA (Chomczynsky and Sachi, 1987), spiked with known amounts of the appropriate control RNA, was reverse transcribed for 45 minutes at 37°C with Gibco BRL Superscript enzyme in a 10 µl reaction containing the manufactures's buffer supplemented with 0.5 mM dNTPs and 10 µM random hexanucleotides. Each reverse transcription reaction was then gently mixed with 40µl PCR solution comprising 1X NBL Taq DNA polymerase buffer, 1 unit of NBL Taq DNA polymerase, 40 µl of 5' end labelled primers and 0.2 mM dNTPs.

In some instances, due to shortage of time, a semi quantitative technique was used. The quantitative RT-PCR reaction was time consuming because it required a number of preliminary reactions to determine the correct amount of control cRNA to use. The semi quantitative technique gives an idea about the comparative level of expression of TrkC mRNA throughout the developmental

period of interest. It consists of amplifying the same amount of target cDNA with labelled primers for L27 and TrkC. The levels of TrkC were estimated by comparison with L27 levels amplified with the same amount of cDNA from the same samples. The products of these separate reaction were run either together or separately in a 7% acrylamide gel and the bands scanned on a densitometer. Relative levels of TrkC mRNA were calculated by normalising TrkC cDNA signals against the amount of L27 mRNA in each cDNA sample.

The initial primers used for PCR were located in the extracellular domain of chicken TrkC. This domain shows the least conservation sequences between the three Trk receptors.

Following the cloning of various isoforms for TrkC (Williams *et al*, 1993) it became evident that the primers within the extracellular domain were recognising various different isoforms of TrkC and not just the full length isoform. In a bid to design an assay specific for the full length TrkC isoform, I then decided to use primers at the 3' prime end of the TrkC cDNA which contains untranslated sequence. However, Okazawa *et al* (1993) showed by Northern Blotting analysis that a probe encompassing this region hybridises to various TrkC mRNAs from 9 kb to 0.4 kb. The bands smaller than 1.2 kb are incapable of encoding the full length and therefore represent truncated isoforms. Therefore, I finally decided to make a very accurate comparative analysis of the highly homologous tyrosine kinase domains of the three different Trks (TrkA, TrkB and TrkC) and design very specific primers in that region for TrkC. In this way I was certain of only amplifying full lenght TrkC. The primers used to amplify the full length form of chicken TrkC were:

5' CCAGCATATTAAGAGAAGA 3'

and

3' GGTCGTAAAACAGTAACC 5'

They hybridise 116 bp apart (Fig 5.3).

In the case of the quantitative RT-PCR assay, chicken TrkC control cRNA transcripts synthesised from the chicken TrkC cDNA that has been cloned by PCR, were included in the initial RT reaction.

In order to compare the relative level of TrkC mRNA in developing sensory neurons at different ages, the level of the mRNA encoding the ubiquitous, constitutively expressed L27 ribosomal protein was also measured in all RNA samples.

The primers used for the quantification of L27 mRNA were:

(5') 5' GGCTGTCATCGTGAACAT (3')

and

(3') 5'CTTCGCTATCTTCTTCTTGCC (5')

These primers hybridise 127 bp apart in the chicken L27 sequence (Lebeau *et al*, 1991) and were radio labelled as described for TrkC primers. The level of L27 mRNA in the extracted RNA was quantified in some cases by co-reverse transcribing cellular RNA with run-off cRNA transcripts synthesised from an L27 cDNA that has an additional 4bp inserted between the primer annealing sites. The results are expressed as the quotient of the amount of TrkC mRNA and the amount of L27 in RNA extracted from various populations of sensory neurons.

3.1. Synthesis of RNA control templates for Quantitative RT-PCR

In order to make the controls in each group of experiments more accurate and reproducible within any particular study, all experiments within a group used the same batch of control RNA templates that were transcribed, purified and quantified in one procedure. The control cDNA template, used to produce the control cRNA for the TrkC RT-PCR assay, was made by cleaving the cloned cDNA at a single internal Sty1 site, filling in the 5' overhangs with Klenow fragment of DNA polymeraseI and religating the resulting blunt ends.

Control RNA transcripts were transcribed in a 200µl reaction containing the following:

40 µl of 5X transcription buffer (40mM Tris, pH 7.9; 6mM $MgCl_2$; 2mM spermidine)
8 µl of 10 mM ATP
8 µl of 10 mM UTP
8 µl of 10 mM GTP
8 µl of 10 mM CTP
8 µl of 100 mM DTT
5 µl of RNA guard (40U/ µl)
3 µl of Sp6 (or T7, or T3) RNA polymerase (16 U/µl)
5 µl of linearized DNA template (1µg/µl)
107 µl of H2O.

Following incubation at 37°C for one hour, a further 3 µl of polymerase were added and the reaction was allowed to proceed at 37°C for another hour. At this point ribonucleoside vanadyl complexes (VRC's) solution were added to a final concentration of 10 mM. Template DNA was removed by the addition of

50 Units of RNase free DNase I (Pharmacia) followed by incubation at 37°C for 60 minutes. The DNase reaction was stopped by the addition of 600 µl of 3M $NaClO_3$ and 50 µl of RNaid matrix (from RNAid kit) . After thorough mixing, the slurry was incubated at room temperature for 5 minutes to allow RNA to bind to the solid matrix. Following the sedimentation of the RNaid matrix by brief centrifugation, the aqueous phase was removed and discarded and the matrix containing the bound DNA was washed twice with ice cold RNaid wash solution. RNA was eluted from the matrix by adding an appropriate volume of water (depending on the starting amount of tissue or cells) and incubating at 60°C for 5 minutes. Following a brief centrifugation to sediment the matrix, the RNA solution was removed to a fresh microfuge tube and placed on ice. An aliquot of the RNA solution was used to determine the RNA concentration spectrophotometrically, after which the RNA was diluted and precipitated by the addition of 0.1 volumes of 3M NaAc, 3 volumes of ETOH and *E. coli* tRNA such that the final mixture contains 1 ng of transcript RNA and 100 ng of E. coli tRNA per microlitre. Finally the ethanolic mixture was aliquoted into individual 0.5 ml Eppendorf tubes in 50 µl aliquots (50 ng each). Ethanol precipitated transcripts were stored at -20°C until required.

3.2. Labelling primers

Primers were labelled with ^{32}P on their 5' ends in a 40 µl reaction containing the following:

4µl of 10X T4 PNK buffer (0.5 M Tris, pH 6, 100mM $MgCl_2$, 1mM spermidine, 50mM DTT, 1mM EDTA)

1µl of each primer (1-2 µg/µl)

30 µl of ^{32}P ATP (3000ci/mmol, 10 mci/ml)

4 µl of T4 polynucleotide kinase (10 units/ µl).

Following thorough mixing, the labelling reaction was carried out at 37° C for 45 minutes. Primers were purified from the labelling reaction mix using the "MERMAID" oligonucleotide purification kit (BIO 101). Basically this purification involves three steps: Three volumes of the binding salt were added to the reaction mix followed by 50 µl of the oligonucleotide binding matrix. After thorough mixing, the slurry was left at room temperature for 10- 15 minutes to allow the primers to bind to the matrix. Following the completion of primer binding, the matrix material was pelleted by brief centrifugation and the high salt solution removed with a pipette. The pellet was then washed twice with 500 µl of "MERMAID" wash solution before final centrifugation and removal of the last drops of the washing solution with a fine micropipette. The matrix pellet was then resuspended in 200 µl of water and the primers were eluted from it by incubating at 60°C for 5 minutes. Following sedimentation of the binding matrix by centrifugation, eluted primers were removed to a fresh Eppendorf tube and stored at -20° C until required

3.3. Reverse Transcription

Reverse transcription reactions were carried out in a 'big scale' reaction. For the quantitative RT-PCR assay, the two different competitors control RNAs (TrkC and L27) appropriately diluted, plus the target RNA were transcribed together. For the semi-quantitative PCR analysis, PCR amplification using different primers (either TrkC or L27) was also performed on cDNA from the same reverse transcription reaction. Basically each reaction has 3 µl of total RNA (or more in case of absence of control RNAs), 1 µl of each of the control RNA, 15 µl of reverse transcription mix, 1 µl of Superscript Reverse

Transcriptase (Gibco BRL). For negative controls RNA was replaced by 2µl of water. Reverse transcription mix was made as a master mix for each batch of reverse transcription reactions being carried out. The use of a master mix ensured uniformity of conditions in all reactions carried out using that mix. The total volume of the master mix that was made up for each set of reverse transcription reactions was given by: v (µl)= (n+1) x 17 where n is the number of reactions being carried out (enough of the master mix was made up to be sufficient for (n+1) reactions to allow for inaccuracies in pipetting when aliquoting the master mix to each individual reaction tube). If the term (n+1) is replaced by the symbol * then the composition of the master mix is:

2 X (*) µl of Gibco Superscript buffer
1 X (*) µl of 0.1M DTT
2 X (*) µl of 5mM dNTP
1 X (*) µl of 100 µM random hexanucleotides
0.25 X (*) µl of RNA Guard
Make up to volume (v) with water.

The actual reaction was done in 0.5 ml Eppendorf tubes where the 17 µl were carefully pipetted followed by the addition of the RNA samples. The tubes were then removed to the PCR machine which should be set at 37°C. After 2 minutes of these tubes being at 37°C 0.5 µl (100 units) of Superscript was added to each reaction and the reactants were carefully mixed by drawing backwards and forwards through a micropipette tip. In the case of a reverse transcription minus control, Superscript was not added to the reactions. Following a brief spin in a microfuge, the Eppendorfs were transferred back to 37°C and incubated for 45 minutes. In some cases RNA secondary structure may result in poor yields of certain cDNA species. If the proposed PCR template results in such cDNA species, it will be necessary to remove secondary structure from the region of RNA concerned, before adding the RNA to the reverse transcription reaction, by heating to 90°C for 5 minutes followed by quenching on ice.

3.4. PCR assay

Following reverse transcription, reactions were centrifuged briefly to return the tube contents to the bottom of the tube. The products of the reverse reaction were prepared for amplification by the addition of 40 µl of PCR mix. PCR mix was a master mix that contained all the additional components required for the PCR amplification, including the thermostable Taq polymerase. The total volume of the PCR master mix required was calculated in a similar way to the reverse transcriptase master mix and was equal to (n+1) X 40 µl where n was the number of PCR reactions being carried out.

The master mix, was composed of:

4X (n+1)µl of 10X PCR reaction buffer (from Promega)

1X (n+1)µl of $MgCl_2$ (25mM)

1X (n+1)µl of 5mM NTP's

4X (n+1)µl of labelled primer mix

0.3X (n+1) µl of Taq polymerase

Made up to a volume V with H_2O

The above PCR mix composition was ideal for amplification of the cDNA template when it was present at 5-100 fg in the reaction. With different target cDNA concentrations it was necessary to adjust the composition of the PCR mix slightly to achieve optimal results.

Following addition of the PCR master mix to the products of the reverse transcription reaction, the two solutions were mixed and overlaid with 40µl of

mineral oil. The reactions were then transferred to the heating block of a PCR machine where they were subjected to a number of cycles of denaturing, annealing and synthesis.

The exact parameters set for denaturing, annealing and synthesis, in terms of temperature, time and numbers of cycles, were dependent on the nature and concentration of the cDNA template to be amplified and largely had to be determined empirically.

Cycling parameters used for the amplification of the 116 bp chicken TrkC cDNA fragment consisted of 27 cycles of 45 seconds at 94°C, 45 seconds at 56°C, 1 minute at 72°C, followed by a final incubation at 72°C for 10 minutes to ensure all single stranded templates were converted to a double stranded form.

L27 cDNA was amplified by 18 cycles of 94°C for 1 min, 60°C for 45 sec and 72°C for 45 sec followed by a final elongation step of 10 min.

Following the completion of amplification, reactions were removed from the PCR room to a room set aside for the analysis of PCR products.

3.5. Electrophoresis of PCR products

The PCR products were separated by electrophoresis on non-denaturing polyacrylamide gels. Because the size difference between the two products was only about 4%, it was important to ascertain the optimum polyacrylamide concentration with regards to maximum separation of the products. This was determined empirically and it was found to be 7% of 19:1 acrylamide-bis. The

vertical gel apparatus used was Gibco V-15.17 that produces gels of 20 cm X 25 cm, 0.8 mm thick and can accept 20 samples (10 µl each). After siliconising one of the glass plates, the gel apparatus was assembled according to the manufactures instructions.

About 100 µl of ammonium persulfate was added to 30 ml of gel solution and the solution was degassed for 20 min. Before pouring the gel solution between the glass plates, 45 µl of TEMED was added to initiate the polymerization of the acrylamide. The polymerised gel was inserted into the gel apparatus and the buffer reservoir were filled with 1X TBE. After flushing the wells of the gel with buffer to remove unpolymerised acrylamide, the samples were loaded into the wells and the gel was run at a constant 350V. To obtain maximum resolution of PCR products, they were run as close as possible to the end of the gel.

At the end of electrophoresis, the gel plates were carefully parted, leaving the gel attached to the non siliconised glass plate. The gel was then transferred to Whatman 3MM paper and subsequently dried for 30 min at 60° C on a slab gel dryer. After the gel was thoroughly dried, it was autoradiographed. The relative intensities of the autoradiographic signals of the control cRNA and mRNA RT-PCR products were ascertained using a Molecular Dynamics laser densitometer with Image Quant software.

5.3. RESULTS

Specificity of the primers

A fragment of chick TrkC cDNA corresponding to the tyrosine kinase domain was cloned by PCR. This unique band (Fig. 5.2) was cut out from the gel and the identity of the cloned cDNA was verified by sequencing.

To select appropriate primers, either for cloning TrkC or for the RT-PCR amplification that will only amplify TrkC and not the others members of the trk family, an alignment of the chicken TrkA, TrkB and TrkC sequences was done using GCG software (Fig.5.3). For the RT-PCR assay primers in the tyrosine kinase domain were used to ensure that only the full length catalytic isoform of the receptor was amplified. The identification of the region in the tyrosine kinase domain that contained most abundant mismatches between TrkC and the other Trks was done manually. Using the computer software, Amplify 2.1., I tested the assay primers chosen against other known nucleotide sequences. A preliminary test was made using chicken TrkA, TrkB and TrkC sequences and only one major and specific product originated from the TrkC sequence.

To allow the construction of a control cDNA species for the RT-PCR assay the cloned chicken TrkC insert was subcloned in pBluescript SK^+ in the SacI and SacII sites, and the unique Sty1 site was filled in. The filling in reaction was checked by sequencing the modified TrkC DNA and then RNA was synthetised *in vitro* .

For quantitative PCR analysis, target mRNA in total RNA from neuronal samples was co-reverse transcribed with a slightly larger control RNA (prepared from TrkC or L27 cDNAs) and the resulting cDNA's were co-amplified by PCR, as described in detail in the Methods section of this Chapter. Co-reverse transcription and co-amplification of target mRNA and a control RNA negates the effects that a number of variables can have on reaction efficiency and on the RT-PCR product yield. For the semi-quantitative PCR analysis, a large reverse transcription reaction was set up only for the target RNA. To determine the reliability of the quantitative RT-PCR technique, reactions were carried out using known quantities of target and control RNA transcripts. During the log-phase of the reaction, the ratio between the reaction products was identical to the initial ratio of target and control RNAs, regardless of the starting quantities of these RNAs (data not shown).

Examples of an autoradiograph showing the separate target and control RT-PCR products for the TrkC mRNA are given in Fig 5.4.

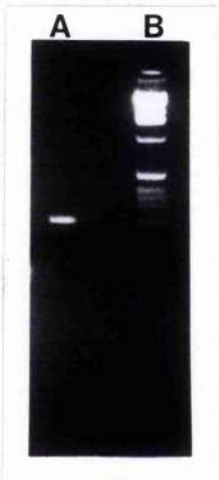
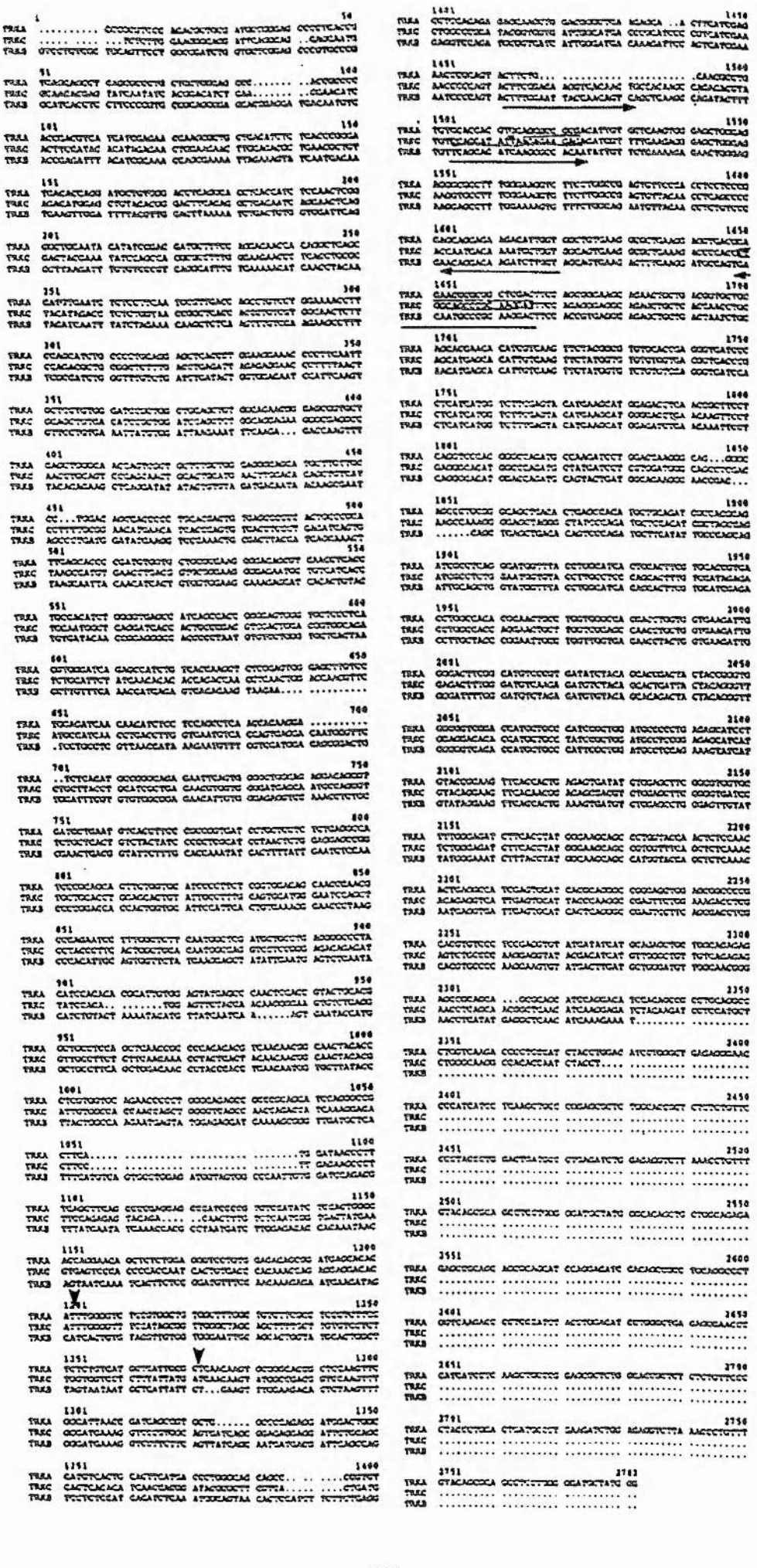


Figure 5.2. Photograph of an agarose mini gel in which the unique TrkC PCR product is resolved (A) 205bp band, (B) 1Kb ladder DNA.

Figure 5.3. Alignment of nucleotide sequences encoding chicken TrkA, TrkB and TrkC. The thin external arrows below the nucleotide sequence (1461-1666) indicate position and orientation of primers used for PCR amplification for cloning of a TrkC fragment. The internal thin arrows below the sequence (1504-1620) indicate the primers used for quantitative and semi-quantitative RT-PCR amplification. The arrowheads denote the transmembrane domain.



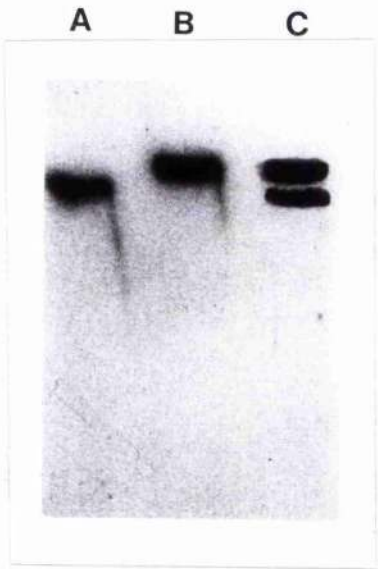


Figure 5. 4. Reliability of the quantitative RT-PCR technique. The autoradiograph shows the product of RT-PCR amplified with TrkC-specific primers. (A) Reaction containing total RNA from E8 DMTG neurons, (B) Reaction containing 1ng of control template, (C) Reaction containing both sets of the respective amplification products.

TrkC mRNA expression in chicken brains.

A semi-quantitative RT-PCR assay was used to measure the levels of TrkC mRNA in total chick brain RNA during development from E6 to P3. Chick brain total RNA was reverse transcribed and an equal amount of the resulting cDNA was amplified either with PCR primers specific for the tyrosine kinase domain of TrkC or with primers specific for the L27 ribosomal protein. TrkC mRNA was abundantly expressed at E6. At this age an additional RT-PCR product of bigger size was detected (Fig 5.5.). The level of TrkC mRNA decreased dramatically in later stages of development (Fig. 5.6.).

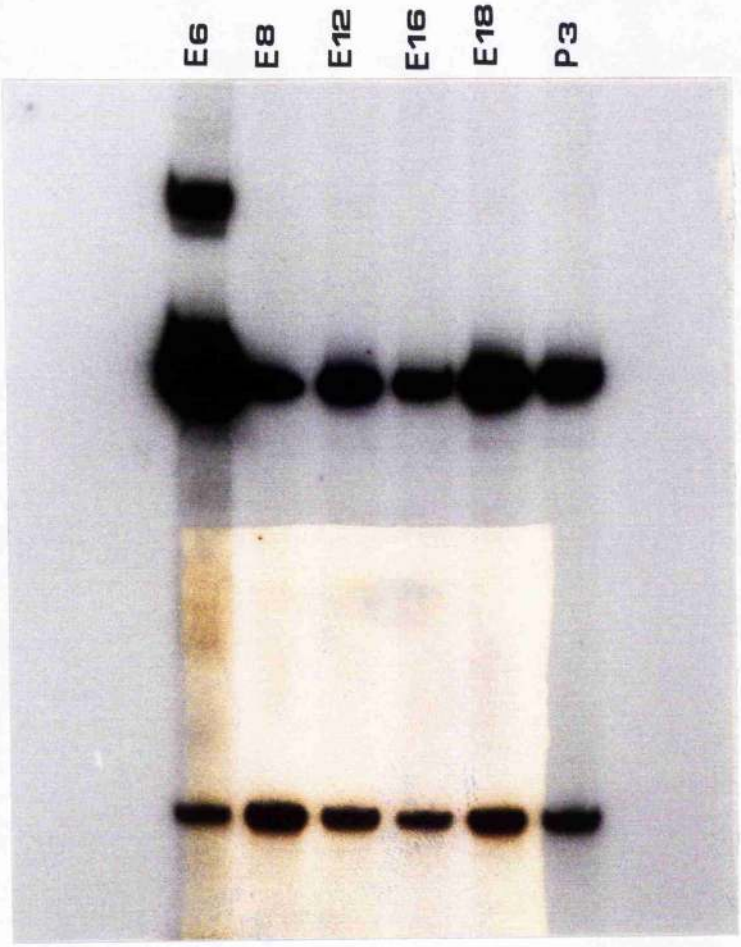


Figure 5.5. Autoradiograph showing full length TrkC mRNA expression in chick brains. PCR reaction using TrkC and L27 primers to amplify cDNA from chicken brains at different ages. The top band represents the RT-PCR products of TrkC mRNA (116 base pairs) and the bottom band represents the RT-PCR product of L27 mRNA (127 base pairs)

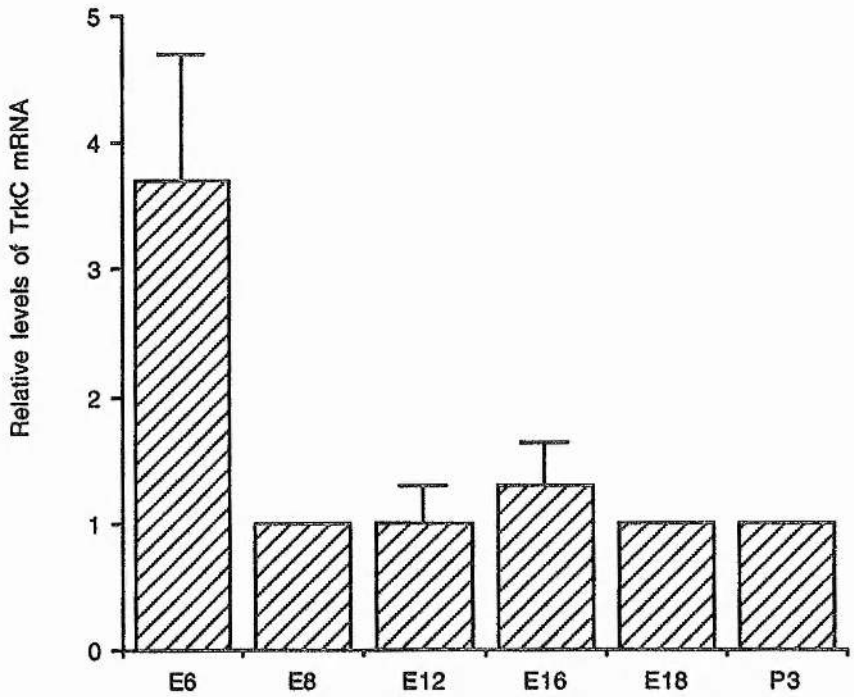


Figure 5. 6. Expression of TrkC mRNA in chicken brains. The bars represent the relative level of TrkC mRNA at each age after normalising to the level of L27 mRNA. Mean ± range between 2 values is shown.

TrkC mRNA in DMTG neurons

Quantitative RT-PCR was used to measure full length TrkC mRNA levels in total RNA extracted from pure preparations of DMTG neurons obtained from E8, E10, E12 and E14 chicken embryos. At E6 it is not possible to separate neuronal from non-neuronal cells via the differential sedimentation technique due to the smaller size of the neurons. Therefore, the sample analysed at E6 contains some RNA from non-neuronal cells as well. There is a high expression level of TrkC mRNA at E6. At this age an additional RT-PCR product of bigger size is produced with the primers used. Perhaps it represents an alternatively spliced isoform of TrkC. From E8 onwards the level of TrkC mRNA is lower compared with the level of expression at E6 (Fig. 5.7.). This result is in accordance with the decrease survival response for NT3 of DMTG neurons during development. However, full length TrkC mRNA can still be detected at later stages of development (E12-E14) when very few of DMTG neurons are responsive to NT3 in culture (Chapter 2). Full length TrkC mRNA was also detected in non neuronal cells. The TrkC levels for purified neurons were expressed relative to mRNA levels for the ubiquitous and constitutively expressed L27 ribosomal protein, which were also determined by RT-PCR.

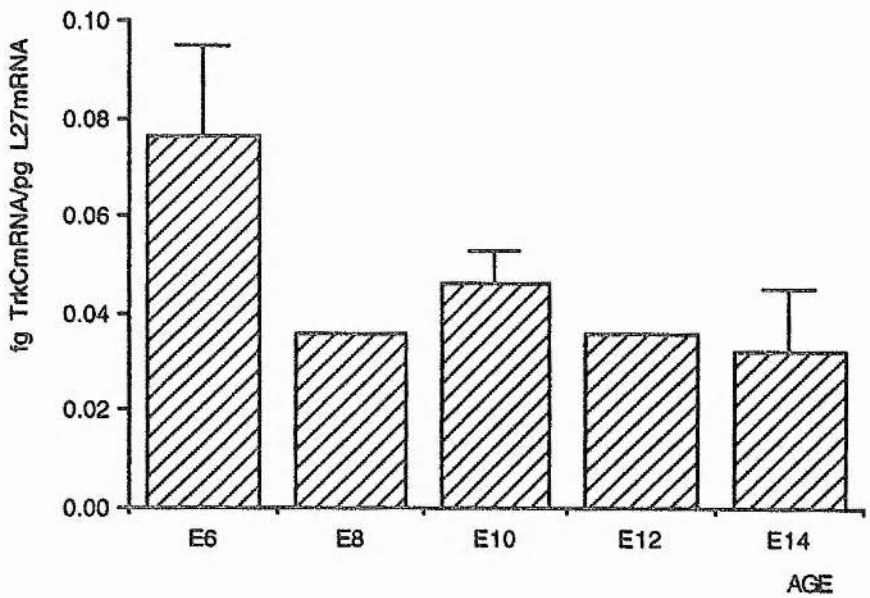


Figure 5. 7. Graph of the level of TrkC mRNA relative to L27 in chicken DMTG neurons during development. The means and standard errors of measurements from three sets of neurons. Mean ± standard errors are shown (n=3).

The regulation of TrkC expression by neurotrophins

In Chapter 2 it was shown that DMTG neurons are responsive to NT3 and BDNF in early stages of development (E6-E8) and become exclusively dependent on NGF in later stages of development (E10 onwards). To investigate the influence of neurotrophins on the regulation of TrkC mRNA expression, quantitative RT-PCR was used to measure the levels of TrkC transcripts in very low density cultures of E6 DMTG neurons growing with or without neurotrophins. The neurotrophins were added at the time of plating and the neurons were left to grow for 48h before RNA was extracted for RT-PCR assay. Figure 5.8. shows that NT3 at low concentration (2ng/ml) up-regulates the expression of TrkC mRNA. No correlation was found between increased concentration of NT3 and higher levels of TrkC mRNA. Also, no increase in TrkC mRNA levels above control cultures was found when NGF or BDNF were administered to these cultures either in low (2ng/ml) or high concentrations (5µg/ml).

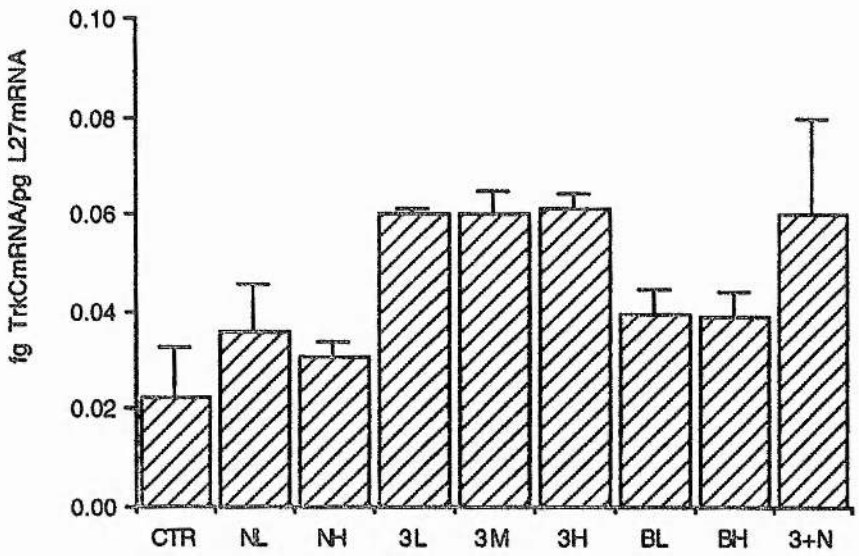


Figure 5. 8. Expression of TrkC mRNA in DMTG neurons in culture. RNA was extracted after 48 hours of cells being in culture in 35 mm dishes supplemented with different neurotrophins (N, NGF; B, BDNF; 3, NT3)at 2ng/ml (L) , 10ng/ml (M) or 5μg/ml (H). Mean ± standard errors are shown (n=3)

TrkC mRNA expression in sympathetic ganglia

Chick sympathetic neurons from the sympathetic chain survive in culture for at least 48h without neurotrophic support (Ernsberger and Rohrer, 1989). Later, at E10, they become highly dependent on NGF for survival *in vitro* . Holtz *et al* (1992) have shown that these neurons do not express TrkA at E7 and the expression of TrkA coincides with the acquisition of NGF dependence by these neurons. An investigation of TrkC mRNA expression was carried out in sympathetic ganglia from E7 to E14. Non-quantitative analysis by RT-PCR shows that initial high levels of TrkC mRNA at E7-E8 are dramatically reduced by E10 (Fig. 5.9.). Surprisingly, the level of TrkC mRNA expression increases after E10, a period when these neurons are not responsive to NT3 (Fig. 5.10.). This later increased expression of TrkC mRNA levels is in accordance with the expression of TrkC mRNA in rat adult sympathetic neurons (Freda-Miller personal communication). However, these neurons do not respond to NT3 at later stages of development (Dechant *et al*, 1993).

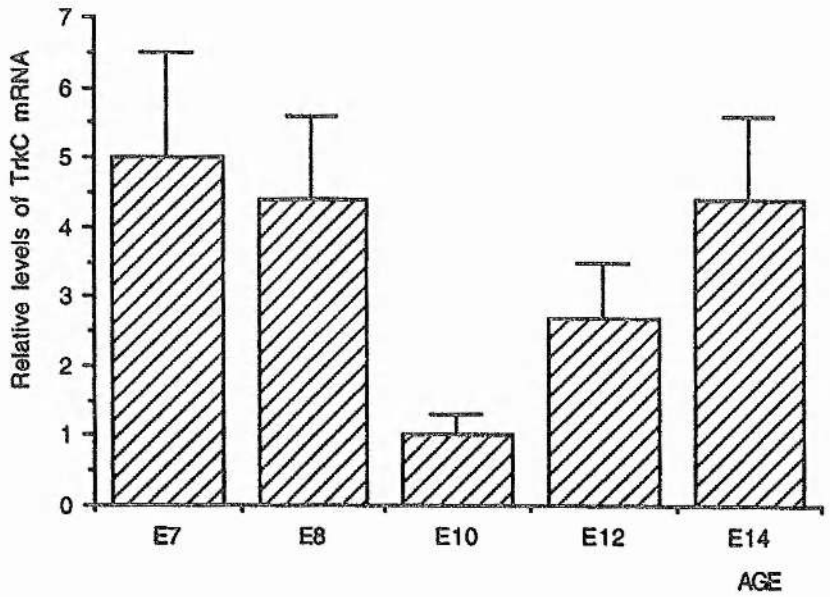


Figure 5. 9. Graph showing the relative levels of TrkC mRNA in chicken sympathetic neurons. during development. Mean ± range between two values is shown. The values shown at each age are normalised to the levels of L27 mRNA.

5. 4. DISCUSSION

The major aim of this study was to investigate the timing and regulation of TrkC mRNA expression in developing sensory and sympathetic neurons. TrkC mRNA levels were measured using both a semi-quantitative RT-PCR assay and also a quantitative RT-PCR assay that used a cRNA control template, added to the reverse transcription reaction, to calibrate the assay. Initially, primers in the extracellular domain of TrkC were used for amplifying TrkC cDNA in the RT-PCR assay. This region has the least homology between the three Trks (≈40%) and therefore facilitated the design of an RT-PCR assay that would detect TrkC mRNA but not TrkA or TrkB mRNAs. However, during the course of study various forms of chick TrkC were discovered by other groups, including an isoform that lacks the tyrosine kinase domain and therefore presumably is incapable of transducing a signal upon NT-3 binding (Garner and Large, 1994; Okazawa *et al.*, 1993). To date, the role that truncated isoforms of TrkC play in NT-3 signalling is largely unknown. However, the isolation of truncated TrkC isoforms both in mammals and in chicken seems to suggest that they serve some function. Clues to the functions of truncated TrkC have been obtained from studies of the TrkB receptor that also exists as a truncated isoform lacking the tyrosine kinase domain (Klein *et al*, 1990). In mouse, the periventricular expression pattern of TrkB is compatible with the idea that the truncated form might function as a scavenger receptor or as a transporter. Alternatively, truncated forms of Trk receptors might act as competitors against the catalytic form in a similar manner to truncated forms of the epidermal growth factor receptor (EGF) (Redemann *et al*, 1992). Data from experiments with mouse trigeminal neurons supports this hypothesis. In these neurons, expression of full

length catalytic TrkB alone confers a survival response to BDNF, whereas co-expression of truncated TrkB substantially reduces the BDNF survival response but has no effect on the NGF survival response. This suggests that BDNF responsiveness in developing sensory neurons is modulated by the relative levels of catalytic and truncated TrkB (Ninkina *et al*, 1996). In accordance with this hypothesis truncated and catalytic full length isoforms of TrkB appear to be co-expressed in the same neurons in the mouse CNS (Armanini *et al*, 1995).

Since the role of truncated TrkC receptors has yet to be fully characterised, I decided to restrict my studies of TrkC mRNA expression to mRNA that encodes the full length isoform of the receptor. For this reason I designed an RT-PCR assay that amplified a region of TrkC cDNA within the cytoplasmic kinase domain. The kinase domain is extremely well conserved between the different Trk receptors, both at the amino acid and the nucleotide level. For this reason, it was necessary to perform a sequence alignment between the different Trk receptors in the kinase domain and choose PCR primers in regions that showed the least homology between TrkC and the other Trk receptors

Timing of expression of TrkC mRNA

Quantitative RT-PCR has shown that full length TrkC mRNA is expressed at high levels at early stages in DMTG neurons and that the level of expression decreases at later ages. The decrease in TrkC mRNA expression that occurs as DMTG neurons mature is reflected by a the loss in responsiveness of DMTG neurons to NT-3 in culture (see Chapter 2) suggesting that the timing of NT3 responsiveness is controlled by the expression of its receptor. The same developmental correlation between neurotrophin responsiveness and

neurotrophin receptor mRNA expression was observed in sympathetic neurons. from the paravertebral sympathetic chain. Although immature E7 chicken sympathetic neurons proliferate and survive in culture for several days independently of neurotrophic factors (Ernsberger *et al*, 1989), NT-3 promotes their long term survival in culture. However, NT-3 does not promote the survival of E11 sympathetic neurons *in-vitro* (Dechant *et al*, 1993). In accordance with this, I have shown that full length TrkC mRNA is expressed at high levels in E7 sympathetic ganglia but is reduced 5-fold by E10. Interestingly, TrkC mRNA levels appear to increase some 4-fold between E10 and E14 so that by E14 they are almost as high as at E7. Sympathetic neurons do not show a survival response to NT-3 during the period E10 to E14. This observation contradicts previously published data showing that in rat sympathetic ganglia at E14.5, a developmental period equivalent to E7 in chick sympathetic ganglia, TrkC mRNA is expressed at high levels but levels are reduced markedly after E17.5 and TrkC mRNA is not detectable in sympathetic ganglia at birth using in-situ hybridisation (Birren *et al*, 1993). A reciprocal pattern of TrkA mRNA expression is observed in rat sympathetic ganglia. TrkA mRNA is not expressed in E14.5 sympathetic ganglia, first appears at low levels at E16.5 and is readily detectable by E17.5 (Birren *et al*, 1993). The timing of neurotrophin receptor expression is in accordance with the neurotrophin responsiveness of developing rat sympathetic neurons. At early developmental stages, rat sympathetic neurons respond to NT-3, but not NGF, with increased survival. However, later in development, when TrkC mRNA levels are low and TrkA mRNA levels are high, sympathetic neurons respond to NGF and not NT-3 (Birren and Anderson, 1993; DiCicco-Bloom *et al*, 1993). It has been suggested that the switch in neurotrophin receptor expression that occurs in developing rat sympathetic ganglia is mediated in part by NT-3 produced by non-neuronal cells surrounding sympathetic ganglia (Verdi and Anderson, 1994; Verdi *et al*, 1996). However, a second study failed to identify a

role for NT-3 in regulating TrkA expression in developing sympathetic neurons (Wyatt and Davies, 1995). Although a study using iodinated NT-3 to detect high affinity NT-3 binding sites in developing chick sympathetic neurons has concluded that TrkC receptors were expressed at high level by E11 neurons, a time when these neurons no longer respond to NT-3 by increased survival, the methodology used does not distinguish between full length catalytically active TrkC receptors and the shorter truncated isoform. My data are therefore novel in that they comprise first demonstration of full length TrkC expression in sympathetic ganglia at a time when sympathetic neurons no longer respond to NT-3 by increased survival. Recently, another group have reported detecting TrkC mRNA expression up to E15.5 in chicken sympathetic ganglia (Holst and Rohrer unpublished observations). Further studies are needed to ensure that the high level TrkC mRNA expression detected in E12 and E14 sympathetic neurons is restricted to neurons and does not reflect expression of TrkC mRNA by non-neuronal cells that are increasing in number in sympathetic ganglia at this time. If this is indeed the case it would signal another role for NT-3 in chick sympathetic neurons apart from promotion of survival. For example, NT-3 may play a role in regulating catecholamine or neuropeptide synthesis or in promoting target innervation.

By treating fertilised quail eggs with an anti-NT3 antibody, Gaese *et al* (1994) demonstrated that NT3 is needed *in vivo* if the full complement of nodose and DRG neurons to be reached in these ganglia. More recent work involving transgenic mice has demonstrated that in the absence of NT-3 there is a marked reduction in the number of proliferating precursors in immature trigeminal, dorsal root and sympathetic ganglia that reflects increased death of neuroblasts rather than a reduction in the rate of neuroblast division (ElShamy and Ernfors, 1996 (a) and 1996 (b); ElShamy *et al*, 1996). This *in vivo* data is in agreement

with *in vitro* data showing that NT-3 supports the long term survival of proliferating sympathetic neuroblasts from rat (Birren and Anderson, 1993; DiCicco-Bloom *et al*, 1993). Proliferating neuronal precursors do not have access to target field neurotrophic factors but rather appear to obtain NT-3 locally. In the case of sympathetic ganglia, the source of NT-3 for proliferating neuroblasts would appear to be non-neuronal cells surrounding the sympathetic ganglia (Verdi *et al*, 1996). In the case of sensory neuroblasts, it appears as if cells within the developing ganglia itself provide NT-3 (ElShamy and Ernfors, 1996b). It is not clear whether neurons, neuronal precursors or non-neuronal cells within early sensory ganglia synthesise NT-3. If the former is the case then it calls into question the supposition that neurons are independent of neurotrophic factors during the period that they grow towards their targets (Vogel and Davies, 1991).

TrkC may mediate the other roles that NT-3 plays during the early stages of neural development. For example NT-3 has been shown to accelerate maturation of sensory neurons and increase differentiation of motor neurons (Wright *et al.*, 1992; Averbuch-Heller *et al*, 1994). Data from experiments with the PC12 cell line support the idea that NT-3 may play a role in regulating some aspect of neuronal differentiation (Valenzuela *et al*, 1993). In addition, in-ovo administration of NT-3 during a specific time window in the development of chick appears to result in the cessation of division of sensory neuron precursors (Ockel *et al.*, 1996). Similarly, the administration of high levels of NT-3 to proliferating sympathetic precursors *in vitro* has been reported to result in exit form the cell cycle (Verdi and Anderson, 1994).TrkC transcripts appear to be expressed very early in neuronal development by subsets of premigratory and early migrating neural crest cells that migrate exclusively on the medial pathway (Henion *et al*, 1995). This suggests that NT-3 may play a role in controlling neural crest-cell migration.

Regulation of the expression of TrkC

I have shown that NT-3 can up-regulate the mRNA for its preferred receptor, TrkC, in E6 DMTG neurons grown in low density cultures. Although regulation of TrkC mRNA by NT-3 does not appear to be dose dependent, it is possible that the lowest concentration of NT-3 (2ng/ml) that I used in my study represents a saturating level. Further experiments would be needed to determine whether this is the case. The regulation of neurotrophin receptor mRNAs by neurotrophins provides a mechanism by which the response of particular neurons to neurotrophins can be coordinated both spatially and temporally with the onset of neurotrophin expression in peripheral and central targets and to the arrival of fibres in their targets. This phenomenon is not new and has been well established *in vitro*, although the role that neurotrophins play in regulating the expression of their receptors *in vivo* is not yet clear. For example, up-regulation of mRNAs for both the low and high affinity NGF receptors in mouse trigeminal sensory neurons occurs in parallel with an increase in NGF levels in the principal peripheral targets of these neurons (Wyatt and Davies, 1993). The expression of mRNA for the low affinity neurotrophin receptor, gp75, is regulated *in vitro* by BDNF and NGF (Wyatt and Davies, 1993). However, gp75 mRNA shows a normal pattern of expression in the developing trigeminal neurons in the absence of a functional NGF gene suggesting that NGF does not play a role in regulating gp75 mRNA expression in sensory neurons *in vivo* (Davies *et al.*, 1995). Although NGF appears to regulate expression of the mRNA for its high affinity receptor TrkA in basal forebrain cholinergic neurons and in PC12 cells (Holtzman *et al*, 1992; Meakin *et al*, 1992), it does not appear to do so either in cultured sympathetic neurons or in sensory neurons both *in vitro* and *in vivo*

(Wyatt and Davies, 1995: Wyatt and Davies, 1993; Davies *et al*, 1995). BDNF has recently been shown to regulate the expression of the mRNA for its high affinity receptor, TrkB in cultured nodose ganglion neurons from chick (Robinson *et al*, 1996), however analysis of TrkB mRNA expression in embryos that contain a null mutation in the BDNF gene will be required to establish whether BDNF regulates the expression of TrkB mRNA during *in vivo* development. Similarly, a study of NT-3 null mutant embryos will determine the role that NT-3 play in regulating the expression of its receptor during development. In addition, further studies analysing the expression of mRNAs encoding different isoforms of the TrkC receptor, both in NT-3 knockout embryos and in culture, will determine whether NT-3 is involved in regulating the splicing of the TrkC message and hence in determining the ratio of full length, truncated and kinase insert isoforms.

Chapter 6

CONCLUSIONS

Taken together, the data presented in this thesis provide evidence for changes in the responses of developing neural crest derived, neurons but not placode derived neurons to neurotrophins.

The chicken tissue culture studies presented in Chapter 2 showed that DMTG and jugular neurons are responsive to BDNF, NT3 and NGF in early stages of development. Subsequently these neurons lose their responsiveness to BDNF and NT3, becoming responsive only to NGF. It is unlikely that the early survival responses of neural crest derived neurons to BDNF and NT3 play a direct role in regulating the final number of neurons that survive in these ganglia because the responses are largely over by the time naturally occurring neuronal cell death begins. It is possible, however, that the function of these early survival responses to BDNF and NT3 may be to sustain the survival of the neurons whose axons reach the target field during the early stages of its innervation. If, in the absence of early BDNF and NT3 survival responses, the supply of NGF from the target field was limiting from the earliest stages of target field innervation, it is possible that too many neurons would be eliminated before the capacity of the growing target field has increased to support the required number of neurons. It may also be an advantage for most of the neurons that innervate a given target field to compete for survival at the same time.

It is also shown that subsets of trigeminal neurons discriminate between neurotrophins at very high concentrations during the period of cell

death, indicating that neurotrophin responses can be far more highly specific than previously thought.

The examination of the timing of neuronal death in mouse trigeminal ganglia of mice lacking each of the three trk receptors by quantification of normal neurons and pyknotic nuclei from E11 to E17 provided convincing supporting evidence *in vivo* for the *in vitro* results showing that neural crest derived neurons undergo a switch in their neurotrophin requirement: early in their development they require signalling through TrkB and later these neurons require signalling through TrkA.

In order to investigate if the loss of the early response of chicken trigeminal neurons to NT3 and BDNF is related to their high affinity receptor expression pattern, quantitative and semi-quantitative PCR assays were developed to investigate the timing and regulation of expression of TrkC mRNA in chicken trigeminal and sympathetic neurons during development. It was observed that there is a high level of TrkC mRNA in early trigeminal neurons which accords with the early survival response of these neurons to NT3. This suggests that the timing of NT3 responsiveness is controlled by the expression of its receptor. The detection of an extra TrkC mRNA transcript using tyrosine kinase primers is an interesting finding. It may represent a novel truncated isoform of TrkC, a possibility which I am currently investigating. The abrupt decrease in the levels of expression of TrkC mRNA at E8 could be due to a transcription of different isoforms of this receptor mRNA instead of the full length form which is known to be the functional one. It is also important to note that the E6 DMTG sample in which TrkC mRNA was investigated comprises a mixed population of neuronal and non-neuronal cells. Therefore the decreased levels of expression could simply reflect the expression of TrkC mRNA only in neuronal cells. The expression and regulation of TrkB mRNA is also under investigation.

It is important to note that this phenomenon of switching neurotrophin responsiveness, although not universal to all types of neurons, is highly conserved between lower (chicken) and higher (mouse) vertebrate neurons of the same origin (neural crest derived neurons), reinforcing the general significance of this phenomenon in neural development.

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Appendix

Media for Tissue Culture

L15

This medium is used for dissecting. It is air buffered.

To one litre of medium (made up with double distilled H_2O)add:
100 mg Streptomycin
60 mg Penicillin
Adjust to pH 7.3 with HCl or NaOH
Millipore filter using 0.22-µm sterile bells.

HAM's F-12

F-12 medium (HAM), carbonate-free, supplemented with L-glutamine. This medium is used for microinjection (do **not** add $NaHCO_3$), or rinsing cells and dishes.
GibcoBRL Cat. No. 21700-026 (1 L unit, powder)

To one litre of medium (made up with double distilled H_2O) add:
100 mg Streptomycin
60 mg Penicillin
Adjust to pH 7.3 with HCl or NaOH
Add heat inactivated horse serum (GibcoBRL) to 10% (defrost in a waterbath at 37°C).
Filter sterilize after adding serum (if serum is required).

HAM's F-14

The F-14 medium is a carbonate-based medium, it is normally used to grow the neurons. It is made up as a 10x concentrate.
The diluted solution is kept not longer than a week at 4°C
Imperial Cat. No. 3-791-35 (5 L unit, powder, with L-glutamine, without $NaHCO_3$).

This is a special formulation for neuronal cell culture
To a 500 ml (10X concentrated stock) add:
500 mg streptomycin
300 mg penicillin
Store in 50 ml aliquots (10x) at -40°C

To make 1x defrost 50 ml 10x aliquot in 37°C waterbath
450 ml ddH_2O
add 1 g $NaHCO_3$
pH to sligthly acid (6.7) with a piece of dry-ice

Filter 0.22-µm into a sterile bottle after serum addition.

SATO STOCK

Note In some formulations, SATO is supplemented with insulin and transferin. These two components are omitted here because of their possible effects on cell differentiation, survival and growth (mitogen).

To make up a 222ml stock, add:

Pathocyte-4-BSA (ICN Biomedicals Cat. No. 810101)	100 ml
Putrescine (Sigma P-7505 (1 g))(160 mg / 100 ml ddH_2O)	100 ml
Progesterone (Sigma P-6149 (1 mg)(1 mg / 1.6 ml EtOH)	1 ml
L-Thyroxine (Sigma T-0397 (4 mg))(4 mg / 10ml EtOH)	10ml
Sodium selenite (Sigma S-9133 (1 mg))(1 mg / 2.6 ml PBS)	1 ml
Tri-iodo-thyronine (Sigma T-6397 (100 mg))(16.8 mg / 50 ml EtOH)	10ml

Mix and aliquot above ingredients in 11.1 ml aliquots, store at -40°C
When preparing the final medium:
Put a 0.22-µm filter + 20 ml syringe on top of a 500 ml F-14 bottle
Add 11.1 ml SATO STOCK to 488.9 ml
5 ml of 200 mM Glutamine (Gibco Cat. No. 25030-024)
Filter using a filter unit with filling bell (Sigma F-9013), 0.22-µm sterile,

pH to 7.3

Solutions

HBSS

Hanks' balanced salts without calcium and magnesium (GibcoBRL Cat. No. 14170-070, 100 ml/bottle).
This medium (serum-free and carbonate-free) is used to coat the plastic dishes with laminin, to trypsinize and triturate the ganglia, and to load the cell suspension on the dropping funnel.

0.1 M PBS

0.1 M phosphate buffered saline. Dissolve 10 tablets (Oxoid, code BR14a) in 1 litre ddH_2O. Autoclave at 115°C for 10 minutes.
To convert to full Dulbecco phosphate saline add 0.5 ml mineral salt solution (code SR39) to the autoclaved solution. pH 7.3 (approx.).

5 M KOH

140 g KOH pellets in 500 ml ddH_2O. This solution is used for making tungsten wire needles.

TUNGSTEN NEEDLES

Tungsten needdles, which are required to complete the dissections, are made from 0.5 mm diameter tungsten wire. The wire is cut into 3 to 5 cm lengths using a grind wheel. The 1cm end is bent at an angle of about 60° and this end is immersed with the bent portion near horizontal in 0.5M KOH. Then, a current of 3 to 12 V AC is passed through to the wire and a second electrode (copper or

steel rod) immersed in the solution. The tungsten is etched away over several minutes forming a taper from the bend to the tip of the needle. The needles should be washed in water to remove any alkali. For dissection, the needles are conveniently held in chuck-grip platinum wire holders.

Trypsin

Use Worthington trypsin, add 50 mg to 5 ml of Ca/Mg free PBS (GibcoBRL). Filter sterilise in laminar flow hood. Aliquot 100 µl into 0.5 ml eppendorf tubes. Used to dissociate neuronal cells.

Laminin

Stored in 20 µl aliquots at 1mg/ml then diluted to 20 µg/ml in PBS, F14 or HBSS.

4% Paraformaldehyde

40 g/l in PBS. Heat in a water bath until temperature reaches 66°C, then add NaOH until all of the paraformaldehyde dissolves. Filter with 0.22-µm filter and 50 ml syringe. (Important! Paraformaldehyde may be carcinogenic, use gloves and avoid breathing dust)

Borate Buffer (0.15M)

4.6g boric acid (Sigma) in 500ml distilled water. pH to 8.4 with 5M NaOH. This buffer is used to dissolve the poly ornithine.

Polyornithin

0.5 mg/ml in 0.15M Boric Acid pH 8.4. Filter sterilised, stored at 4°C and used within one week.