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**Functions of different neurotrophic factors in  
regulating neuronal survival in the developing  
peripheral nervous system**

**A thesis submitted to the University of  
St. Andrews  
for the degree of  
Doctor of Philosophy (PhD)**

**by**

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## Abstract

The main aim of this thesis was to clarify the significance of the GDNF family of neurotrophic factors in neural development. The ability of GDNF, neurturin, persephin and artemin to promote the survival of embryonic chicken parasympathetic, sympathetic and sensory neurons was studied *in vitro*. All of these neurons responded to GDNF and neurturin, but failed to respond to artemin and persephin. The sensitivity to GDNF and neurturin differed for each type of neuron and varied with age. Competitive RT-PCR revealed correlations between sensitivity to GDNF and neurturin and the level of mRNAs encoding the GPI-linked receptor components for these factors. An attempt was made to generate antibodies against these receptor components so that the relationship between receptor expression and responsiveness could be examined. Although antibodies were successfully generated, they were of limited use due to a lack of specificity.

To clarify the ligand specificity of the GPI-linked receptors for members of the GDNF family, the response of neurons from wild-type and *GFR $\alpha$ 1*<sup>-/-</sup> embryos was studied *in vitro*. Whereas *GFR $\alpha$ 1*-deficient nodose neurons failed to respond to GDNF and neurturin, *GFR $\alpha$ 1*-deficient submandibular neurons retained normal responsiveness. These results suggest that GFR $\alpha$ 1 mediates GDNF and neurturin signalling in nodose sensory neurons, whereas an alternative receptor mediates the response of submandibular parasympathetic neurons to these factors.

Finally, in the course of studying the survival responses of PNS neurons to the GDNF family, compelling evidence for the existence of an NGF-dependent subpopulation of nodose neurons was obtained. In addition to *in vitro* survival data, RT-PCR demonstrated that TrkA mRNA is expressed

at relatively high levels in the developing nodose ganglion and histological analysis of nodose ganglia in *NGF*<sup>-/-</sup> mice revealed a significant loss of neurons in the absence of NGF.

## Declaration of candidate

I, Alison Jane Forgie, hereby certify that this thesis, which is approximately 55,000 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.

Signature of candidate

Date 6.10.99

I was admitted as a research student in October 1995 and as a candidate for the degree of Doctor of Philosophy in October 1996; the higher study for which this is a record was carried out in the University of St. Andrews between 1995 and 1999.

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## Declaration of supervisor

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

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# Chapter 1

## General Introduction

### **1.1. Development of the vertebrate nervous system**

The complexity of the nervous system is unparalleled within vertebrate tissues. This is attributed to the large number and diversity of neurons, coupled with the extensive and precise pattern of connections they establish. It is considered that the various functions of the mature human nervous system, such as motor coordination, memory, perception, emotion, consciousness and learning, depend on the establishment and consolidation of specific patterns of neuronal connections. Hence, as a first insight into this functional network, it is important to understand the development of the nervous system.

During the earliest stages of vertebrate development, the monolayered blastula is transformed into a three-layered structure through a process of morphogenetic movements termed gastrulation. These three layers comprise the ectoderm which later gives rise to the epidermis and nervous system, the mesoderm which gives rise to connective tissue elements, muscles, vascular and urogenital systems, and the endoderm from which the epithelial structures of the respiratory and digestive systems and associated organs are derived. During gastrulation, a process termed neural induction initiates the development of the nervous system. Dorsal ectoderm is induced by the underlying notochord and adjacent mesoderm to thicken and form the neural plate. A series of morphogenetic movements, termed neurulation, causes the lateral edges of the neural plate to rear up, forming the neural folds. As the midline of the neural plate sinks deeper, these folds come into

apposition and fuse forming the intact neural tube. The neural tube then sinks beneath the surface, leaving a covering layer of ectoderm. The neural tube gives rise to the central nervous system (CNS), comprising the brain and spinal cord.

Along the line where the neural tube separates from the future epidermis, a number of cells, termed neural crest cells, break loose from the ectoderm and migrate through the mesoderm along well defined pathways to reach diverse regions of the vertebrate embryo where they subsequently give rise to a number of derivatives. Neural crest cells form almost all of the peripheral nervous system (PNS), including the autonomic sympathetic, parasympathetic and enteric ganglia, most sensory ganglia, Schwann cells and satellite cells. Moreover, they give rise to chromaffin cells of the adrenal medulla, melanocytes of the skin and craniofacial connective tissue elements.

## **1.2. The peripheral nervous system**

The peripheral nervous system is composed of the sensory and autonomic ganglia and their associated peripheral nerves that lie outwith the brain and spinal cord.

### **1.2.1. Sensory ganglia**

Sensory ganglia are classified into cranial and dorsal root ganglia. Cranial sensory ganglia are found on five of the twelve pairs of cranial nerves and comprise the trigeminal ganglion (located on cranial nerve V), geniculate ganglion (VII), jugular ganglion (X), nodose ganglion (X), superior glossopharyngeal ganglion (IX), petrosal ganglion (IX) and vestibulo-cochlear ganglion (VIII). An exception to this is the trigeminal mesencephalic nucleus (TMN), which is located in the midbrain and contains

the cell bodies of proprioceptive neurons that innervate the muscles of mastication. This is the only central nervous system site in which primary sensory neuron cell bodies, derived from neural crest, are found. The dorsal root ganglia (DRG) are located on the dorsal roots of the spinal nerves immediately adjacent to the spinal cord. The innervation targets of the neuronal populations studied in this thesis are discussed within the relevant chapters.

### **1.2.2. Autonomic ganglia**

The autonomic nervous system has three principal divisions: sympathetic (thoracolumbar), parasympathetic (craniosacral) and enteric. The sympathetic division comprises paravertebral and prevertebral sympathetic ganglia. Paravertebral sympathetic ganglia include the superior cervical ganglion (SCG), middle cervical ganglion, stellate ganglion and sympathetic chain ganglia. Prevertebral sympathetic ganglia include the coeliac ganglion, aorticorenal ganglion and superior and inferior mesenteric ganglia. Additionally, the chromaffin cells of the adrenal medulla can be considered to be a prevertebral sympathetic ganglion as these cells are developmentally and functionally related to postganglionic sympathetic neurons. Examples of parasympathetic ganglia include the ciliary ganglion, pterygopalatine ganglion, submandibular ganglion, otic ganglion, terminal ganglia of the vagus nerve and the pelvic plexus. The enteric nervous system (ENS) is composed of a collection of autonomic ganglia, comprising sensory neurons, interneurons, motoneurons and astrocyte-like glia arranged in interconnected plexuses within the wall of the gastro-intestinal tract. The two major plexuses are the submucosal (Meissner's) and the myenteric (Auerbach's) plexuses. The ENS provides innervation to the smooth muscle of the gut wall and to the vessels and glands of the mucosa.

### 1.2.3. Origins of the peripheral nervous system

Sensory neurons differentiate from progenitor cells that originate from two regions of the embryonic ectoderm, the neural crest and neurogenic placodes (Noden, 1978; Le Douarin, 1986; D'Amico-Martel, 1982; D'Amico-Martel and Noden, 1983). The neurogenic placodes are epithelial thickenings in the cephalic ectoderm of the early vertebrate embryo. Detailed mapping experiments involving transplantation of neural crest- and placode-derived cells have determined the origins of cranial sensory neurons in avians. Whilst the neurons of the ventrolateral part of the trigeminal ganglion and the vestibulo-cochlear, geniculate, petrosal and nodose ganglia are derived from neurogenic trigeminal, epibranchial and otic placodes, those of the dorsomedial part of the trigeminal ganglion, trigeminal mesencephalic nucleus, jugular ganglion and dorsal root ganglia are derived from different regions of neural crest. The Schwann cells and satellite cells associated with all sensory ganglia arise exclusively from the neural crest (D'Amico-Martel and Noden, 1983). Heterotopic grafting experiments have demonstrated that the presumptive placodal ectoderm is not specified to differentiate into particular sensory neurons. Rather, signals acting in the vicinity of this ectoderm commit the cells to a particular neuronal fate (Vogel and Davies, 1993).

Both neuronal and glial cells of sympathetic and parasympathetic ganglia are derived from neural crest cells of the trunk region (Le Douarin, 1980; D'Amico-Martel and Noden, 1983; Bronner and Fraser, 1988), whereas neurons and glia of the ENS arise from three regions of neural crest, vagal, sacral and truncal (Le Douarin and Teillet, 1973, 1974; Bronner and Fraser, 1988; Serbedzija *et al.*, 1991; Epstein *et al.*, 1994). Cell lineage studies have shown that premigratory crest cells are not generally committed to a single fate (Bronner and Fraser, 1988). Instead, factors such as the

microenvironment of their migratory pathway and destination site influence their differentiation.

### 1.3. Apoptosis

The generation of the correct number of cells in the nervous system is a highly controlled and coordinated process that is the consequence of both cell proliferation and cell death. Within the vertebrate nervous system, neurons are initially generated in excess. Approximately half of the neurons generated will die during embryonic and early postnatal development to ensure that the correct number of neurons innervate particular targets (see section 1.4.1.) (Oppenheim, 1991). This programmed cell death occurs by apoptosis which is associated with several characteristic morphological and biochemical changes that distinguish it from necrotic cell death (reviewed in Sanders and Wride, 1995). These include preservation of membrane integrity, cytoplasmic shrinkage and chromatin condensation (Kerr *et al.*, 1972; Wyllie, 1997).

There are two major triggers of neuronal cell death during development: deprivation of appropriate neurotrophic support or stimulation of receptors that activate programmed cell death directly. However, these two processes cannot be entirely separated, since stimulation of receptors that activate cell death may induce the requirement for neurotrophic support in some cell types. While neuronal cell death resulting from an inadequate supply of neurotrophic factors has been extensively investigated, the presence of death-inducing receptors on neurons has only recently been elucidated. For example, the p75 neurotrophin receptor, a member of the tumour necrosis factor (TNF) and Fas family, has been reported to transduce ligand-dependent apoptotic signals in developing retinal neurons, basal forebrain

cholinergic neurons and trigeminal mesencephalic neurons (Frade *et al.*, 1996; Van der Zee *et al.*, 1996; Yeo *et al.*, 1997; Davey and Davies., 1998). Additionally, neonatal oligodendrocytes in culture are killed by a p75-dependent mechanism (Casaccia-Bonnel *et al.*, 1996). The intracellular events leading to p75-mediated apoptosis are unclear, however, they are well documented in non-neuronal tissues following activation of other death-inducing receptors of the TNF/Fas family, namely CD95, p55 tumour necrosis factor receptor and death receptors-3, 4 and 5 (reviewed in Yuan, 1997).

Following initial activation of the cell death program, metabolic changes occur within the cell including decreased glucose uptake and decreased RNA and protein synthesis. Once the stereotypical morphological changes in cell structure associated with apoptosis begin, the process is irreversible and the cell enters a commitment phase. Although any one of many intracellular signalling pathways can be employed during the induction phase of apoptosis, depending on the apoptotic trigger and cell type, these pathways converge during the commitment and execution phases of this process (reviewed in Green and Kroemer, 1998).

Extensive studies of the nematode, *Caenorhabditis elegans* found that three genes, *ced-3*, *ced-4* and *ced-9*, are responsible for the programmed death of 131 somatic cells during nematode development (reviewed in Hengartner and Horvitz, 1994, 1994a). The *ced-3* gene is involved in the execution of apoptosis and encodes a cysteine protease (Yuan *et al.*, 1993; Xue *et al.*, 1996), while the product of *ced-4* promotes the activation of *ced-3* (Ellis and Horvitz, 1986). *Ced-9*, on the other hand, works upstream of *ced-3* and *ced-4* (Hengartner *et al.*, 1992). By physically interacting with *ced-4*, and thus preventing the activation of *ced-3*, *ced-9* has an anti-apoptotic role

(Chinnaiyan *et al.*, 1997; Spector *et al.*, 1997; Irmeler *et al.*, 1997; Wu *et al.*, 1997, 1997a).

Although the components of cell death machinery are remarkably well conserved between species, the death pathway has acquired higher levels of complexity within mammals. The interleukin-1 $\beta$ -converting enzyme (ICE) family of cysteine proteases (or caspases), currently 13 in number, constitute the mammalian counterparts of *ced-3* (reviewed in Bergeron and Yuan, 1998), while Apaf-1 is the mammalian homologue of *ced-4* (Zou *et al.*, 1997). During the commitment stage of the apoptosis process, mitochondrial permeability pores are opened releasing cytochrome *c*. In the presence of cytochrome *c* and ATP, Apaf-1 binds to and activates procaspase-9, which in turn cleaves and activates caspase-3. Caspase-3 then cleaves and activates other caspases in the death cascade resulting in the cleavage of numerous cellular substrates, including poly(ADP-ribose) polymerase (PARP) and lamins, inducing cellular disassembly (Zou *et al.*, 1997; Li *et al.*, 1997; Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997; Villa *et al.*, 1997; Wang *et al.*, 1996, 1998; Cohen, 1997; Friedlander and Yuan, 1998).

Because caspases are constitutively present in the cytoplasm as inactive proenzymes, their activation must be held in check if cells are to survive. The *Bcl-2* family of genes are the mammalian homologues of the *ced-9* gene and as such, they act upstream of the caspases and regulate their activity (Chinnaiyan *et al.*, 1996). The *Bcl-2* family is comprised of 15 genes, encoding proteins that either inhibit or accelerate cell death (reviewed in Kelekar and Thompson, 1998). *Bcl-2* family members with anti-apoptotic action include the founder member, *Bcl-2*, as well as *Bcl-x<sub>L</sub>*, *Bcl-w*, *Bfl-1*, *A-1* and *Mcl-1*. Those with pro-apoptotic activity are *Bcl-x<sub>S</sub>*, *Bax*, *Bad*, *Bak*, *Bik*, *Bid*, *Bim* and *Hrk*. A striking feature of the *Bcl-2* family is their ability to homodimerise

and heterodimerise through their conserved regions, BH1, BH2, BH3 and BH4 (Bcl-2 homology regions 1-4). The level of expression of pro-apoptotic and anti-apoptotic proteins and the interactions between them seem to regulate cell death (reviewed in Rao and White, 1997). Furthermore, post-translational phosphorylation of some Bcl-2 family members controls apoptosis by preventing caspase activation. It has recently been shown that Bcl-2 interferes with the activation of caspase-3 by preventing the release of cytochrome *c* from mitochondria (Du *et al.*, 1997, 1997a; Kluck *et al.*, 1997; Reed, 1997; Yang *et al.*, 1997). Additionally, anti-apoptotic Bcl-2 proteins such as Bcl<sub>x</sub>L, may act by holding the pro-apoptotic Apaf-1/caspase-3 complex inactively bound to the mitochondrial membrane (Hengartner, 1997; Golstein, 1997; Reed, 1997). Conversely, Bax, a pro-apoptotic homologue of Bcl-2, heterodimerises with Bcl<sub>x</sub>L thus displacing it from the inactive Apaf-1/caspase-3 complex and inducing cytochrome *c* release and activation of caspase-3 (Golstein, 1997; Reed, 1997; Rosse *et al.*, 1998).

## **1.4. Role of neurotrophic factors in neuronal development**

### **1.4.1. The neurotrophic hypothesis**

Neurons are generated in excess in the developing vertebrate nervous system, and those that are superfluous to requirements or inappropriately connected die shortly after their axons reach their targets, during a period of naturally occurring developmental cell death. Studies of the distribution of nerve growth factor (NGF), the first neurotrophic factor to be identified (Levi-Montalcini and Angeletti, 1968), and experimental manipulation of its availability to neurons during development has led to the formulation of the neurotrophic hypothesis. This classic paradigm for neurotrophic action proposes that the survival of developing neurons is dependent on the supply

of a neurotrophic factor that is synthesised in limiting amounts in their target fields.

In support of the neurotrophic hypothesis is the finding that certain populations of developing sensory and sympathetic neurons are dependent on NGF for survival *in vitro* and *in vivo*. Administration of anti-NGF antibodies during the phase of target field innervation eliminates these neurons whereas exogenous NGF rescues neurons that would otherwise die (Levi-Montalcini and Angeletti, 1968; Johnson *et al.*, 1980). These same neurons are also lost in mice that have targeted null mutations in the *NGF* gene (Crowley *et al.*, 1994) or NGF receptor tyrosine kinase (*TrkA*) gene (Smeyne *et al.*, 1994). Furthermore, studies of the site, timing and level of expression of NGF in development provided additional support for this hypothesis. NGF is synthesized in the peripheral target fields of these neurons coinciding with the beginning of target field innervation (Davies *et al.*, 1987; Korsching and Thoenen, 1988). Additionally, the level of NGF expression is proportional to the final innervation density. For example, at the onset of neuronal death in the trigeminal ganglion, high levels of NGF mRNA are detected in future densely innervated cutaneous territories, whereas only low levels of NGF mRNA are found in future sparsely innervated territories (Harper and Davies, 1990). Following uptake by sensory and sympathetic fibres in the target field, NGF is conveyed by fast axonal transport to the cell bodies of the innervating neurons where it exerts its survival-promoting effects (Hendry *et al.*, 1974; Korsching and Thoenen, 1983).

NGF is the founder member of a family of secreted proteins which in mammals includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Extensive *in vitro* and *in vivo*

studies of the effects of these neurotrophins on neurons and studies of mice with targeted null mutations in genes encoding these proteins or their receptors have revealed the generality of the neurotrophic hypothesis with regard to a number of neurotrophin growth factors (reviewed in Davies, 1994; Lewin and Barde, 1996). Furthermore, a number of other growth factors not belonging to the neurotrophin family have also been shown to promote neuronal survival in accordance with the neurotrophic hypothesis (as discussed below).

Studies using neurons of chicken cranial sensory ganglia have provided useful information on the timing and regulation of neurotrophin dependence in neurons dependent on target-derived neurotrophic factors for survival. Different populations of cranial sensory neurons, derived from the neurogenic placodes, are born during the same period of development and start extending axons to their targets at the same time. However, the distance their axons must grow to reach their respective targets varies. When these neurons are cultured at a stage of development when they are first starting to extend axons *in vivo*, the duration of neurotrophin independent survival and the onset of neurotrophin dependence *in vitro* correlates with the distance and time taken to reach their targets *in vivo* (Davies, 1987, 1989; Vogel and Davies, 1991). Furthermore, studies of the survival of neurons that differentiate *in vitro* from neurogenic placodal cells suggest that the duration of neurotrophin independence is already programmed in sensory neuron progenitor cells (Vogel and Davies, 1991).

Despite the overwhelming evidence for the neurotrophic hypothesis, recent data suggests that this is not the only paradigm by which neurotrophic factors influence neurons (reviewed in Davies, 1999; Davies *et al.*, 1999). Some neurons require trophic support, not just during the period of target

field innervation, but at stages prior to and after this. Indeed, neurotrophic factors have been shown to influence neuron precursor cell proliferation and differentiation, promote the maturation of newly generated neurons, regulate process outgrowth and neurotransmitter and neuropeptide synthesis and influence neuronal form and synaptic function throughout life (reviewed in Davies, 1994; Averbuch, 1994; Thoenen, 1995; Lewin and Barde, 1996; Maina *et al.*, 1998). Also, some neurons can procure neurotrophic factors from sites other than their peripheral and central target fields, such as cells encountered by their axons whilst en-route to their targets. Convincing evidence that Schwann cells or their precursors provide trophic support to sensory neurons at early stages in their development comes from the finding that most DRG neurons die early in development in mice lacking Schwann cells in peripheral nerves as a result of a targeted mutation in the *erbB3* gene (Riethmacher *et al.*, 1997). Alternatively, some neurons are capable of producing neurotrophic factors to sustain their own survival at certain stages in development. For example, chicken DRG neurons at an early stage in their development express BDNF, which, although without effect on survival, seems to enhance an early maturational change in these neurons (Wright *et al.*, 1992). The survival of immature sympathetic neurons is promoted by hepatocyte growth factor (HGF) produced in an autocrine manner (Maina *et al.*, 1998). Furthermore, a subset of adult DRG neurons are reported to be maintained via a BDNF-autocrine route (Acheson *et al.*, 1995). Experimental evidence also exists to suggest that the survival of neurons can be promoted by neuronally-produced growth factors in a paracrine manner (Robinson *et al.*, 1996).

Several different neurotrophic factors may participate in regulating the survival of certain developing neurons, either by acting in concert or in a sequential manner. *In vitro* survival studies of TMN proprioceptive neurons

demonstrate that during the period of naturally occurring neuronal death, the survival of these neurons is promoted by both BDNF and NT-3 (Davies, 1986, Hohn *et al.*, 1990). Accordingly, there is a 50 % reduction in total neuronal number in the TMN of neonatal *BDNF*<sup>-/-</sup> mice (Ernfors *et al.*, 1994; Jones *et al.*, 1994) and a 50 % reduction in *NT-3*<sup>-/-</sup> mice (Ernfors *et al.*, 1994a) compared to wild-type littermates. Additional evidence for the cooperation of two different neurotrophic factors in regulating the survival of neurons during the phase of naturally occurring neuronal death comes from studies of sympathetic neurons of the superior cervical ganglion which demonstrate a late survival requirement for NT-3 *in vivo* at a stage when these neurons are also dependent on NGF for survival (Zhou and Rush, 1995; Wyatt *et al.*, 1997; Francis *et al.*, 1999).

It is now becoming clear that some neurons switch their trophic requirements from one factor to another during development (reviewed in Davies, 1994a, 1997, 1999; Davies *et al.*, 1999). Compelling evidence for neurotrophin switching comes from detailed *in vitro* and *in vivo* studies of the developing mouse trigeminal system (reviewed in Davies, 1997a). The *in vitro* survival requirements of mouse trigeminal neurons switch from BDNF and/or NT-3, at E10 and E11, to NGF at E12 (Buchman and Davies, 1993; Paul and Davies, 1995). This switch is correlated with a change in expression of the respective neurotrophin receptors (Wyatt and Davies, 1993; Ninkina *et al.*, 1996) and a change in the neurotrophin mRNA expression in the peripheral trigeminal territory (Davies *et al.*, 1987; Buchman and Davies, 1993; Arümae *et al.*, 1993). An examination of the timing of cell death in the trigeminal ganglia of mice with null mutations in the genes encoding the neurotrophin receptors suggests that neurotrophin switching occurs *in vivo* (Piñón *et al.*, 1996). Recent data directly illustrates that some early-born neurons of the trigeminal ganglion do indeed switch neurotrophin

dependence from BDNF to NGF during development, whereas late-born neurons respond to NGF from the outset (Enokido *et al.*, 1999). Unlike the onset of BDNF-responsiveness in placode-derived sensory neurons, which is controlled by an intrinsic timing mechanism (Vogel and Davies, 1991), the switch from BDNF/NT-3 dependence to NGF requires a signal to act on the neurons during the switch-over period. Studies in explant culture demonstrate that a signal from the cellular environment of the early trigeminal ganglion itself is sufficient to enable early-born trigeminal neurons to switch neurotrophin dependence (Enokido *et al.*, 1999).

It is unclear why some populations of sensory neurons switch their neurotrophin dependence during an early stage in development. Interestingly, sensory neurons that switch neurotrophin dependence can survive only very briefly in cultures without neurotrophins during the earliest stages of their development (Ernsberger and Rohrer, 1988; Wright *et al.*, 1992; Buchman and Davies, 1993), unlike those that do not switch which have a regulated period of neurotrophin independence related to target distance (Vogel and Davies, 1991). The availability of BDNF and NT-3 to the peripheral axons (Buchman and Davies, 1993; Arümae *et al.*, 1993) of sensory neurons that undergo early neurotrophin switching may suggest that these neurotrophins sustain these neurons until their peripheral axons reach the target tissues where NGF is produced (Davies *et al.*, 1987; Harper and Davies, 1990). It is possible that neurons that do not switch, and which have a programmed period of neurotrophin independence, do not require such intermediate support before reaching their targets.

It is also interesting to note that the neurotrophin dependence of many sensory neurons, during and after the peak of naturally occurring cell death, is related to sensory modality. For example, proprioceptive neurons are

dependent on NT-3 and BDNF, thermo-and nociceptive neurons are dependent on NGF and visceral sensory neurons are dependent on BDNF and NT-4. Evidence for this is provided from detailed *in vitro* studies of the neurotrophin responses of cranial sensory neurons which are anatomically segregated into groups serving distinct sensory modalities (reviewed in Davies, 1994, 1999). Analysis of the neurotrophin receptors expressed by different functional classes of sensory neurons and studies of the types of neurons eliminated in mice with null mutations in genes encoding neurotrophins and their receptors have substantiated the *in vitro* results (reviewed in Davies, 1999).

Several members of other families of trophic factors have been found to promote the survival of developing neurons. In addition to the neurotrophins, these include neuropoietic cytokines and TGF- $\beta$ -related proteins, including members of the GDNF family. Each of these families will be discussed in the following sections. As the majority of this thesis describes investigations into the role of the GDNF family in developing peripheral neurons, this family of neurotrophic factors will be reviewed in the greatest detail.

## **1.5. The family of neurotrophins and their receptors**

The neurotrophin family comprises six structurally and functionally related proteins: nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, neurotrophin-4/5, neurotrophin-6 and neurotrophin-7. In order to transduce their signals into the cell, neurotrophins interact with two distinct classes of receptor: a specific high affinity receptor tyrosine kinase (RTK) of the Trk family (TrkA, TrkB or TrkC) and a common receptor, p75. The neurotrophins and their receptors are discussed in turn below.

### 1.5.1. Nerve growth factor

Following the initial discovery that a fraction of snake venom had nerve growth promoting activity, the protein responsible for this activity was isolated and purified (Cohen and Montalcini, 1956; Cohen, 1959). Subsequently, the submandibular salivary gland of the adult male mouse was found to be an extremely rich source of this protein, which was termed nerve growth factor (NGF) (Cohen, 1960). The determination of the mouse NGF amino acid sequence (Angeletti and Bradshaw, 1971) led to the cloning of the mouse *NGF* cDNA (Scott *et al.*, 1983). Thereafter, human, bovine and chicken *NGF* genes have been cloned demonstrating that NGF has been highly conserved in evolution (Ullrich *et al.*, 1983; Meier *et al.*, 1986; Ebendal *et al.*, 1986). The mouse *NGF* gene encodes a 307-amino acid precursor polypeptide that is cleaved to release a 118-residue mature protein (Berger and Shooter, 1977; Scott *et al.*, 1983; Edwards *et al.*, 1988). Analysis of the three-dimensional structure of mature NGF, using X-ray diffraction, has revealed that it contains a motif of three intrachain disulphide bridges, known as the cysteine knot motif. Non-covalent homodimerisation through extensive hydrophobic interactions gives rise to the biologically active form of NGF (McDonald *et al.*, 1991).

NGF mRNA is found in several regions of the CNS, including the hippocampus, olfactory bulb and neocortex, all of which are innervated by cholinergic neurons of the basal forebrain. Additionally, NGF mRNA is detected in regions of the brain where the cell bodies of these neurons are located (septum, nucleus of the diagonal band of Broca and the nucleus basalis of Meynert) (Korsching *et al.*, 1985). NGF is also detected in other brain regions, but at significantly lower levels (Whittemore *et al.*, 1986; Maisonpierre *et al.*, 1990). Outwith the brain, NGF mRNA is found in the target tissues of NGF-responsive neurons (Korsching and Thoenen, 1983,

1988; Heumann *et al.*, 1984; Shelton and Reichardt, 1984; Korsching *et al.*, 1985). The exact identity of target cells that secrete NGF during development was first elucidated in a detailed study of the developing mouse whisker pad (Davies *et al.*, 1987). Enzymatic dissociation of the whisker pad into its main components, epithelium (presumptive epidermis) and mesenchyme (presumptive dermis), demonstrated that the highest levels of NGF mRNA are expressed in the epithelial component.

Extensive *in vitro* and *in vivo* studies have demonstrated that NGF is a target-derived neurotrophic factor for sympathetic neurons and subpopulations of neural crest-derived sensory neurons. NGF does not exert neurotrophic effects on placode-derived cranial sensory neurons, enteric neurons, parasympathetic neurons or spinal motoneurons. Within the CNS, NGF is a survival factor for basal forebrain cholinergic neurons *in vitro*. (Chun and Patterson, 1977; Gorin and Johnson, 1980; Hamburger *et al.*, 1981; Davies and Lindsay, 1985; Levi-Montalcini, 1987; Hartikka and Hefti, 1988)

The generation of mice with a targeted null mutation in the *NGF* gene enabled clarification of the role of NGF in the nervous system *in vivo*. *NGF*<sup>-/-</sup> mice are born with virtually no sympathetic neurons and a very substantial loss of neurons in the trigeminal and dorsal root ganglia (Crowley *et al.*, 1994). Detailed examination of the neuronal loss in the DRG demonstrates that it is a selective loss, affecting only small diameter neurons thought to mediate nociceptive information. This is reflected in the observation that *NGF*<sup>-/-</sup> mice fail to respond to noxious mechanical stimuli. In the converse transgenic experimental paradigm, over-expression of NGF in the epidermis results in decreased neuronal loss in certain sensory and sympathetic ganglia compared to wild-type counterparts (Albers *et al.*, 1994). Whilst the severe PNS phenotype confirms the requirement of certain populations of

peripheral neurons for NGF, the normal complement of basal forebrain cholinergic neurons and other central neurons indicates that NGF is not an essential survival factor in the embryonic CNS. Since *NGF<sup>-/-</sup>* mice die soon after birth, it is not clear whether NGF is an essential survival factor for postnatal CNS neurons. Interestingly, choline acetyltransferase (ChAT) immuno-reactivity in the basal forebrain is reduced in mice homozygous for the null mutation compared to wild-type littermates, suggesting that NGF may play a role in regulating the functional status of these cells.

### **1.5.2. Brain-derived neurotrophic factor**

Brain-derived neurotrophic factor (BDNF) was first isolated from adult pig brain based on its ability to promote the survival of E10 chicken DRG neurons in culture (Barde *et al.*, 1982). Data obtained from partial amino acid sequencing was used to clone the porcine *BDNF* cDNA (Leibrock *et al.*, 1989). *BDNF* has subsequently been cloned in several other species including human, mouse and rat (Hofer *et al.*, 1990; Jones and Reichardt, 1990; Maisonpierre *et al.*, 1991). BDNF is a basic, 12.3 kDa protein which, like NGF, is synthesized as a precursor polypeptide of 252 amino acids that is cleaved to yield a 119-amino acid mature protein. BDNF displays approximately 50% amino acid sequence identity with mature NGF and contains the six cysteine residues involved in forming the cysteine knot motif. Biologically active BDNF exists as a tightly associated homodimer (Radziejewski *et al.*, 1992).

BDNF mRNA is largely restricted to the CNS with relatively low levels of expression in the embryonic brain giving way to more widespread, higher levels of expression as the CNS matures. In the adult CNS, substantial amounts of BDNF mRNA are found in the cortex, hippocampus and cerebellum. Lower levels of BDNF mRNA are detected in the striatum,

olfactory bulb, midbrain, hindbrain, and spinal cord, the latter two being the central targets of BDNF-responsive sensory neurons (Leibrock *et al.*, 1989; Hofer *et al.*, 1990). In the developing PNS, BDNF mRNA is expressed in NGF-dependent neurons of the dorsal root, trigeminal and jugular ganglia (Robinson *et al.*, 1996; Schecterson and Bothwell, 1992). BDNF is also expressed by a subpopulation of adult DRG neurons and promotes their survival in an autocrine manner (Acheson *et al.*, 1995). BDNF mRNA is also detected, albeit at low levels, in the peripheral target tissues of BDNF-responsive neurons, such as the heart, skin, muscle and lung (Maisonpierre *et al.*, 1990; Schecterson and Bothwell, 1992).

In the PNS, BDNF has been shown to support the survival of both embryonic neural crest- (trigeminal mesencephalic nucleus and dorsal root ganglia) and placode-derived (ventrolateral part of the trigeminal ganglion, geniculate, petrosal, vestibular and nodose ganglia) sensory neurons *in vitro* (Lindsay *et al.*, 1985; Davies *et al.*, 1986, 1987a; Barde *et al.*, 1987; Hofer and Barde, 1988). In addition, BDNF transiently supports the survival of neurons from the dorsomedial part of the trigeminal ganglion and the jugular ganglion (Buj-Bello *et al.*, 1994). Likewise, within the CNS, BDNF enhances the survival of several classes of neurons in culture. These include embryonic rat basal forebrain cholinergic neurons, mesencephalic dopaminergic neurons, motoneurons, hippocampal neurons, cerebellar granule cells and retinal ganglion cells (Johnson *et al.*, 1986a; Alderson *et al.*, 1990; Hyman *et al.*, 1991; Knüsel *et al.*, 1991; Oppenheim *et al.*, 1992; Lindholm *et al.*, 1993; Vejsada *et al.*, 1995). In addition to its survival promoting effects, BDNF is also known to influence the differentiation and maturation of developing sensory neurons (Kalcheim and Gendreau, 1988; Wright *et al.*, 1992) and certain central neurons (Marty *et al.*, 1996).

The physiological relevance of BDNF has been elucidated from the analysis of mice with a targeted null mutation in the *BDNF* gene. These mice develop symptoms of nervous system dysfunction and on histological examination, are found to have severe deficits in the number of cranial and spinal sensory neurons (Ernfors *et al.*, 1994; Jones *et al.*, 1994). Particularly substantial neuronal losses are found in the trigeminal, geniculate, vestibular and nodose-petrosal ganglia. In contrast to the PNS, the CNS develops with no apparent gross structural abnormalities. Nevertheless, the expression of neuropeptide Y and calcium binding proteins is altered in many neurons which may be an indication of abnormal functioning. Hence, BDNF is clearly an essential survival factor for many populations of sensory neurons, whereas, it does not appear to be critical for the development of sympathetic, dopaminergic and motoneurons. However, as these mice die at an early stage in postnatal life, they may simply not survive long enough for a wider phenotype to be observed.

### **1.5.3. Neurotrophin-3**

Neurotrophin-3 (NT-3), the third member of the neurotrophin family to be identified, was cloned in the mouse on the basis of sequence homology between NGF and BDNF (Hohn *et al.*, 1990). Subsequently, human and rat NT-3 cDNAs were also isolated (Jones and Reichardt, 1990; Rosenthal *et al.*, 1990; Maisonpierre *et al.*, 1990a, 1991). NT-3 is synthesised as a 258-amino acid precursor which is cleaved to release the 119-residue mature NT-3 polypeptide. The mature NT-3 protein is a 13.6 kDa basic protein that shares between 50 % and 60 % amino acid sequence identity with NGF and BDNF, including all six cysteine residues, which in NGF and BDNF are involved in the formation of disulphide bridges (Hohn *et al.*, 1990). Like NGF and BDNF, NT-3 is biologically active as a homodimer (Radziejewski *et al.*, 1992).

Unlike NGF and BDNF, NT-3 has a very widespread distribution in peripheral tissues. In developing peripheral tissues, the expression of NT-3 appears to coincide with the onset of neuritogenesis. In both mice and chicken, *in situ* hybridisation studies have shown that NT-3 expression peaks very early in embryonic development and declines thereafter (Maisonpierre *et al.*, 1990; Rosenthal *et al.*, 1990; Buchman and Davies, 1993; Hallböök *et al.*, 1993). Particularly high levels of NT-3 mRNA are found in the developing trigeminal target field, especially the epithelium of the whisker follicles (Hallböök *et al.*, 1993; Buchman and Davies, 1993). In the adult, widespread expression of NT-3 has been demonstrated in muscle innervated by proprioceptive sensory neurons of the dorsal root ganglia (Hohn *et al.*, 1990; Maisonpierre *et al.*, 1990; Rosenthal *et al.*, 1990; Henderson *et al.*, 1993). Other areas expressing high levels of NT-3 mRNA in the adult include: the heart, lung, skin, kidney, liver, intestine and spleen, suggesting that NT-3 may act as a target-derived trophic factor for sympathetic and sensory neurons innervating these structures. NT-3 is highly expressed in developing regions of the CNS, however, as these regions mature, NT-3 expression decreases dramatically. This is in stark contrast to BDNF expression within the CNS, which increases with maturity, and NGF expression, which is developmentally regulated within discrete CNS regions. Within the adult CNS, the cerebellum, medulla oblongata and hippocampus express the highest levels of NT-3 (Hohn *et al.*, 1990; Maisonpierre *et al.*, 1990; Rosenthal *et al.*, 1990).

Several studies have demonstrated a role for NT-3 in the development of sensory and sympathetic neurons. Subpopulations of sensory neurons from the nodose, cochlear and dorsal root ganglia and proprioceptive neurons of the trigeminal mesencephalic nucleus respond to NT-3 *in vitro* with enhanced survival (Ernfors *et al.*, 1990; Maisonpierre *et al.*, 1990a; Hohn *et al.*, 1990;

Rosenthal *et al.*, 1990; Fariñas *et al.*, 1994; Ockel *et al.*, 1996). Furthermore, NT-3 transiently supports the survival of neural crest-derived sensory neurons in the early stages of development before they switch their dependence to NGF (Buchman and Davies, 1993; Buj-Bello *et al.*, 1994; Wilkinson *et al.*, 1996; Fariñas *et al.*, 1996). NT-3 has also been shown to induce survival, differentiation and proliferation of neuronal precursors (Kalcheim *et al.*, 1992; Pinco *et al.*, 1993; DiCicco-Bloom *et al.*, 1993; Verdi and Anderson, 1994; Karavanov *et al.*, 1995; Mømberg and Hall, 1995; Elshamy and Ernfors, 1996; Elshamy *et al.*, 1996). Within the CNS, NT-3 promotes the survival of embryonic motoneurons *in vitro* (Henderson *et al.*, 1993), however, it cannot rescue neonatal motoneurons from axotomy-induced degeneration (Sendtner *et al.*, 1992; Koliatsos *et al.*, 1993). NT-3 promotes the survival of cultured embryonic noradrenergic neurons (Friedman *et al.*, 1993) and prevents 6-hydroxydopamine-induced degeneration of adult noradrenergic neurons of the locus coeruleus *in vivo* (Areñas and Persson, 1994). Additionally, NT-3 promotes the proliferation and survival of oligodendrocyte precursor cells *in vitro* and *in vivo* (Barres *et al.*, 1993, 1994).

Neurotrophin-3 deficient mice display substantial neuronal cell losses in all peripheral sensory ganglia including the trigeminal ganglion, nodose ganglion and the trigeminal mesencephalic nucleus, clearly demonstrating the physiological relevance of NT-3 in the development of peripheral sensory neurons. Deficits in sympathetic neurons are also evident in these mice (Ernfors *et al.*, 1994a; Fariñas *et al.*, 1994, 1996; Tessarolo *et al.*, 1994; Wilkinson *et al.*, 1996; Wyatt *et al.*, 1997; Elshamy *et al.*, 1996; Francis *et al.*, 1999). Significantly, the main components of the proprioceptive system, namely the muscle spindles and Golgi tendon organs, are absent in *NT-3<sup>-/-</sup>* mice, as are the large diameter proprioceptive DRG neurons (Ernfors *et al.*, 1994a). Cutaneous afferents innervating one type of peripheral sense organ, the

Pacinian corpuscle, are unaffected by the mutation. However, NT-3 appears to be required for the survival of low threshold mechanoreceptors innervating hair follicles and Merkel cells (Airaksinen *et al.*, 1996). In contrast to the severe phenotype within the PNS, there are no deficits in CNS neuronal populations in NT-3 null mutant mice, however, early postnatal lethality precludes the examination of these populations at later developmental stages.

#### 1.5.4. Neurotrophin-4/5

Neurotrophin-4/5 was the fourth member of the neurotrophin family to be isolated, based on its homology to NGF, BDNF and NT-3. It was originally cloned from *Xenopus laevis* and designated neurotrophin-4 (Hallböök *et al.*, 1991). Subsequently, human and rat clones, designated neurotrophin-5, were identified and found to share 65 % amino acid identity with *Xenopus* NT-4 (Berkemeier *et al.*, 1991; Ip *et al.*, 1992). It is currently believed that *Xenopus* NT-4 and mammalian NT-5 are the same molecule and hence are collectively referred to as NT-4/5. Mature NT-4/5 is a basic protein containing 123 amino acids and all six conserved cysteine residues found throughout the neurotrophin family. Mature NT-4/5 shares over 50 % amino acid homology with NGF, BDNF and NT-3 (Ip *et al.*, 1992).

NT-4/5 is widely distributed in both embryonic and adult tissues, including: the testis, prostate, ovary, kidney, muscle, heart, lungs, thymus and placenta (Berkemeier *et al.*, 1991; Ip *et al.*, 1992; Timmusk *et al.*, 1993). Developmentally regulated expression of NT-4/5 mRNA in the trigeminal target territory suggests a role for NT-4/5 in the development of trigeminal neurons (Ibáñez *et al.*, 1993). Similarly, NT-4/5 mRNA is present in the developing limb bud, suggesting a role for this factor in the development of spinal motoneurons (Henderson *et al.*, 1993). NT-4/5 mRNA is also detected

within many regions of the developing and adult brain including the cerebral cortex, cerebellum, pons, medulla, hypothalamus, olfactory bulb and hippocampus (Timmusk *et al.*, 1993). Despite its widespread expression pattern, the total amount of NT-4/5 mRNA expression in the adult rat brain is over 10-fold less than that of NGF, BDNF and NT-3 mRNAs (Ip *et al.*, 1992).

Several populations of neurons from both the peripheral and central nervous systems respond to NT-4/5, both during development and in adulthood. Within the PNS, NT-4/5 transiently supports the *in vitro* survival of cultured murine trigeminal and jugular ganglion neurons at a stage corresponding to the beginning of target field innervation (Davies *et al.*, 1993a). NT-4/5 also supports the *in vitro* survival of nodose ganglion neurons, during the phase of naturally occurring cell death (Davies *et al.*, 1993a). Significantly, all of these neurons are also BDNF-responsive during the same developmental periods. NT-4/5 also supports the survival of sympathetic neurons, albeit at high concentrations (Hallböök *et al.*, 1991; Berkmeier *et al.*, 1991; Huber, personal communication). It is interesting to note that mammalian NT-4/5 has only very limited survival promoting activity on embryonic chicken neurons, suggesting that NT-4/5 may not be well conserved between mammals and birds (Davies *et al.*, 1993a; Ibáñez *et al.*, 1993). In the CNS, NT-4/5 supports embryonic spinal motoneurons *in vitro* (Henderson *et al.*, 1993) and rescues neonatal facial motoneurons from injury-induced cell death *in vivo* (Koliatsos *et al.*, 1994). Additionally, NT-4/5 maintains the phenotype of adult axotomised spinal motoneurons (Friedman *et al.*, 1995), as well as inducing sprouting of intact adult motor nerves (Funakoshi *et al.*, 1995). NT-4/5 also promotes the survival of embryonic cholinergic basal forebrain and noradrenergic locus coeruleus neurons *in vitro* (Friedman *et al.*, 1993).

Mice with a targeted disruption in the *NT-4/5* gene display severe neuronal losses of sensory neurons of the nodose-petrosal and geniculate ganglia. Conversely, sympathetic neurons of the superior cervical ganglion, facial motoneurons and midbrain dopaminergic neurons are unaffected, suggesting that additional factors sustain these neurons during development (Conover *et al.*, 1995; Liu *et al.*, 1995; Erickson *et al.*, 1996).

#### 1.5.5. Neurotrophin-6

Neurotrophin-6 (NT-6) was cloned from the teleost fish, *Xiphophorus maculatus*, and alignment of its sequence with that of *Xiphophorus* NGF or BDNF revealed that it did not represent a teleostean homologue of a known neurotrophin, but a new family member (Götz *et al.*, 1994). In accordance with the features of the neurotrophin family, NT-6 is synthesised as a 286-amino acid precursor which is cleaved to yield a 143-residue mature basic protein, containing all six cysteine residues conserved throughout the family. However, in contrast, to NGF, BDNF, NT-3 and NT-4/5, NT-6 contains an insertion sequence of 22 amino acids between the second and third cysteine residues of the mature molecule. Also contrary to the other members, NT-6 is not detected in the medium of producing cells indicating that it is likely to be bound to proteoglycans of the cell surface and/or extracellular matrix (Götz *et al.*, 1994).

NT-6 mRNA is expressed in the developing and mature brain, with the highest levels in the developing cerebellum. NT-6 mRNA is also expressed in the gill, liver, eye, skin, spleen, heart and skeletal muscle of adult fish (Götz *et al.*, 1994). Like NGF, NT-6 supports the survival of embryonic chicken dorsal root ganglion and sympathetic neurons, however, NT-6 is less potent than NGF in this respect. Additionally, the finding that NT-6 cannot

support the survival of cultured nodose or ciliary neurons suggests that the spectrum of activity of NT-6 is similar to that of NGF (Götz *et al.*, 1994).

#### 1.5.6. Neurotrophin-7

Neurotrophin-7 (NT-7), cloned from a genomic library of the teleost common carp *Cyprinus carpio*, is the most recent neurotrophin to be isolated (Lai *et al.*, 1998). Subsequently, zebrafish NT-7 was cloned (Nilsson *et al.*, 1998). NT-7 shares 66 % amino acid homology with *Xiphophorus* NGF and NT-6, and possesses the six conserved cysteine residues characteristic of the neurotrophin family. Like NT-6, but unlike the other neurotrophins, NT-7 has a 15-residue insertion between the second and third cysteines of the mature molecule.

NT-7 mRNA is expressed at high levels in adult skin, heart and intestine and at low levels in the brain and testis (Lai *et al.*, 1998). NT-7 is reported to promote the survival of and enhance neurite outgrowth from chicken dorsal root ganglia neurons (Lai *et al.*, 1998).

#### 1.5.7. The p75 receptor

Cloning of *p75* in human, rat and chicken (Chao *et al.*, 1986; Radeke *et al.*, 1987; Large *et al.*, 1989) and subsequent sequence analysis has revealed that *p75* encodes a 75 kDa transmembrane glycoprotein that contains a long extracellular domain with four cysteine repeats, responsible for ligand recognition (Welcher *et al.*, 1991; Yan and Chao, 1991). *p75* shares several features with the Fas antigen and tumour necrosis factor receptors, type I and II (TNFR-I and TNFR-II). These features include the extracellular cysteine repeats and a short intracellular domain that is homologous to a small segment of the death domain of Fas and TNFR-I (Nagata and Golstein, 1995; Chapman, 1995). *p75* was first described as a low affinity receptor for

NGF, however, it has since been shown to bind BDNF, NT-3 and NT-4/5 with equal affinity (Rodrigues-Tebar *et al.*, 1990, 1992). More recently, p75 has been shown to bind NT-3 with high affinity under some circumstances (Dechant *et al.*, 1997). A soluble truncated form of p75, comprising only the extracellular domain, has been isolated from the conditioned medium of p75-producing cells (DiStefano and Johnson, 1988). Although truncated p75 can bind NGF, BDNF and NT-3 with equal affinity to the full length receptor, its biological relevance is unclear.

The p75 receptor is widely expressed in both the developing and adult central nervous system (Ernfors *et al.*, 1988; Yan and Johnson, 1989). p75 mRNA is also present in developing sensory and sympathetic neurons, being substantially upregulated as the neurons establish target contact (Heuer *et al.*, 1990; Hallböök *et al.*, 1990; Wyatt *et al.*, 1990). Although several studies of p75 expression in developing and adult peripheral and central neurons have indicated that NGF upregulates p75 mRNA expression (Doherty *et al.*, 1988; Lindsay *et al.*, 1990; Verge *et al.*, 1992; Miller *et al.*, 1991, 1994; Wyatt and Davies, 1993), levels of p75 mRNA in the trigeminal ganglion of NGF null mutant mice are unaffected compared to wild-type littermates (Davies *et al.*, 1995). Several populations of non-neuronal cells, including glial cells, Schwann cells and mesenchymal cells also express p75 (DiStefano and Johnson, 1988a; Heuer *et al.*, 1990).

Despite the fact that p75 was the first neurotrophin receptor to be isolated, its functional role and/or signalling capacity are still not fully elucidated. p75 is thought to play a role in modifying the function of the Trk receptors, acting to increase the affinity of the Trk receptors for neurotrophin binding. This has been demonstrated for TrkA, where p75 increases the affinity of NGF binding to TrkA and enhances TrkA phosphorylation (Hempstead *et al.*,

1991; Verdi *et al.*, 1994; Barker and Shooter, 1994; Ip *et al.*, 1993). This is proposed to occur either by p75 concentrating NGF within the TrkA local environment or by p75 forming a molecular complex with TrkA that modifies ligand binding. Data from *p75*<sup>-/-</sup> mice suggest that p75 can enhance the sensitivity of NGF-responsive neurons to NGF. Mice homozygous for a null mutation in the *p75* gene display deficits in populations of NGF-dependent sensory and sympathetic neurons with no major effects on neurons of the CNS (Lee *et al.*, 1992). Embryonic and postnatal sensory and sympathetic neurons derived from *p75*<sup>-/-</sup> mice exhibit a decreased sensitivity to NGF, but not to other neurotrophins, *in vitro* compared to wild-type counterparts (Davies *et al.*, 1993; Lee *et al.*, 1994).

p75 also appears to play a role in ligand discrimination by the Trk receptors. For example, the ability of NT-3 to activate TrkA in PC12 cells is enhanced when the binding of NT-3 to p75 is blocked by antibodies or when the expression of p75 is very low (Clary and Reichardt, 1994; Benedetti *et al.*, 1993). Similarly, sympathetic neurons from *p75*-deficient mice are more sensitive to NT-3 than wild-type sympathetic neurons (Lee *et al.*, 1994; Brennan *et al.*, 1999). These findings suggest that p75 reduces the ability of NT-3 to signal via TrkA.

The initial characterisation of p75 indicated that alone, it was insufficient to mediate neurotrophin high affinity binding and NGF responsiveness (Chao *et al.*, 1986; Radeke *et al.*, 1987; Hempstead *et al.*, 1989). Nevertheless, later studies report that p75 may transduce signals in the absence of the Trks by increasing intracellular ceramide levels, and activating NFκ-B and JNK kinases (Dobrowsky *et al.*, 1994, 1995; Carter *et al.*, 1996; Casaccia-Bonnel *et al.*, 1996; Hamanoue *et al.*, 1999). It has recently been shown that ceramide accumulation and JNK activation are correlated with apoptotic stimuli in

some cells (Brugg *et al.*, 1996; Weisner and Dawson, 1996; Hartfield *et al.*, 1997, 1998). It is now clear that p75 can promote apoptosis both in the presence and absence of ligand binding. For example, increased expression of p75 in PC12 cells, grown in the absence of NGF, accelerates apoptosis (Rabizadeh *et al.*, 1993), whereas reduced expression of p75 in postnatal rat dorsal root ganglion neurons grown without NGF promotes their survival (Barrett and Bartlett, 1994). Additionally, several reports have shown that neurotrophins can induce apoptosis by binding to p75 (Casaccia-Bonofil *et al.*, 1996; Frade *et al.*, 1996; Van der Zee *et al.*, 1996; Yeo *et al.*, 1997; Bamji *et al.*, 1998). A recent study has shown that activation of p75 by NGF in neurons not expressing TrkA leads to cell death. This cytotoxic effect of NGF can be prevented by activation of TrkB signalling pathways, suggesting that competitive signalling between the Trks and p75 can determine cell survival (Davey and Davies, 1998). The apoptotic role of p75 *in vivo* has been confirmed in mice with a null mutation in the *BDNF* gene. In the absence of BDNF, sympathetic neurons, which normally express BDNF but not TrkB, do not die during the normal period of developmental neuronal death, suggesting that BDNF normally acts through p75 to cause apoptosis in these cells (Bamji *et al.*, 1998). Also, transgenic mice overexpressing p75 display substantially reduced numbers of sympathetic, sensory and neocortical neurons (Majdan *et al.*, 1997).

#### **1.5.8. Trk receptor tyrosine kinases**

*Trk* (tropomyosin kinase) was first identified as a human oncogene resulting from a DNA rearrangement between truncated tropomyosin and protein tyrosine kinase sequences (Martin-Zanca *et al.*, 1986, 1989). The *Trk* proto-oncogene encodes a 140 kDa transmembrane glycoprotein that contains a cytoplasmic tyrosine kinase domain and is designated gp140<sup>trkA</sup> or TrkA (Martin-Zanca *et al.*, 1989). Low stringency hybridisation of cDNA libraries

using a *TrkA* probe led to the isolation of cDNAs of two related tyrosine kinases, *TrkB* and *TrkC*. *TrkB* encodes a 145 kDa transmembrane protein tyrosine kinase, designated gp145<sup>trkB</sup>, that shares around 70 % homology with *TrkA* (Klein *et al.*, 1989). *TrkC* also encodes a 145 kDa transmembrane glycoprotein with tyrosine kinase activity, termed gp145<sup>trkC</sup>, that shares about 65 % homology with *TrkA* and *TrkB* (Lamballe *et al.*, 1991). The intracellular tyrosine kinase domains of these receptors share more than 85 % sequence homology. Although clearly members of the receptor tyrosine kinase (RTK) family, certain amino acid substitutions in highly conserved kinase motifs and the presence of a short carboxy-terminal tail distinguishes Trks from other subfamilies of RTKs (Barbacid, 1994). Additionally, the extracellular domain of the Trk receptors exhibits two distinct subsets of cell adhesion-related motifs (Schneider and Schweiger, 1991): two immunoglobulin like domains and an array of three tandem leucine-rich motifs.

Extensive studies have demonstrated that these receptors, acting in conjunction with the common neurotrophin receptor p75, are responsible for mediating most of the biological activities of the neurotrophins. For example, *TrkA* is highly expressed in NGF-responsive neurons in the developing mouse (Martin-Zanca *et al.*, 1990). NGF stimulates tyrosine phosphorylation of *TrkA* in PC12 cells, transfected fibroblasts and sensory neurons (Kaplan *et al.*, 1991, 1991a). Furthermore, ectopic expression of *TrkA* in neurons that do not normally respond to NGF confers an NGF-mediated survival response (Allsopp *et al.*, 1993). In addition to mediating signal transduction, Trk receptors impart ligand/receptor specificity to neurotrophin signalling. NGF, BDNF and NT-3 preferentially bind to *TrkA*, *TrkB* and *TrkC*, respectively and NT-4/5 signals through *TrkB* (Kaplan *et al.*, 1991, 1991a; Klein *et al.*, 1991, 1991a; Glass *et al.*, 1991; Soppet *et al.*, 1991;

Squinto *et al.*, 1991; Lamballe *et al.*, 1991; Berkemeier *et al.*, 1991; Ip *et al.*, 1992; Klein *et al.*, 1992). The receptor specificities of NT-6 are thus far undefined. NT-7 had been reported to bind to TrkA but with a considerably lower affinity than that of the NGF-TrkA interaction (Lai *et al.*, 1998).

It appears that some neurotrophins can interact with more than one neurotrophin receptor. Thus, NT-3 can also bind to TrkA and TrkB (Klein *et al.*, 1991a; Soppet *et al.*, 1991; Squinto *et al.*, 1991), and NT-4/5 can activate TrkA (Berkemeier *et al.*, 1991; Ip *et al.*, 1992), however, the activation of TrkA by NT-3 and NT-4/5 and TrkB by NT-3 is less efficient than activation by their respective preferred ligands (depicted in Figure 1.1.). For example, NT-4/5 can promote the *in vitro* survival of embryonic trigeminal and sympathetic neurons derived from *TrkB* null mutant mice. This response, mediated by TrkA, is only possible if NT-4/5 is present at very high concentrations (Huber and Davies, unpublished data). Similarly, high concentrations of NT-3 can promote the survival of sympathetic and sensory neurons lacking TrkC (Davies *et al.*, 1995a). Although high levels of NT-3 are required to activate TrkA and TrkB and induce neuronal survival *in vitro*, comparison of the phenotypes of *NT-3* and *TrkC* null mutant mice suggests that these interactions are functionally relevant *in vivo* (Wilkinson *et al.*, 1996; Piñón *et al.*, 1996; Fagan *et al.*, 1996; Wyatt *et al.*, 1997).

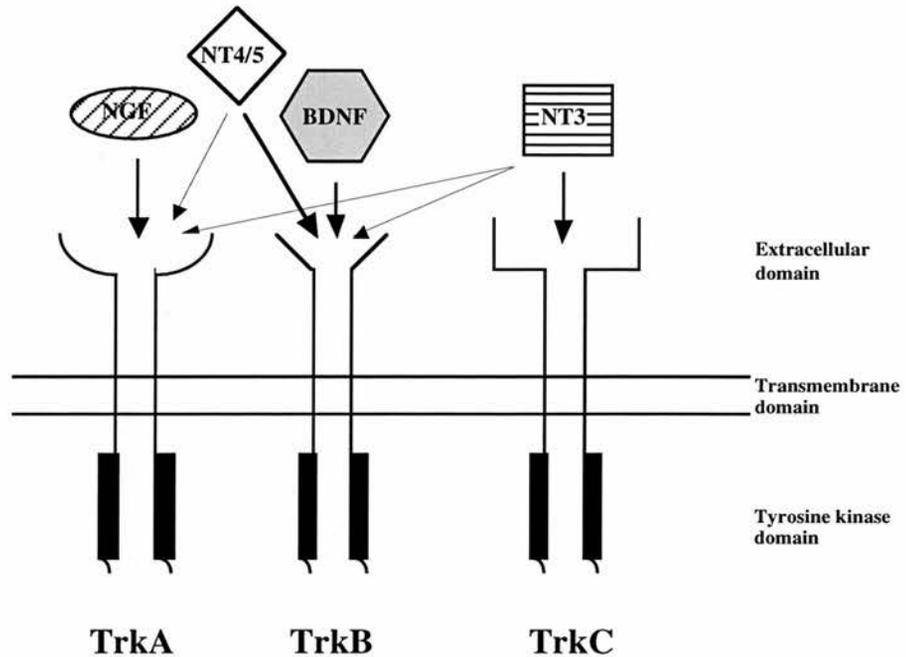


Figure 1.1. Schematic diagram summarising the interactions of neurotrophins with members of the Trk family of receptor tyrosine kinases.

The thick arrows demonstrate preferred ligand/receptor interactions while the thin arrows denote other interactions known to occur. Adapted from Davies, 1994a.

Widespread expression of Trk mRNA is detected in the central and peripheral nervous systems. Within the CNS, TrkA expression is limited to cholinergic neurons of the basal forebrain and striatum (Vasquez and Ebendal, 1991; Holtzman *et al.*, 1992; Merlio *et al.*, 1992; Steininger *et al.*, 1993). Conversely, virtually all central neurons express TrkB or TrkC mRNAs or both (Klein *et al.*, 1989, 1990, 1990a; Merlio *et al.*, 1992; Tessarollo *et al.*, 1993; Lamballe *et al.*, 1994). Similarly, TrkB and TrkC are expressed to varying degrees in the neurons of all ganglia of the PNS (Klein *et al.*, 1989; Ninkina *et al.*, 1996; Carroll *et al.*, 1992; Tessarollo *et al.*, 1993; Lamballe *et al.*, 1994), with the exception of parasympathetic neurons of the ciliary ganglion. TrkA is restricted to a subset of neurons of neural crest origin (Martin-Zanca *et al.*,

1990; Tessarollo *et al.*, 1993; Schröpel *et al.*, 1995). Trk receptors have also been reported in various non-neuronal tissues. TrkA mRNA is found in cells of the immune system (Ehrhard *et al.*, 1993, 1993a), TrkB mRNA is detected in lung, muscle and ovary and TrkC mRNA is found in facial structures and various tissues within the body cavity (Tessarollo *et al.*, 1993; Lamballe *et al.*, 1994).

Interestingly, several isoforms of the Trk receptor tyrosine kinases have been isolated. *TrkA* encodes two protein isoforms, with similar biological properties, that differ in the presence of six amino acid residues located in their extracellular domain near the transmembrane region (Barker *et al.*, 1993; Horigome *et al.*, 1993). The *TrkB* gene generates several different transcripts that in addition to the full length gp145<sup>trkB</sup>, encode two truncated receptors, each lacking the entire intracellular catalytic domain (Klein *et al.*, 1990, 1990a; Middlemas *et al.*, 1991), and several other isoforms that contain deletions in the leucine-rich motifs of the extracellular domain (Ninkina *et al.*, 1997). *TrkC* encodes several distinct protein isoforms including the full length gp145<sup>trkC</sup> receptor, truncated receptors lacking the intracellular tyrosine kinase domain and three isoforms with different insertions in the tyrosine kinase domain. Although these insert-containing isoforms of TrkC retain the ability to autophosphorylate in response to NT-3, they cannot mediate a biological response to this factor (Valenzuela *et al.*, 1993; Tsoulfas *et al.*, 1993).

The physiological relevance of the splice variants of the Trk receptors has not been fully determined. While full length Trk receptors are widely expressed within neuronal elements of the nervous system, in general, truncated Trk isoforms are predominately expressed within non-neuronal tissues. Interestingly, the expression of truncated TrkB isoforms, relative to full length TrkB, increases as embryonic development proceeds (Allendoerfer *et*

*al.*, 1994). Several functions for truncated TrkB have been proposed including a role in potentiating BDNF action by presenting BDNF to full length TrkB receptors (Klein *et al.*, 1990; Middlemas *et al.*, 1991; Beck *et al.*, 1993). Conversely, truncated TrkB has been implicated as an inhibitor of full length TrkB signalling due to the formation of functionally inactive receptor heterodimers (Eide *et al.*, 1996; Ninkina *et al.*, 1996). This is supported by results demonstrating that the loss of responsiveness of early trigeminal neurons to BDNF is correlated with an increase in expression of truncated TrkB receptors (Ninkina *et al.*, 1996).

Detailed studies of Trk-mediated signalling have elucidated the intracellular signalling pathways involved in various cellular responses. Ligand binding facilitates receptor dimerisation and resultant autophosphorylation of tyrosine residues within the intracellular catalytic domain (reviewed in Kaplan and Stephens, 1994). This activation of catalytic activity enables the binding of particular SH2 domain-containing adaptor proteins to key tyrosine residues, each of which activate distinct downstream signalling cascades culminating in altered gene expression within the nucleus (reviewed in Barbacid, 1994; Greene and Kaplan, 1995; Kaplan and Miller, 1997; Gunn-Moore and Tavaré, 1998). Binding of the adaptor protein, Shc, and subsequent activation of PI-3 kinase and Ras/MAP kinase pathways are implicated in effecting neuronal differentiation and survival responses (Minichiello *et al.*, 1998). Binding of PLC $\gamma$ 1 increases the levels of protein kinase-C resulting in elevated intracellular calcium (Guiton *et al.*, 1995). Increased calcium levels have been associated with cell survival as well as plasticity and response to injury (reviewed in Jiang and Guroff, 1997). Two additional adaptor proteins of importance in Trk-mediated neurotrophin signalling are SH2-containing tyrosine phosphatase-1 (SHP-1) (Vambutas *et al.*, 1995) and the Suc1-associated neurotrophic factor target (SNT) (Rabin *et*

*al.*, 1993). Although activation of SNT has been implicated in effecting cell proliferation, the downstream signalling pathways of SNT and SHP-1 are unknown.

The physiological relevance of the neurotrophin receptors has been elucidated by analysing the phenotype of mice with targeted null mutations in the *TrkA*, *TrkB* or *TrkC* genes. Mice lacking functional TrkA are insensitive to pain and display substantial neuronal losses in the trigeminal, dorsal root and sympathetic ganglia. Interestingly, cholinergic neurons of the basal forebrain, known to express TrkA, are unaffected by this mutation (Smeyne *et al.*, 1994). In mice homozygous for a targeted disruption of the *TrkB* gene, there is a marked reduction in nodose-petrosal, trigeminal and dorsal root ganglion neurons (Klein *et al.*, 1993). Finally, *TrkC* null mutant mice display severe proprioceptive defects, due to the elimination of Ia muscle afferent projections to spinal motoneurons, which is reflected by the loss of a subpopulation of DRG neurons (Fariñas *et al.*, 1994; Klein *et al.*, 1994; Minichiello *et al.*, 1995). Collectively, these findings confirm the role of the Trk receptors in mediating neurotrophin function. The similarities in the phenotype of mice carrying a null mutation in *TrkA* with that of the *NGF* null mutant (Crowley *et al.*, 1994), and also *TrkB*<sup>-/-</sup> mice compared with *BDNF*<sup>-/-</sup> mice (Jones *et al.*, 1994; Ernfors *et al.*, 1994) substantiates the view that these are the preferred ligand/receptor interactions *in vivo*. In general, the phenotype of *TrkC*<sup>-/-</sup> mice is less severe than that in *NT-3*<sup>-/-</sup> mice (Ernfors *et al.*, 1994a; Klein *et al.*, 1994; Wilkinson *et al.*, 1996; Piñón *et al.*, 1996; Fagan *et al.*, 1996; Wyatt *et al.*, 1997), demonstrating the more promiscuous nature of NT-3 and its ability to signal via TrkA or TrkB *in vivo*.

## 1.6. The family of neurotrophic cytokines and their receptors

Neurotrophic cytokines constitute a family of distantly related proteins with extensive pleiotropy in several biological systems. Within the developing and mature nervous system, cytokines influence many processes including proliferation, survival, differentiation, regeneration and maintenance of neurotransmitter phenotype (reviewed in Heller *et al.*, 1996; Horton *et al.*, 1996; Jonakait, 1997; Murphy *et al.*, 1997). The cytokine family includes ciliary neurotrophic factor (CNTF), growth promoting activity (GPA), leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), interleukin-6 (IL-6) and interleukin-11 (IL-11). Although the cytokines share very little sequence identity (<30 %), they all adopt a very similar tertiary structure and analysis of their gene structure indicates that they may have evolved from a common ancestral gene (Bazan, 1991, Bruce *et al.*, 1992). Members of the cytokine family bind to related multicomponent receptors that contain a common signal transducing subunit, gp130 (Sato and Miyajima, 1994).

### 1.6.1. Ciliary neurotrophic factor

CNTF is the most comprehensively characterised neurotrophic cytokine. It was first identified as a neurotrophic factor, present in eye tissues, based on its ability to promote the survival of parasympathetic neurons of the chicken ciliary ganglion *in vitro* (Adler *et al.*, 1979). Rabbit, rat, human and mouse cDNAs have since been cloned and sequence analysis has shown that CNTF is a 200-residue, highly acidic protein with a molecular mass of 20-24 kDa (Lin *et al.*, 1989; Stöckli *et al.*, 1989; Masikowski *et al.*, 1991; Kaupmann *et al.*, 1991; Lam *et al.*, 1991). In contrast to other neurotrophic factors, CNTF appears to be a cytosolic protein as it lacks a signal peptide sequence for secretion and a consensus sequence for glycosylation. Accordingly, CNTF is

not released into the medium of transfected cell lines (Lin *et al.*, 1989; Stöckli *et al.*, 1989).

High levels of CNTF mRNA are found only in the adult mammalian nervous system, with particularly large quantities in the sciatic and optic nerves, olfactory bulb and spinal cord (Stöckli *et al.*, 1991; Dobrea *et al.*, 1992). In addition to ciliary neurons, a wide variety of peripheral and central neurons respond to CNTF *in vitro*. These include neurons of the chicken nodose, trigeminal, dorsal root and sympathetic lumbar ganglia, spinal motoneurons, hippocampal neurons and Purkinje cells (Barbin *et al.*, 1984; Manthorpe and Varon, 1985; Arakawa *et al.*, 1990; Ip *et al.*, 1991; Lärkfors *et al.*, 1994). Furthermore, CNTF protects embryonic spinal motoneurons *in ovo* from naturally occurring cell death and protects facial and spinal motoneurons and dopaminergic neurons *in vivo* from axotomy-induced cell death (Oppenheim *et al.*, 1991; Sendtner *et al.*, 1990; Hagg and Varon, 1993; Li *et al.*, 1994; Vejsada *et al.*, 1995). In addition to survival-promoting effects, CNTF inhibits the proliferation and induces cholinergic differentiation of developing sympathetic neurons (Ernsberger *et al.*, 1989; Saadat *et al.*, 1989). CNTF also displays actions on non-neuronal cells of the nervous system, promoting the *in vitro* differentiation of astrocyte progenitor cells and microglia (Hughes *et al.*, 1988; Sendtner *et al.*, 1994) and the maturation and survival of oligodendrocytes (Louis *et al.*, 1993).

Despite the multiple roles of CNTF *in vitro*, its role *in vivo* remains unclear. In accordance with the fact that CNTF mRNA is only expressed postnatally, CNTF null mutant mice display no overt abnormal phenotype at birth (Masu *et al.*, 1993). Nevertheless, CNTF does appear to be essential for the maintenance of a subset of adult motoneurons, as in CNTF<sup>-/-</sup> mice, there is a progressive degeneration of motoneurons during the postnatal period.

### 1.6.2. Other neuropoietic cytokines

GPA, LIF, OSM, CT-1, and IL-6 are structurally and functionally related to CNTF. Although it is beyond the scope of this thesis to discuss these factors in detail, their activities within the nervous system will be briefly reviewed.

Since chicken GPA shares 50 % sequence homology with mammalian CNTF, it was initially suggested to be the avian homologue of CNTF (Leung *et al.*, 1992). However, unlike CNTF, GPA is released in a soluble form from the cells that synthesise it (Leung *et al.*, 1992) and it is expressed in the chicken embryo, most notably in the target tissue of ciliary neurons (Leung *et al.*, 1992; Finn and Nishi, 1996). This suggests that, unlike CNTF, GPA may act as a target-derived neurotrophic factor for chicken ciliary neurons. Additionally, GPA promotes the survival of chicken sensory and autonomic neurons in culture (Eckenstein *et al.*, 1990; Finn *et al.*, 1998) and induces the cholinergic differentiation of sympathetic neuroblasts (Heller *et al.*, 1993).

LIF enhances the survival of several populations of embryonic and postnatal sensory neurons in culture (Horton *et al.*, 1996), as well as regulating transmitter phenotype and neuropeptide expression (Nawa *et al.*, 1991; Fan and Katz, 1993). LIF also exerts effects on non-neuronal cells, promoting the generation, maturation and survival of oligodendrocytes *in vitro* (Mayer *et al.*, 1994; Vos *et al.*, 1996). Similarly, OSM supports the survival of peripheral sensory neurons *in vitro* (Horton *et al.*, 1996) and promotes oligodendrocyte cell survival and differentiation (Vos *et al.*, 1996). CT-1 has been shown to act as a survival factor for cultured sensory and parasympathetic neurons, hindbrain dopaminergic neurons and motoneurons (Pennica *et al.*, 1995, 1995a; Horton *et al.*, 1998; Arce *et al.*, 1998). CT-1 can also protect motoneurons *in vivo* from axotomy-induced degeneration (Pennica *et al.*, 1996). Within the CNS, IL-6 promotes the survival of cultured embryonic

and postnatal midbrain catecholaminergic neurons and basal forebrain cholinergic neurons, and also stimulates astrocyte proliferation (Kushima *et al.*, 1992; Hama *et al.*, 1989; Fattori *et al.*, 1995). Additionally, IL-6 is a survival factor for peripheral sensory neurons in culture (Horton *et al.*, 1996).

### 1.6.3. Cytokine signalling

The biological functions of the neuropoietic cytokines are mediated by multicomponent cell surface receptors that share a common subunit, the glycoprotein, gp130. CNTF and IL-6 first bind to  $\alpha$ -receptor subunits, termed CNTFR $\alpha$  and IL-6R $\alpha$ , respectively. Each complex then interacts with gp130, which in the case of CNTF signalling then recruits LIFR- $\beta$ . In the case of IL-6 signalling, the IL-6 $\alpha$ -gp130 complex recruits an additional gp130 subunit. In contrast, direct ligand binding of CT-1, LIF or OSM is sufficient to induce heterodimerisation of gp130 and LIFR $\beta$ , in the absence of an  $\alpha$ -component. Ligand-induced homodimerisation, of two gp130 receptors, or heterodimerisation, of gp130 and LIFR $\beta$ , activates a rapid phosphorylation of tyrosine residues on these  $\beta$ -components. As these cytokine receptors lack intrinsic enzymatic activity, tyrosine phosphorylation is mediated by JAK kinases, which are associated with gp130 and LIFR $\beta$  prior to receptor dimerisation. Several SH2 domain-containing proteins have been identified as substrates for JAK phosphorylated receptor components. These include PLC $\gamma$ , PI3 kinase, Shc, Grb2, Raf-1 and the MAP kinases, ERK1 and ERK2. The STAT family of SH2-bearing proteins are also activated, upon which they translocate to the nucleus and influence gene expression (reviewed in Frank and Greenberg, 1996; Inoue *et al.*, 1996). Recent data has also implicated NF $\kappa$ B as an important component of the cytokine signalling cascade (Middleton *et al.*, 1999).

## 1.7. The superfamily of TGF- $\beta$ related cytokines

The TGF- $\beta$  family constitutes a large group of cytokines that exert many and varied roles throughout development. The principal subfamilies within this superfamily are the TGF- $\beta$ s, activins, *decapentaplegic-Vg1*-related (DVR) proteins and the GDNF family. Of these subfamilies, the TGF- $\beta$ s and the GDNF family are most well characterised with respect to their roles in the developing nervous system. It is beyond the scope of this thesis to discuss, other than briefly, the role of the TGF- $\beta$ s. As the GDNF family constitute the main focus of this work, the individual family members and the signalling mechanisms employed will be discussed in detail.

### 1.7.1. TGF- $\beta$ s and their receptors

Within the nervous system, members of the TGF- $\beta$  subfamily promote the survival of motoneurons, sensory neurons and midbrain dopaminergic neurons (Martinou *et al.*, 1990; Chalazonitis *et al.*, 1992; Krieglstein and Unsicker, 1994; Poulsen *et al.*, 1994). Newly emerging evidence suggests that TGF- $\beta$ s may require cofactors, such as GDNF, neurotrophins or CNTF, to exert their full neurotrophic potential (Krieglstein *et al.*, 1996, 1998, 1998a).

With the exception of the GDNF family, all members of the TGF- $\beta$  superfamily signal via transmembrane serine/threonine kinase receptors, types I, II and III. Binding of the ligand dimer to the type I and II receptors, either sequentially or simultaneously, results in the formation of a heterotetrameric complex. Type III proteoglycan receptors also exist and may be involved in ligand presentation to type I and II receptors. Constitutive activation of the type II receptor phosphorylates serine and threonine residues of the type I receptor leading to activation of Smad

proteins and signal transduction to the nucleus (reviewed in Massagué, 1998).

## 1.8. The GDNF family and their receptors

The GDNF family comprises four structurally and functionally related proteins: glial cell-line derived neurotrophic factor, neurturin, persephin and artemin. GDNF ligands signal via a receptor complex comprised of a ligand binding component, termed the GDNF family receptor alpha (GFR $\alpha$ ) (GDNF Receptor Nomenclature Committee, 1997) and a shared signal transducing component, the transmembrane receptor tyrosine kinase, Ret. The GDNF ligands and their receptors are discussed in turn below.

### 1.8.1. Glial cell line-derived neurotrophic factor

Glial cell line-derived neurotrophic factor (GDNF) was initially purified from the conditioned medium of the rat B49 glial cell line, based on its ability to promote the survival of embryonic dopaminergic neurons from the rat mesencephalon *in vitro* (Lin *et al.*, 1993). On the basis of data obtained from partial amino acid sequencing of purified B49-derived GDNF, the rat and human *GDNF* genes were cloned (Lin *et al.*, 1993), demonstrating that the *GDNF* gene is highly conserved between species. More recently, murine *GDNF* has also been cloned (Hellmich *et al.*, 1996). GDNF is a typical secretory protein first synthesized as an inactive prepro-protein of 211 amino acids. The secreted pro-GDNF undergoes proteolytic cleavage yielding a mature protein of 134 amino acids. Whilst mature GDNF has less than 20 % amino acid sequence homology with any other TGF- $\beta$  member, it contains all seven conserved cysteine residues in the same relative spacing as found throughout the entire TGF- $\beta$  superfamily, making it a distant member of this family. GDNF is, however, also a member of the cysteine knot growth factor

superfamily (Eigenbrot and Gerber *et al.*, 1997). This structural property relates GDNF to the neurotrophins. The biologically active form of this basic, heparin-binding protein appears to be a disulphide-bonded homodimer with an apparent molecular mass of 32-42 kDa on non-reducing SDS gels. GDNF is a heterogeneously N-glycosylated protein, with the molecular mass of the monomer decreasing from 18-22 kDa to 15 kDa after N-glycanase treatment (Lin *et al.*, 1993, 1994). Glycosylation of mature growth factors is an unusual feature not seen in the neurotrophins, however, the functional significance of these carbohydrates in GDNF is unclear. Human GDNF has been mapped to chromosome 5p13.1 (Schindelhauer *et al.*, 1995 and Bermingham *et al.*, 1995).

GDNF mRNA has a widespread tissue distribution, both during development and in adulthood. Within the CNS, expression of GDNF is found predominantly, but not exclusively, within neuronal cells. GDNF mRNA is detected within many regions of the developing brain including: the olfactory tubercle, olfactory bulb, basal ganglia, thalamus, locus coeruleus, cerebellar Purkinje cells and trigeminal brainstem nuclei. GDNF transcripts are also found in Clarke's column and the dorsal horns of the spinal cord. Similarly, GDNF is widely expressed in the adult CNS with the highest levels in the neurons of the striatum, substantia nigra, thalamus, hippocampus, cortex, cerebellum, and spinal cord (Schaar *et al.*, 1993; Springer *et al.*, 1994; Choi-Lundberg and Bohn, 1995; Nosrat *et al.*, 1996; Pochon *et al.*, 1997; Trupp *et al.*, 1997; Golden *et al.*, 1998). GDNF mRNA is also expressed within cortical, nigral and basal forebrain astrocytes. The distribution of GDNF in the CNS demonstrates the availability of this ligand as a target-derived and/or locally-acting neurotrophic factor for many classes of CNS neurons. Within the PNS, GDNF is expressed in dorsal root and superior cervical sympathetic ganglia and in Schwann cells. In general, GDNF mRNA expression is significantly higher in peripheral organs than in

neuronal tissues. These include the developing limb bud, metanephric kidney, gut, testes, skin, skeletal muscle, vibrissae and teeth (Henderson *et al.*, 1994; Choi-Lundberg and Bohn, 1995; Trupp *et al.*, 1995; Suvanto *et al.*, 1996; Wright and Snider, 1996). In the adult periphery, GDNF mRNA expression is more restricted with the highest levels in the lung, liver and ovary (Suter-Crazzolaro and Unsicker, 1994; Nosrat *et al.*, 1996; Golden *et al.*, 1999). The peripheral expression pattern of GDNF suggests a role as a target-derived neurotrophic factor for neurons of the PNS, however, the possibility that GDNF also serves as a growth factor for non-neuronal cells cannot be discounted.

The expression of GDNF in sites of embryonic induction suggests a role for this factor in epithelial-mesenchymal inductive interactions, especially in the developing kidney (Suvanto *et al.*, 1996, Hellmich *et al.*, 1996). Experiments with embryonic kidney explants in organ culture have demonstrated that GDNF promotes supernumerary ureteric budding from the Wolffian duct (an essential early stage in metanephric kidney development) and increases cell proliferation in the ureter tips (Vega *et al.*, 1996; Pepicelli *et al.*, 1997; Sainio *et al.*, 1997). Once ureteric bud induction is initiated by a GDNF signal, a well controlled branching process occurs, regulated by reciprocal interactions between the nephrogenic mesenchyme (a source of GDNF) and the tips of the ureteric tubules (expressing GDNF receptors) (reviewed in Sariola and Sainio, 1997). GDNF has also been shown to regulate bud orientation, ureteric cell adhesion and synthesis of extracellular matrix (Sainio *et al.*, 1997).

The potent effects of GDNF on the survival and morphological differentiation of cultured embryonic mesencephalic dopaminergic neurons (Lin *et al.*, 1993) have been extensively investigated. Although many other

neurotrophic factors can support embryonic dopaminergic neurons *in vitro*, GDNF has shown the unique ability to protect dopaminergic neurons in several *in vivo* injury models. For example, pre-treatment with GDNF significantly protects nigrostriatal dopaminergic neurons from the effects of the specific neurotoxin methylphenyl-tetrahydropyridine (MPTP), and it also induces regeneration of dopamine nerve terminals in adult mice when administered after MPTP treatment (Tomac *et al.*, 1995). GDNF prevents the death of axotomised dopaminergic neurons in the adult mesencephalon, however, it cannot fully prevent the lesion-induced loss of tyrosine hydroxylase (TH), the rate limiting enzyme for dopamine synthesis (Beck *et al.*, 1995; Lu and Hagg, 1997). GDNF can also protect midbrain dopaminergic neurons against the neurotoxic effects of 6-hydroxydopamine (6-OHDA) (Hoffer *et al.*, 1994; Bowenkamp *et al.*, 1995; Kearns and Gash, 1995; Sauer *et al.*, 1995; Kearns *et al.*, 1997; Choi-Lundberg *et al.*, 1997). In this experimental paradigm, treatment with GDNF restores the levels of dopamine in the lesioned substantia nigra to normal with a resultant improvement in behavioural function. Moreover, a recent study has demonstrated that GDNF can maintain postnatal nigral dopaminergic neurons in culture, during the period of natural cell death *in vivo* (Burke *et al.*, 1998). A physiological role for GDNF in the adult nigrostriatal dopaminergic system is further suggested by the finding that mesencephalic dopaminergic neurons specifically convey striatal GDNF by retrograde axonal transport (Tomac *et al.*, 1995a).

GDNF is also a potent neurotrophic factor for embryonic spinal and cranial motoneurons in culture, increasing survival, neurite outgrowth and cholinergic differentiation (Henderson *et al.*, 1994; Zurn *et al.*, 1994, 1996; Houenou *et al.*, 1996; Arce *et al.*, 1998). These *in vitro* results are substantiated by the finding that lumbar spinal motoneurons bind, internalise and

transport GDNF in a receptor-mediated fashion (Yan *et al.*, 1995). Furthermore, GDNF rescues developing avian motoneurons from naturally occurring cell death *in vivo* (Oppenheim *et al.*, 1995). GDNF prevents cell death and atrophy of axotomised neonatal facial motoneurons *in vivo* (Henderson *et al.*, 1994; Zurn *et al.*, 1994) as well as maintaining the cholinergic phenotype of adult, axotomised facial motoneurons (Yan *et al.*, 1995). Although GDNF can prevent, in part, the severe loss of adult motoneurons observed after spinal avulsion (Li *et al.*, 1995), it cannot prevent the rapid death of adult motoneurons in dissociated culture, demonstrating the importance of trophic support from surrounding glial cells and afferent neurons (Oorschot and McLennan, 1998). GDNF has also been shown to be a survival factor for neonatal corticospinal motoneurons in culture and is capable of preventing the axotomy-induced death and atrophy of adult corticospinal motoneurons *in vivo* (Junger and Varon, 1997; Giehl *et al.*, 1997).

GDNF also has trophic effects on several other CNS neuronal populations. For example, GDNF prevents the 6-OHDA-induced degeneration of neurons in the noradrenergic nucleus of the locus coeruleus, maintains their neuronal phenotype and stimulates sprouting and hypertrophy (Areñas *et al.*, 1995). Similar effects are elicited by GDNF treatment on adult axotomised basal forebrain cholinergic neurons *in vivo* (Ha *et al.*, 1996; Williams *et al.*, 1996). Additionally, GDNF promotes the survival and morphological differentiation of embryonic cerebellar Purkinje cells *in vitro* (Mount *et al.*, 1995).

In the peripheral nervous system, GDNF promotes the survival of chicken embryonic sympathetic, parasympathetic and sensory neurons *in vitro* (Buj-Bello *et al.*, 1995; Ebendal *et al.*, 1995; Trupp *et al.*, 1995; Forgie *et al.*, 1999; this thesis). GDNF has also been shown to promote neurite outgrowth from

adult mouse DRG explants (Leclere *et al.*, 1998). Evidence of a physiological role for this factor comes from the observation that GDNF is retrogradely transported to subpopulations of DRG neurons in the neonatal rat (Yan *et al.*, 1995). Moreover, in a sciatic nerve axotomy-induced cell death model, GDNF influences adult rat DRG neuronal survival *in vivo* (Matheson *et al.*, 1997; Bennett *et al.*, 1998). GDNF also promotes the survival, proliferation and differentiation of ENS precursors and enhances the survival of embryonic and postnatal differentiated enteric neurons *in vitro* (Hearn *et al.*, 1998; Heuckeroth *et al.*, 1998; Schäfer and Mestres, 1999).

The physiological role of GDNF in mammalian development has been investigated by the generation and analysis of mice with a null mutation in the *GDNF* locus (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996). Mice homozygous for this mutation die in the first postnatal day due to a bilateral renal agenesis or severe dysgenesis and a complete absence of the enteric nervous system with resultant pyloric stenosis. The absence of neurons of the myenteric and submucosal plexuses is evident as early as E12.5, suggesting that GDNF may be required for the commitment, migration and differentiation of enteric neuron precursors and/or their survival thereafter.

Despite the widespread expression pattern of GDNF in the embryonic substantia nigra and striatum, no losses in the number of midbrain dopaminergic neurons or alterations in their innervation patterns are apparent in the *GDNF*<sup>-/-</sup> mouse. Similarly, a normal complement of dopaminergic neurons in the adult heterozygote suggests that GDNF is not a limiting survival factor for these neurons as they mature postnatally. Likewise, no deficits are found in the facial motor nucleus, however, neuronal losses are evident in the trigeminal motor nucleus and in lumbar

spinal motoneurons, indicating that GDNF is a neurotrophic factor for a subset of voluntary motoneurons during development (Moore *et al.*, 1996; Sanchez *et al.*, 1996). In accordance with this, overexpression of GDNF in muscle fibres of transgenic animals results in a dose-dependent hyperinnervation of the neuromuscular junction (Nguyen *et al.*, 1998). Detailed immunohistochemical analysis of the CNS in *GDNF*<sup>-/-</sup> mice indicates a role for GDNF in the development of noradrenergic neurons of the locus coeruleus (Granholtm *et al.*, 1997). Consistent with *in vitro* survival results, there are deficits in the neuronal complement of the nodose, dorsal root and superior cervical sympathetic ganglia in *GDNF*<sup>-/-</sup> mice (Moore *et al.*, 1996).

Parkinson's disease is a chronic, neurodegenerative condition characterised by a loss of dopaminergic neurons of the substantia nigra pars compacta with concomitant depletion of dopamine in their axons terminating in the striatum. The progressive nature of the degenerative process affords the opportunity for neuroprotective therapeutic intervention to halt these changes and also for neurorestorative therapy to improve the function of those neurons that are spared, but compromised. Several studies have investigated whether the ability of GDNF to promote the survival of midbrain dopaminergic neurons in culture (Lin *et al.*, 1993) and in rodent nigrostriatal lesion models (Hoffer *et al.*, 1994; Tomac *et al.*, 1995) can be extended to the more complex system of the non-human primate brain, with a view to the therapeutic potential of GDNF in Parkinson's disease (reviewed in Björklund *et al.*, 1997; Lapchak *et al.*, 1997; Gash *et al.*, 1998).

Administration of MPTP in primates causes the pathophysiological and behavioural features of Parkinsonism. Intracerebral administration of GDNF induces sustained hypertrophy, enhanced neurite outgrowth and an

upregulation of phenotypic markers in injured primate dopaminergic neurons. Striatal dopamine levels and accordingly, motor function (including bradykinesia, rigidity and postural instability) are also improved (Gash *et al.*, 1996; Zhang *et al.*, 1997). Side effects of GDNF administration are limited to weight loss, however, the long term effects of neurotrophic factor treatment on the mature nervous system are unclear. Although intracerebral administration of GDNF is necessary because of the blood-brain barrier, various routes of administration are found to be effective in rodents including injection of an adenovirus vector encoding GDNF (Bilang-Bleuel *et al.*, 1997; Choi-Lundberg *et al.*, 1997) and implantation of genetically engineered GDNF-producing cells (Lindner *et al.*, 1995; Emerich *et al.*, 1996; Tseng *et al.*, 1997) adjacent to the substantia nigra. These preclinical studies suggest that GDNF may have therapeutic applications in the treatment of Parkinson's disease, indeed, preliminary clinical testing in humans is currently underway.

As GDNF can promote the survival of and upregulate phenotypic markers in central noradrenergic neurons *in vivo* (Areñas *et al.*, 1995), GDNF may also have a therapeutic application in neurodegenerative disorders of the locus coeruleus, including Alzheimer's and Huntington's diseases (reviewed in Connor and Dragunow, 1998). Furthermore, a neuroprotective role for GDNF is suggested by the observation that GDNF mRNA is upregulated in several regions of the rat brain after epileptic seizure (Humpel *et al.*, 1994; Schmidt-Kastner *et al.*, 1994). Additionally, GDNF administration can suppress seizure activity and thus prevent the associated neuronal loss in the hippocampal and thalamic regions (Martin *et al.*, 1995; reviewed in Connor and Dragunow, 1998).

In addition to its protective and restorative roles in the CNS, several studies suggest that GDNF has an important role during regeneration after nerve damage in the adult. Following a sciatic nerve crush injury, GDNF mRNA is rapidly upregulated in the distal part of the nerve and in the soleus and gastrocnemius muscles. GDNF mRNA is also upregulated in Schwann cells at and distal to the site of nerve injury and also within the satellite cells of the affected DRG (Trupp *et al.*, 1995; Hammarberg *et al.*, 1996; Naveilhan *et al.*, 1997; Bär *et al.*, 1998). Similarly, expression of GDNF receptors is upregulated, with an increase in GFR $\alpha$ 1 mRNA in the distal part of the nerve and an increase in Ret mRNA in spinal cord motoneurons and DRG neurons (Naveilhan *et al.*, 1997). The post-lesion expression pattern in the adult is reminiscent of expression during embryonic development. Moreover, exogenous GDNF has been shown to accelerate regeneration after nerve injury, suggesting a potential therapeutic role for locally applied GDNF in nerve repair (Naveilhan *et al.*, 1997).

### **1.8.2. Neurturin**

Neurturin (NTN) was originally identified based on its ability to promote the long term survival of sympathetic neurons in culture. The protein responsible for this survival-promoting activity was purified from the conditioned medium of Chinese hamster ovary (CHO) cells, and after cloning, found to be structurally related to GDNF. Neurturin cDNA encodes a 195-amino acid protein with a 76-amino acid pro-region. Proteolytic cleavage of the pro-protein yields a 100-residue mature protein. The amino acid sequence of mature neurturin shares 42 % similarity with mature GDNF and less than 20 % similarity with other TGF- $\beta$  family members, hence a new subfamily of TGF- $\beta$  growth factors, the GDNF family, was established. Despite the relatively low amino acid sequence homology with other TGF- $\beta$  family members, mature neurturin contains all seven cysteine residues found

in the same relative spacing across the family. The mature neurturin protein has a predicted molecular weight of 11.5 kDa, half that of the purified survival-promoting activity, suggesting that neurturin is biologically active as a homodimer (Kotzbauer *et al.*, 1996). The human neurturin gene has been localised to chromosome 19p13.3 (Heuckeroth *et al.*, 1997).

Like GDNF, neurturin is widely expressed in the developing and adult brain and peripheral tissues. Developmentally regulated expression of neurturin mRNA is detected in several brain regions including: the ventral midbrain, brain stem, basal forebrain, olfactory bulb, thalamus, neocortex, hippocampus and striatum (Widenfalk *et al.*, 1997; Lenhard and Suter-Crazzolaro, 1998; Golden *et al.*, 1999). In the adult brain, neurturin mRNA is present in: the hypothalamus, thalamus, hippocampus, piriform cortex, neocortex, cingulate cortex, striatum and granule and Purkinje cell layers of the cerebellum (Golden *et al.*, 1998). Interestingly, neurturin mRNA in the adult rat brain is upregulated after brain insults such as ischaemia or seizure (Kokaia *et al.*, 1999). The most prominent sites of neurturin mRNA expression in the embryonic periphery include the developing limbs, urogenital and digestive systems. A strong neurturin mRNA signal is detected in many trigeminal neuron targets including the nasal epithelium, teeth and whisker follicles, suggesting that neurturin may be a target-derived factor for a subset of trigeminal neurons (Luukko *et al.*, 1998). In addition, high levels of neurturin mRNA are found at sites of mesenchymal-epithelial induction, such as the kidneys, teeth and submandibular glands (Golden *et al.*, 1999). Interestingly, high levels of neurturin are found in neonatal blood, suggesting that it may be distributed in the circulation (Kotzbauer *et al.*, 1996). Within the periphery of the adult, neurturin mRNA has a more restricted expression with the highest levels found in the gut, prostate, testis, oviduct, pituitary and salivary glands (Golden *et al.*, 1999; Xian *et al.*, 1999).

Neurturin may act as a target-derived neurotrophic factor for neurons innervating these tissues or it may play a part in the maintenance of non-neuronal cells of these adult organs.

Although the biological activities of GDNF and neurturin share many common features, there are some interesting differences. Neurturin, which is expressed in the developing kidney, has been shown to act as a collecting duct morphogen in culture, inducing branch initiation and promoting subsequent epithelial arborisation. The expression of neurturin in the collecting ducts themselves suggests neurturin may be an autocrine regulator of renal collecting duct development (Davies *et al.*, 1999; Davies and Davey, 1999). This is in contrast to other kidney morphogens, including GDNF, which act in a paracrine manner (reviewed in Sariola and Sainio, 1997).

In accordance with its developmentally regulated expression in the nigrostriatal system, neurturin promotes the survival of developing dopaminergic neurons in culture (Klein *et al.*, 1997; Horger *et al.*, 1998). Additionally, neurturin protects mature dopaminergic neurons from degeneration in two different experimental paradigms of Parkinson's disease: administration of the neurotoxin 6-OHDA (Horger *et al.*, 1998; Akerud *et al.*, 1999; Rosenblad *et al.*, 1999) and medial forebrain bundle axotomy (Tseng *et al.*, 1998). Nevertheless, whilst neurturin can rescue dopaminergic cell bodies from degeneration, it cannot exert the marked effects on neuronal sprouting, maintenance of the TH phenotype and behavioural corrections observed with GDNF treatment. Interestingly, intrastriatal administration of neurturin in intact adult animals causes behavioural and biochemical changes associated with a functional up-regulation of the nigro-striatal pathway (Horger *et al.*, 1998).

Neurturin promotes the survival of embryonic motoneurons *in vitro* (Klein *et al.*, 1997) as well as postnatal motoneurons in spinal cord explant culture (Bilak *et al.*, 1999). Like GDNF, neurturin increases motoneuron choline acetyltransferase (ChAT) activity, neurite outgrowth from the spinal cord and protects these neurons from degeneration mediated by chronic glutamate exposure (Bilak *et al.*, 1999). Given the fact that Ret and at least GFR $\alpha$ 1 mRNAs are highly expressed in adult motoneurons (Widenfalk *et al.*, 1997; Yu *et al.*, 1998), neurturin may be beneficial in the treatment of motoneuron diseases and spinal cord trauma.

The effects of neurturin within the CNS may not be restricted solely to neuronal cells. The demonstration that neurturin (and GDNF) can stimulate DNA synthesis in a dose-dependent manner in an oligodendrocyte precursor cell line suggests that neurturin may have a role in the development of these supporting cells (Strelau and Unsicker, 1999). Additional evidence indicating that CNS glia are targets for GDNF and neurturin comes from the widespread expression of their receptors in the developing brain (Franke *et al.*, 1998). The trophic actions of neurturin on the nervous system are not restricted to the CNS. Neurturin also exerts neurotrophic effects within the PNS, supporting the survival of subpopulations of sensory and autonomic neurons during development (Kotzbauer *et al.*, 1996; Forgie *et al.*, 1999; this thesis). Furthermore, neurturin potently promotes the survival of enteric neurons as well as the survival, proliferation and differentiation of multipotential ENS progenitors (Heuckeroth *et al.*, 1998; Taraviras *et al.*, 1999).

The role of neurturin as an essential neurotrophic factor *in vivo* has recently been elucidated with the generation of mice with a targeted null mutation in the neurturin gene (Heuckeroth *et al.*, 1999). Mice homozygous for this null

mutation are viable and fertile but display deficits in the enteric and parasympathetic nervous systems. Although the number of enteric neurons is not depleted, as in *GDNF*<sup>-/-</sup> and *Ret*<sup>-/-</sup> mice, there is a reduction in enteric neuronal size, myenteric plexus innervation density and neuropeptide release, culminating in reduced gastro-intestinal motility. Hence, neurturin is a critical neurotrophic factor for post-mitotic enteric neurons. *NTN*<sup>-/-</sup> mice also display peri-orbital abnormalities reflecting profound deficits in parasympathetic innervation to the lacrimal gland. Reductions in parasympathetic cell number and/or cell size are also apparent in the submandibular, ciliary and otic ganglia, demonstrating that neurturin is an essential neurotrophic factor for certain populations of parasympathetic neurons both during development and postnatally.

### 1.8.3. Persephin

Persephin (PSP) was cloned by PCR amplification of genomic DNA using degenerate primers designed to bind to regions of GDNF and neurturin that share a high degree of similarity. Persephin is synthesised as a prepro-protein of 156 residues, which is cleaved to yield a mature protein 96 amino acids long that lacks the N-terminal region as compared to GDNF. Mature persephin is 40 % identical to the mature regions of GDNF and neurturin and contains the characteristic cysteine residue spacing of TGF- $\beta$  family members. The molecular mass of the persephin monomer is 10-12 kDa. However, like other cysteine knot proteins, persephin is likely to be biologically active as a homodimer (Milbrandt *et al.*, 1998). The human persephin gene has been assigned to chromosome 19p13.3 (Chadwick *et al.*, 1998).

Initial studies of persephin mRNA expression using Northern blotting and *in situ* hybridisation failed to detect transcripts in any of the tissues examined.

However, subsequent RT-PCR analysis has shown that persephin is ubiquitously expressed, albeit at low levels, in both embryonic and adult tissues. The levels of persephin mRNA expressed are relatively similar in different tissues and are constant throughout development, with only a slight fall in adult tissues (Milbrandt *et al.*, 1998). Persephin mRNA is expressed in numerous regions of the CNS including: the cortex, hippocampus, striatum, diencephalon, mesencephalon, cerebellum, hindbrain and spinal cord. Persephin mRNA also shows widespread expression in the PNS including the SCG, DRG and adrenal gland. Additionally, persephin mRNA is detected in several non-neuronal elements of the nervous system including sciatic nerve, optic nerve, astroglial cells and oligodendrocyte cell lines (Jaszai *et al.*, 1998; Strelau and Unsicker, 1999). The motoneuron-muscle axis has also been investigated, where it is found that persephin is synthesized in both skeletal muscle and spinal motoneurons (Jaszai *et al.*, 1998). This expression pattern does not support a role for persephin as a typical target-derived neurotrophic factor for motoneurons.

Like GDNF and neurturin, persephin promotes branching of embryonic kidney ureteric buds. The role of persephin as a kidney morphogen may indicate redundancy in the regulation of kidney morphogenesis by GDNF family members and may explain the variability in renal phenotypes of *GDNF*<sup>-/-</sup> mice (Milbrandt *et al.*, 1998; Davies and Davey, 1999). Persephin also promotes the survival of ventral midbrain dopaminergic neurons *in vitro* and *in vivo*, preventing their degeneration after 6-OHDA treatment. Similarly, persephin promotes the survival of cultured embryonic spinal motoneurons as well as rescuing neonatal motoneurons *in vivo* after sciatic nerve axotomy (Milbrandt *et al.*, 1998). Interestingly, unlike GDNF and neurturin, persephin does not exhibit neurotrophic or neurite-promoting effects on postnatal motoneurons *in vitro* and cannot protect these neurons

from chronic glutamate-mediated degeneration, in fact persephin potentiates the toxicity (Bilak *et al.*, 1999). Also, in stark contrast to GDNF and neurturin, persephin does not support the survival of peripheral neurons including sympathetic neurons of the superior cervical sympathetic ganglion and sensory neurons of the nodose, trigeminal and dorsal root ganglia (this thesis; Milbrandt *et al.*, 1998). Additionally, persephin is unable to promote the proliferation or survival of enteric neuron precursors *in vitro* (Heuckeroth *et al.*, 1998; Milbrandt *et al.*, 1998).

#### **1.8.4. Artemin**

Artemin (ARTN) was recently isolated by searching nucleotide sequence databases using the mature neurturin sequence as a query. Like other GDNF family members, artemin contains a signal sequence for secretion and is synthesized as a precursor protein that on cleavage, yields a 113-amino acid mature protein. Sequence alignment shows that artemin shares most similarity with neurturin and persephin (45 % identity) and least with GDNF (36 % identity). All seven cysteine residues characteristic of the TGF- $\beta$  family are conserved in mature artemin and, like the other members of the GDNF family, artemin appears to be a cysteine knot protein that is active as a homodimer. The human artemin gene is located on chromosome 1p32-33 (Baloh *et al.*, 1998).

Despite very low levels of artemin expression in both foetal and adult rat brain, artemin transcripts are detected in the basal ganglia and thalamus, suggesting a role for artemin in influencing subcortical motor systems. *In situ* hybridisation has detected high levels of artemin mRNA in the nerve roots exiting the dorsal root ganglia, reflecting high levels of artemin production in immature Schwann cells. Interestingly, artemin expression is up-regulated in the distal nerve segment after sciatic nerve transection,

indicating that artemin may influence axon regeneration. Artemin mRNA is detected in several peripheral tissues in the developing rat with the highest levels of expression in the kidney and lung. In the adult, the trachea, placenta and pituitary gland express the highest levels of artemin (Baloh *et al.*, 1998).

The expression pattern of artemin is consistent with a role as a paracrine and/or target-derived factor for developing peripheral neurons. This hypothesis is substantiated by the finding that, like GDNF and neurturin, artemin is a survival factor for peripheral neurons *in vitro*, supporting subpopulations of neonatal rat sensory neurons of the trigeminal, nodose, and dorsal root ganglia and sympathetic neurons of the superior cervical sympathetic ganglion. Artemin also promotes the survival of embryonic ventral midbrain dopaminergic neurons (Baloh *et al.*, 1998).

#### **1.8.5. GFR $\alpha$ receptors**

The first member of the GFR $\alpha$  receptor family, GFR $\alpha$ 1, was isolated by expression cloning and screening for GDNF binding proteins. It was found to be a novel protein with no significant homology to other receptors (Jing *et al.*, 1996; Treanor *et al.*, 1996). Subsequently, conventional cloning techniques using a portion of the GFR $\alpha$ 1 nucleotide sequence as a probe and searches of nucleic acid sequence databases for sequences homologous to GFR $\alpha$ 1 have isolated cDNAs encoding three further members of this family of structurally related receptors: GFR $\alpha$ 2 (Baloh *et al.*, 1997; Buj-Bello *et al.*, 1997; Creedon *et al.*, 1997; Jing *et al.*, 1997; Klein *et al.*, 1997; Sanicola *et al.*, 1997; Suvanto *et al.*, 1997), GFR $\alpha$ 3 (Baloh *et al.*, 1998a; Masure *et al.*, 1998; Naveilhan *et al.*, 1998; Nomoto *et al.*, 1998; Trupp *et al.*, 1998; Worby *et al.*, 1998) and GFR $\alpha$ 4 (Thompson *et al.*, 1998). GFR $\alpha$ 4 has thus far been found only in chicken, however, a putative mouse GFR $\alpha$ 4 gene has recently been identified (Gunn *et*

*al.*, 1999). Sequence analysis of *GFR $\alpha$ 1-4* cDNAs has revealed that they encode secreted proteins of 468, 464, 397 and 431 amino acids, respectively. *GFR $\alpha$ 1-4* have molecular masses in the region of 38-65 kDa, dependent on the degree of glycosylation, which is greatest in *GFR $\alpha$ 2*.

Sequence alignment studies have found the translated sequences to be more than 40 % homologous, with the exception of *GFR $\alpha$ 3* which is the most divergent family member. In particular, the carboxyl terminal of *GFR $\alpha$ 3* lacks a relatively large region corresponding to two cysteine residues and the intervening domains in *GFR $\alpha$ 1* and *GFR $\alpha$ 2*. This deleted region shows the lowest homology between the *GFR $\alpha$ 's* and, as such, has been suggested to be an important region for the direct association of the *GFR $\alpha$*  receptor with its ligand. Each protein has a highly conserved cysteine backbone, not related to that found in extracellular cysteine-rich domains of other receptors. Internal homologies within these conserved cysteine-rich sequences suggest a putative domain structure for these receptors. In this model, based on secondary structure analysis, three globular cysteine-rich domains in *GFR $\alpha$ 1*,  $\alpha$ 3 and  $\alpha$ 4, and four domains in *GFR $\alpha$ 2* are joined together by less conserved hinge sequences (reviewed in Airaksinen *et al.*, 1999).

*GFR $\alpha$ 1-4* all possess a hydrophobic C-terminus suggesting attachment to the outer cell membrane via a glycosyl-phosphatidylinositol (GPI)-linkage. Treatment of GDNF ligand-responsive cell lines and neurons expressing *GFR $\alpha$*  and Ret, with phosphoinositide-specific phospholipase C (PIPLC), an enzyme that specifically cleaves GPI linkages, selectively abolishes responsiveness to these factors, leaving neurotrophin signalling unaffected (Treanor *et al.*, 1996; Baloh *et al.*, 1997; Buj-Bello *et al.*, 1997; Creedon *et al.*, 1997; Klein *et al.*, 1997). These experiments not only confirm the attachment of *GFR $\alpha$ 's* to the cell membrane via a GPI-linkage, but also demonstrate the

essential role of these receptors in mediating GDNF ligand signalling as the response was restored on addition of soluble GFR $\alpha$  (Jing *et al.*, 1996; Treanor *et al.*, 1996; Klein *et al.*, 1997; Sanicola *et al.*, 1997). It is interesting to note that whilst GFR $\alpha$ 3 has been shown to be anchored to the cell membrane via a GPI-tail, it is insensitive to cleavage by PIPLC (Worby *et al.*, 1998).

The existence of splice variants of GFR $\alpha$ 1 (GFR $\alpha$ 1a and GFR $\alpha$ 1b) (Sanicola *et al.*, 1997; Dey *et al.*, 1998) and GFR $\alpha$ 2 (GFR $\alpha$ 2a, GFR $\alpha$ 2b and GFR $\alpha$ 2c) (Baloh *et al.*, 1997; Wong and Too, 1998) have been reported. Alternative splice isoforms have been reported for many growth factor receptors including TrkA (Barker *et al.*, 1993; Meakin *et al.*, 1997), TrkB (Strohmaier *et al.*, 1996; Ninkina *et al.*, 1996, 1997) and TrkC (Tsoulfas *et al.*, 1993), and the alternative spliced forms of these receptors show differences in specificities and affinities to their cognate ligands (Strohmaier *et al.*, 1996; Tsoulfas *et al.*, 1996; Ninkina *et al.*, 1997; Baxter *et al.*, 1997). Therefore, the existence of isoforms of the GFR $\alpha$ 's and their differential distributions may significantly influence the biological responses of tissues to GDNF family ligands. Both GFR $\alpha$ 1a and GFR $\alpha$ 1b bind GDNF with high affinity and phosphorylate Ret efficiently (Sanicola *et al.*, 1997), however, the functional interactions of GFR $\alpha$ 2 splice isoforms with Ret are currently unknown.

The physiological relevance of the GFR $\alpha$  receptors has been determined by analysis of the phenotypes of mice with a null mutation in the *GFR $\alpha$ 1* (Cacalano *et al.*, 1998, Enomoto *et al.*, 1998), *GFR $\alpha$ 2* (Rossi *et al.*, 1999) and *GFR $\alpha$ 3* (Nishino *et al.*, 1999) genes. Mice with a targeted null mutation in the *GFR $\alpha$ 1* gene die soon after birth due to kidney agenesis. *GFR $\alpha$ 1* null mutants also display absence of the enteric nervous system below the level of the stomach and have deficits in spinal motoneurons and sensory neurons. In contrast, mice lacking GFR $\alpha$ 2 are viable and fertile, but have dry eyes and

grow poorly after weaning. Additionally, *GFR $\alpha$ 2*<sup>-/-</sup> mice exhibit profound deficits in parasympathetic innervation to the lacrimal and salivary glands and to the small bowel. Sympathetic innervation, on the other hand, is normal. Mice with a disruption of the *GFR $\alpha$ 3* gene grow normally and are fertile. In *GFR $\alpha$ 3*<sup>-/-</sup> mice, the SCG is missing or markedly reduced containing only a few degenerating neurons, whereas parasympathetic and peripheral sensory ganglia appear normal. Accordingly, these mice exhibit marked ptosis. No gross defects of CNS neurons have been detected in any of these null mutants. The distinct phenotypes of each *GFR $\alpha$*  null mutation demonstrates the critical role of each receptor in the development of specific tissues and neuronal populations.

In accordance with the importance of *GFR $\alpha$*  receptors in the development of multiple neuronal tissues, *GFR $\alpha$ 1-4* mRNAs are widely expressed in the developing CNS, PNS and a variety of other tissues. Transcripts of *GFR $\alpha$ 1-4* mRNAs are differentially regulated during development. For example, *GFR $\alpha$ 1* mRNA expression in whole mouse embryos first appears at E11, declines by E15 and then rises at E17, whereas *GFR $\alpha$ 2* mRNA is barely detectable at E11, after which it rises to peak at E17. The expression of both *GFR $\alpha$ 1* and *GFR $\alpha$ 2* is maintained into adulthood. In contrast, *GFR $\alpha$ 3* mRNA expression is strongest at E11, thereafter declining to barely detectable levels in the adult (Jing *et al.*, 1997; Worby *et al.*, 1998).

During development, *GFR $\alpha$ 1* is widely expressed in peripheral tissues including: liver, intestine, kidney, heart, lung, skin, bone, muscle and endocrine glands (Treanor *et al.*, 1996; Buj-Bello *et al.*, 1997; Thompson *et al.*, 1998, Golden *et al.*, 1999). *GFR $\alpha$ 1* transcripts are also detected in neuronal tissues of the developing PNS, including subpopulations of DRG neurons, trigeminal neurons and SCG neurons, and CNS, including the forebrain,

midbrain, pons, medulla, cerebellum and spinal cord (Treanor *et al.*, 1996; Nosrat *et al.*, 1997; Buj-Bello *et al.*, 1997; Horger *et al.*, 1998; Worby *et al.*, 1998; Yu *et al.*, 1998; Widenfalk *et al.*, 1999). Particularly high levels of GFR $\alpha$ 1 mRNA are expressed in the ventral midbrain (dopaminergic neurons) and the ventral gray horn of the spinal cord (spinal motoneurons), both of which contain populations of neurons highly responsive to GDNF *in vitro*. In the adult, GFR $\alpha$ 1 mRNA expression is maintained at high levels in numerous regions of the CNS including: the olfactory tubercle, hippocampal formation, cerebellum, ventral striatum, substantia nigra, habenula, caudate nucleus and spinal cord (Sanicola *et al.*, 1997; Masure *et al.*, 1998). GFR $\alpha$ 1 transcripts are also detected in several peripheral tissues and organs in adulthood with the highest levels detected in components of the male reproductive system (Jing *et al.*, 1997; Golden *et al.*, 1999).

During development, GFR $\alpha$ 2 transcripts, like GFR $\alpha$ 1 transcripts, are abundantly expressed in the brain and many other tissues and organs (Buj-Bello *et al.*, 1997; Baloh *et al.*, 1997; Klein *et al.*, 1997; Widenfalk *et al.*, 1997; Thompson *et al.*, 1998; Yu *et al.*, 1998; Golden *et al.*, 1999). One important exception is the substantia nigra pars compacta where only GFR $\alpha$ 1 mRNA is expressed by the dopaminergic neurons (Horger *et al.*, 1998). In adulthood, the expression of GFR $\alpha$ 2 mRNA within the brain is less extensive than GFR $\alpha$ 1 and distinct expression patterns of these two receptors are apparent. High levels of GFR $\alpha$ 2 mRNA are restricted to the inferior and superior colliculi, pineal gland, lateral septum, deep layers of the neocortex, olfactory tubercle and hippocampal formation (Sanicola *et al.*, 1997; Masure *et al.*, 1998; Trupp *et al.*, 1998). The expression pattern of GFR $\alpha$ 2 mRNA in adult peripheral tissues does not differ markedly from that of GFR $\alpha$ 1 mRNA (Sanicola *et al.*, 1997; Golden *et al.*, 1999).

The expression of GFR $\alpha$ 3 mRNA, unlike GFR $\alpha$ 1 and GFR $\alpha$ 2, appears to be predominately restricted to neuronal tissues. Although low levels of GFR $\alpha$ 3 transcripts are detected in the developing urogenital tract, intestinal mucosal epithelium, thymus and lung, they are thought to be associated with chromaffin cells or other neuronal elements present in these tissues. The expression of GFR $\alpha$ 3 mRNA within the nervous system is also restricted compared to GFR $\alpha$ 1 and GFR $\alpha$ 2 mRNAs. In the CNS, GFR $\alpha$ 3 transcripts are only detected during the earliest period of neurogenesis (Naveilhan *et al.*, 1998). The highest levels of GFR $\alpha$ 3 mRNA expression are associated with neuronal subpopulations of developing peripheral and cranial ganglia including the trigeminal, dorsal root, pre- and para-vertebral sympathetic ganglia and, most strongly, the SCG (Worby *et al.*, 1998; Baloh *et al.*, 1998a; Widenfalk *et al.*, 1998, 1999; Yu *et al.*, 1998). GFR $\alpha$ 3 transcripts are not detected in parasympathetic ganglia. Additionally, high levels of GFR $\alpha$ 3 mRNA are expressed, in the absence of Ret, in Schwann cells of peripheral nerves and in the ensheathing cells of the olfactory nerve (Widenfalk *et al.*, 1998). It is interesting to note that the expression pattern of GFR $\alpha$ 3, almost exclusively confined to neural crest-derived tissues, parallels the expression of Ret (Pachnis *et al.*, 1993), much more closely than the other GFR $\alpha$  family members.

GFR $\alpha$ 4 mRNA is expressed in several developing chicken tissues and organs, including: the brain, kidney, liver, heart, lung, muscle, intestinal myenteric plexus and mucosal epithelium. At E10, the highest levels of GFR $\alpha$ 4 transcripts are found in the kidney and spinal cord. *In situ* hybridisation has shown that at E18, GFR $\alpha$ 4 mRNA expression within the CNS is localised to neurons of the dorsal and ventral gray columns of the spinal cord, Purkinje cells, granule cells and neurons of the deep nuclei of the cerebellum (Thompson *et al.*, 1998).

GFR $\alpha$ 1 was first identified as a receptor for GDNF in studies using cell lines transfected with GFR $\alpha$ 1 (Jing *et al.*, 1996; Treanor *et al.*, 1996). As new GDNF ligands and GFR $\alpha$  receptors were identified, studies were undertaken to assess the ligand specificity of the GFR $\alpha$ 's. Although the results of *in vitro* studies and analyses of mice with null mutations in the GDNF ligand and receptor genes indicate that GFR $\alpha$ 1, GFR $\alpha$ 2, GFR $\alpha$ 3 and GFR $\alpha$ 4 are the preferred receptors for GDNF, neurturin, artemin and persephin, respectively, other ligand/receptor interactions can occur. This is discussed in detail in Chapter 3.

#### 1.8.6. Ret receptor tyrosine kinase

*Ret* (rearranged during transfection) was first identified as a proto-oncogene when NIH3T3 cells were transfected with human T-cell lymphoma DNA and a rearrangement between a DNA segment of *Ret* and an unlinked DNA segment activated its transforming potential (Takahashi *et al.*, 1985). Cloning and sequence analysis of the *Ret* gene indicated that the predicted 170 kDa Ret protein has all the characteristics of a receptor tyrosine kinase (RTK), i.e. an extracellular ligand binding domain, a single hydrophobic transmembrane region and an intracellular segment containing the catalytic tyrosine kinase domain separated into two parts by a small insertion sequence (Takahashi and Cooper, 1987; Takahashi *et al.*, 1989). Despite this overall structural homology with other RTKs, the extracellular domain of Ret is unique in containing a domain homologous to the intermolecular binding regions of the cadherin family of cell adhesion molecules (Schneider, 1992; Iwamoto *et al.*, 1993). In accordance with the fact that the amino acid residues required for homophilic binding in N-cadherin are absent from Ret, no adhesive properties have been described. The putative calcium binding sites, also required for cell adhesion, are conserved in Ret (Robertson and Mason, 1995). Differential splicing at the 3' end of *Ret* results in transcripts

encoding three isoforms of 1114, 1106 and 1072 amino acids respectively, with the differences located in the C-terminal region of the protein only (Ivanchuk *et al.*, 1998).

In accordance with the role of *Ret* as a proto-oncogene in transfected fibroblasts, germline, "gain of function" mutations in the human *Ret* gene are associated with the inherited cancer syndromes multiple endocrine neoplasia type 2A and 2B (MEN2A & 2B) and familial medullary thyroid carcinoma (FMTC) (Eng, 1996, 1996a; Pasini *et al.*, 1996; Edery *et al.*, 1997). In MEN2A and FMTC, a mutation in a cysteine residue in the extracellular cysteine-rich domain causes constitutive activation of *Ret* by ligand-independent dimerisation of the protein (Mulligan *et al.*, 1993, 1994; Santoro *et al.*, 1995). In MEN2B, a single mutation in the tyrosine kinase domain of *Ret* (met<sup>918</sup> → thr) changes substrate specificity and increases catalytic activity independently of receptor dimerisation (Carlson *et al.*, 1994; Hofstra *et al.*, 1994; Santoro *et al.*, 1995). Unlike the activating *Ret* mutations caused by constitutive homodimerisation, MEN2B *Ret* activity and hence the disease phenotype, can be modulated by GDNF family ligands (Carlomagno *et al.*, 1998).

In addition to a role in cellular transformation and tumorigenesis, *Ret* plays an important part in normal mammalian embryonic development. This is illustrated by the phenotype of *Ret* kinase-deficient mice in which the kidneys, enteric nervous system below the stomach and the SCG are all absent (Schuchardt *et al.*, 1994). Similarly, germline or sporadic inactivating mutations of the *Ret* gene in humans can cause Hirschsprung's disease (aganglionic megacolon) which is characterised by the absence of enteric ganglionic cells in the submucosal and myenteric plexuses of variable segments of the distal gastro-intestinal tract (Romeo *et al.*, 1994; Edery *et al.*,

1994; Pasini *et al.*, 1996). Mutations in the *Ret* gene are also associated with congenital central hypoventilation syndrome (Amiel *et al.*, 1998). Interestingly, *Ret* null mutant mice and the diseases that result from *Ret* mutations are all characterised by neurocristopathies, demonstrating the crucial role of *Ret* in the development of neural crest derivatives.

*Ret* mRNA is detected in many tissues from early stages in mouse embryogenesis (Pachnis *et al.*, 1993; Nosrat *et al.*, 1997; Widenfalk *et al.*, 1997; Golden *et al.*, 1999). Expression in the developing PNS, CNS and excretory system is observed as early as E8.5 and by the mid-embryonic stages all cranial ganglia, as well as autonomic and dorsal root ganglia of the trunk, express *Ret* mRNA. *Ret* mRNA is also expressed in developing dopaminergic neurons, motoneurons and adrenal chromaffin cells. The strongest signals are associated with the developing kidneys (Wolffian duct, ureteric bud epithelium and the growing tips of the renal collecting ducts) and the gastro-intestinal tract (in the presumptive enteric neuroblasts of the vagal neural crest and in myenteric ganglia of the gut). In these tissues, the expression of *Ret* and GDNF mRNAs are precisely complementary (Nosrat *et al.*, 1996, 1997). The levels of *Ret* mRNA expression seen in the adult are much lower than during development, being restricted to neurendocrine tissues only. The fact that *Ret* is primarily found in cells and lineages derived from neural crest as well as in urogenital precursors reiterates the role of *Ret* in the development of neural crest derivatives and the kidney.

The discovery of *Ret*, in conjunction with  $GFR\alpha$ , as a receptor for GDNF was unexpected, as most TGF- $\beta$  family members signal via heterodimeric receptors of transmembrane serine/threonine kinases (reviewed by Massagué, 1996, 1998). This raises the possibility that other TGF- $\beta$  family members may activate alternative signalling pathways via receptor tyrosine

kinases, or that GDNF ligands may be able to utilise serine-threonine kinase receptors in certain cell types. Ret is not alone amongst receptor tyrosine kinases in requiring an accessory receptor component. Other examples include the FGF receptor which requires heparin-containing proteoglycans (Schlessinger *et al.*, 1995) and the novel RTK, MuSK, which transduces the agrin signal at the neuromuscular junction and requires an accessory component found only on the surface of muscle cells (Glass *et al.*, 1996).

Ret, a former orphan receptor tyrosine kinase, was initially proposed as a functional GDNF receptor based on the similarity in phenotypes of *Ret* and *GDNF* null mutant mice (Schuchardt *et al.*, 1994; Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996) as well as the striking complementary expression patterns of GDNF and Ret in several embryonic tissues (Nosrat *et al.*, 1996, 1997; Pachnis *et al.*, 1993). Experiments involving chemical cross-linking of GDNF to a motoneuron hybrid cell line identified a high molecular weight complex that could be immunoprecipitated with anti-phosphotyrosine antibodies or Ret antisera (Trupp *et al.*, 1996). Further experiments demonstrated that treatment of Ret- (and GFR $\alpha$ 1-) expressing cell lines with GDNF phosphorylated Ret in a dose dependent manner (Treanor *et al.*, 1996; Trupp *et al.*, 1996; Vega *et al.*, 1996), and ectopic expression of Ret in *Xenopus* animal caps enabled them to differentiate into mesoderm (indicative of MAPK activation) in the presence of GDNF (Durbec *et al.*, 1996). In addition, autonomic neurons associated with nephrogenic tissue derived from *Ret*<sup>-/-</sup> mice did not respond to GDNF with enhanced neurite outgrowth, as did their wild-type counterparts (Durbec *et al.*, 1996). The additional demonstration that GDNF/NTN, GFR $\alpha$ 1 and Ret can form a physical complex on the cell surface indicates that Ret is an integral part of the GDNF receptor system rather than a downstream signalling molecule (Treanor *et al.*, 1996; Jing *et al.*, 1996; Klein *et al.*, 1997). The rapid induction of

Ret phosphorylation by GDNF and the ability of soluble Ret-Fc fusion protein to block the autophosphorylation also suggests that Ret is activated directly (Jing *et al.*, 1996).

In one model of GDNF signalling, it has been proposed that the initial step is the binding of disulphide-linked dimeric GDNF to GFR $\alpha$ 1. It has been suggested that GFR $\alpha$ 1 may be in monomeric or dimeric form resulting in different binding affinities for GDNF (two classes of binding site have been identified in fibroblasts transfected with GFR $\alpha$ 1 (Jing *et al.*, 1996). According to this model, in the next step of the signalling process a GDNF-GFR $\alpha$ 1 heterotetramer is presented to two molecules of the signalling component of the receptor, Ret, triggering dimerisation and transphosphorylation of their kinase domains (Jing *et al.*, 1996). There are still uncertainties about the topology and stoichiometry of the interactions between GDNF, the GFR $\alpha$  coreceptor and Ret, and other models for GDNF signalling are possible. For example, rather than the ligand first binding to a GPI-linked receptor and this complex interacting with Ret, GDNF may stabilise preformed complexes between Ret and GFR $\alpha$ 's at the cell membrane. Various observations do suggest the existence of preformed Ret-GFR $\alpha$  receptor complexes in the absence of ligand. For example, Ret-GFR $\alpha$ 1/2 can be co-immunoprecipitated from unstimulated cells (Treanor *et al.*, 1996; Klein *et al.*, 1997) and soluble Ret extracellular domain can interact with soluble GFR $\alpha$ 1 *in vitro* (Sanicola *et al.*, 1997). Furthermore, the high levels of constitutive tyrosine phosphorylation of Ret in unstimulated fibroblasts are negatively regulated by coexpression of GFR $\alpha$  receptors (Trupp *et al.*, 1998). This ligand-independent interaction between Ret and GFR $\alpha$  may be relevant in regulating oncogenic forms of Ret. The presence of the cadherin-like motif in the Ret extracellular domain suggests that Ret function may be Ca<sup>2+</sup> dependent. Recent data supports this hypothesis demonstrating that GDNF-

and neurturin-mediated Ret autophosphorylation and subsequent MAPK activation are induced in a  $\text{Ca}^{2+}$  dependent manner and that GDNF/neurturin-Ret complex formation requires the presence of  $\text{Ca}^{2+}$  (Nozaki *et al.*, 1998).

The specific intracellular signalling pathways leading to proliferation, survival and neurite outgrowth in neurons following GDNF-dependent Ret activation are now beginning to be characterised. Specific phosphotyrosine residues on the intracellular domain of Ret act as docking sites for SH2 domain-containing adaptor proteins, for example,  $\text{tyr}^{905}$  binds Grb7/Grb10,  $\text{tyr}^{1015}$  binds PLC $\gamma$ ,  $\text{tyr}^{1062}$  binds Shc and  $\text{tyr}^{1096}$  binds Grb2 (Asai *et al.*, 1996; Borrello *et al.*, 1996; Durick *et al.*, 1996; Liu *et al.*, 1996; Arighi *et al.*, 1997; Lorenzo *et al.*, 1997; Ohiwa *et al.*, 1997; Alberti *et al.*, 1998; Xing *et al.*, 1998). Experiments utilising cell lines and primary neurons suggest that the Ras-MAPK pathway is essential for the survival and neurite growth promoting activities of GDNF and neurturin (Creedon *et al.*, 1997; van Weering and Boss, 1998; Xing *et al.*, 1998). GDNF-induced formation of large lamellipodia in neuritogenesis and differentiation of cultured dopaminergic neurons appears to involve the PI3K/Akt pathway (van Weering and Boss, 1997, 1998; Pong *et al.*, 1998). GDNF has also been shown to activate the JNK pathway (Chiariello *et al.*, 1998) and to stimulate PLC $\gamma$ -dependent IP3 production with a subsequent increase in intracellular free calcium (Airaksinen *et al.*, 1999). It is unclear whether Ret activation by each GDNF ligand and coreceptor stimulates a distinct signalling profile as suggested for the activation of TrkB by its two ligands, BDNF and NT-4 (Minichiello *et al.*, 1998).

Ret is normally coexpressed with one or several GFR $\alpha$ 's, often in a complementary manner to the expression of their ligand mRNAs (Luukko *et al.*, 1997; Trupp *et al.*, 1997, 1998; Widenfalk *et al.*, 1997; Golden *et al.*, 1998;

Naveilhan *et al.*, 1998; Yu *et al.*, 1998). Nevertheless, several cells are known to express Ret without any GFR $\alpha$  or vice versa (Trupp *et al.*, 1997; Widenfalk *et al.*, 1998; Yu *et al.*, 1998; Golden *et al.*, 1999). The functional significance of this expression pattern may lie in Ret-independent signalling, the existence of unidentified receptor components or the formation of receptor complexes *in trans*.

Recent data suggest that the GFR $\alpha$  subunits alone may be capable of transducing the GDNF signal into the cell, independently of Ret. GDNF has been shown to promote the survival of postnatal rat cochlear neurons which express GFR $\alpha$ 1 but not Ret (Ylikoski *et al.*, 1998) and also to promote survival and fos activation in a cell line expressing GFR $\alpha$ 1 but lacking Ret (Trupp *et al.*, 1998). Unlike Ret-expressing cell lines, the signalling pathway utilised is thought not to be Ras-MAPK, but rather a stimulation of a GFR $\alpha$ 1-associated Src-like kinase (Trupp *et al.*, 1999). Similarly, sensory neurons from *Ret*<sup>-/-</sup> mice, show a GDNF-induced activation of Src-type kinases resulting in elevated intracellular calcium levels. Hence, a model has been proposed suggesting GFR $\alpha$ 1, located in a membrane lipid raft, can recruit and activate Src-type kinases upon GDNF binding, causing phosphorylation of PLC $\gamma$ , production of IP3 and elevation of intracellular calcium (reviewed in Airaksinen *et al.*, 1999). The mechanism of GFR $\alpha$ 1-mediated Src activation and the significance of this signalling *in vivo* is unclear. Whether other GFR $\alpha$  coreceptors can similarly signal independently of Ret remains to be elucidated as does the relationship between Ret-dependent and independent signalling systems in cells expressing both Ret and GFR $\alpha$ 1.

The discovery of GFR $\alpha$  receptor expression in the projection targets of Ret positive neurons (Yu *et al.*, 1998) has led to the proposal that Ret may be able to interact with a GFR $\alpha$  receptor located on a target neuron or as a soluble

protein. Such an interaction *in trans* is possible *in vitro*, as treatment of Ret expressing cell lines with GDNF and soluble GFR $\alpha$ 1 fusion protein leads to a strong activation of Ret tyrosine kinase (Treanor *et al.*, 1996; Klein *et al.*, 1997; Jing *et al.*, 1997; Sanicola *et al.*, 1997; Yu *et al.*, 1998), however, as yet there is no direct evidence of a *trans* interaction *in vivo*.

## 1.9. Aims of this project

The initial aim of this study was to investigate the *in vitro* responsiveness of various populations of developing neurons from the peripheral nervous system to the newly isolated neurotrophic factor, neurturin, and to make a comparison with the effects of its well characterised relative, GDNF. In the course of this project, two additional members of this ligand family were identified, hence, this study was extended to consider the neurotrophic effects of all family members. In addition, this first study aimed to correlate the neuronal responsiveness to these factors *in vitro* with the expression of their respective receptors.

Having established a comprehensive picture of GDNF responsiveness *in vitro*, in both avian and mammalian developing neurons, the second phase of this project aimed to consider further the receptors involved in mediating the GDNF signal. The first aim was to investigate the specificity of the GDNF receptor, GFR $\alpha$ 1, by analysing mice with a null mutation in this gene. Secondary aims were to generate polyclonal antibodies against members of the GFR $\alpha$  receptor family, to characterise these antibodies and to utilise them in a number of experimental approaches to determine aspects of GFR $\alpha$  expression and ligand binding.

During the course of these studies, a surprising result indicated a potential role for NGF in the development of the nodose ganglion. As a strong body of evidence argues against a role for NGF in this neuronal population, the final phase of this project aimed to use mice with a targeted disruption of the *NGF* gene as the definitive means of addressing this issue.

## Chapter 2

### Differences and developmental changes in responsiveness of PNS neurons to members of the GDNF ligand family

#### 2.1. Introduction

The GDNF family of neurotrophic factors comprises GDNF (Lin *et al.*, 1993), neurturin (Kotzbauer *et al.*, 1996), persephin (Milbrandt *et al.*, 1998) and artemin (Baloh *et al.*, 1998). GDNF promotes the survival of several populations of developing avian peripheral neurons in culture. These include sympathetic, parasympathetic, proprioceptive, enteroceptive and small and large cutaneous sensory neurons (Buj-Bello *et al.*, 1995; Ebendal *et al.*, 1995; Trupp *et al.*, 1995). Additionally, GDNF mRNA is expressed in the tissues innervated by these neurons, suggesting that GDNF may be important for regulating the survival of various populations of avian neurons at different stages in their development. In contrast to the well characterised neurotrophic effects of GDNF within the PNS, very limited information existed at the start of this project regarding the role of neurturin within the developing PNS. This was also true for persephin and artemin which were isolated during the course of this study.

The study of neurons from the PNS in culture provides invaluable information on fundamental aspects of neurotrophic factor biology as well as clarifying the functions of neurotrophic factor receptors. The use of avian embryonic PNS neurons to investigate the trophic actions of newly discovered growth factors presents clear benefits as sensory and autonomic neurons can be easily obtained free of contaminating non-neuronal cells for use in *in vitro* studies (Davies, 1988). Cranial sensory neurons are especially

useful for ascertaining which classes of sensory neurons respond to a particular factor, as functionally distinct classes of sensory neurons are segregated into anatomically discrete groups (Davies, 1987a), unlike for example, the functional heterogeneity observed in the dorsal root ganglia.

The aim of the study described in this chapter was to undertake a systematic study of the responsiveness of different populations of developing avian PNS neurons *in vitro* to the newly discovered members of the GDNF ligand family, neurturin, persephin and artemin, and to make a comparison with the established neurotrophic effects of GDNF. Using competitive RT-PCR, the levels of mRNAs encoding GDNF family receptors from purified neurons were also ascertained, allowing correlations between the levels of receptor expression and responsiveness to GDNF ligands to be made. The results demonstrate that neurturin, like GDNF, does promote the *in vitro*, survival of several populations of chicken PNS neurons. After a comprehensive, comparative study, the responsiveness of various neuronal populations to these two factors was found to differ and indeed, to change throughout development. In general, such changes were correlated with differences in receptor expression. Persephin and artemin, on the other hand, were found to have no survival promoting effects within the chicken PNS in culture.

## 2.2. Methods

### 2.2.1. Neuronal cultures

In this study, the responses of several well characterised populations of avian parasympathetic, sympathetic and sensory neurons to members of the GDNF ligand family, at intervals throughout embryonic development, were investigated. Dissociated cultures were established because, unlike explant cultures, the effects of various factors on neuronal survival are easily quantified in low density cultures. Also, because non-neuronal cells can be effectively removed from these cultures, the analysis of the effects of a particular factor on neurons is not complicated by any indirect effects mediated by non-neuronal cells.

White Leghorn fertile chicken eggs were incubated in a forced-draft, humidified incubator at 38 °C for the required period. All dissections and subsequent preparations of neuronal cultures were carried out in a laminar flow hood using standard sterile technique. Dissections of cranial sensory ganglia were performed under a stereomicroscope using a fibre-optic light source for illumination as this prevents overheating of the specimen. Embryos were dissected in sterile plastic Petri dishes (60 and 100 mm, Sterilin) in Liebowitz-15 (L-15) medium without sodium bicarbonate. Toothed forceps and straight and curved watchmaker's forceps, sterilized by flaming in alcohol, were used in the initial stages of the dissections. Tungsten needles were required to complete the procedure and to remove adherent connective tissue from the dissected neural tissue. These were made from 0.5 mm diameter tungsten wire, electrolytically sharpened in 1 M KOH and held in chuck-grip platinum wire holders (Figure 2.1.).

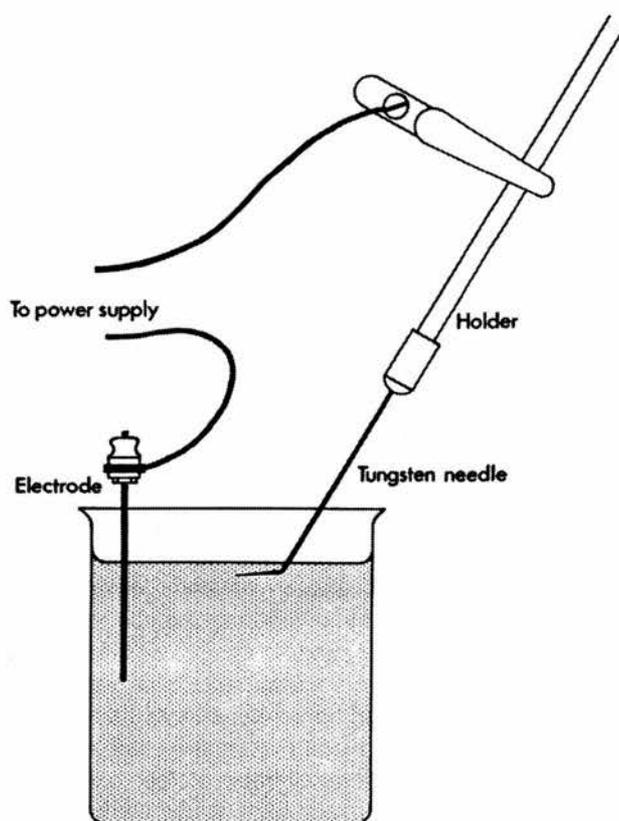


Figure 2.1. Method for making tungsten needles.

A 3-12 V AC current passes through the wire and a second electrode immersed in the KOH solution. The tungsten is etched away over several minutes forming a taper from the bend to the tip of the needle. The needles are washed in water to remove alkali before use. From Davies, 1988.

The trigeminal, nodose, ciliary, vestibular, paravertebral sympathetic and superior cervical ganglia were dissected from chicken embryos ranging from 6 to 16 days of incubation (E6-E16). This represented stages 29 through 42 in chicken development (Hamburger and Hamilton, 1951)(Figure 2.2.). Figure 2.3. shows the location of the cranial sensory ganglia from the lateral aspect of the head.

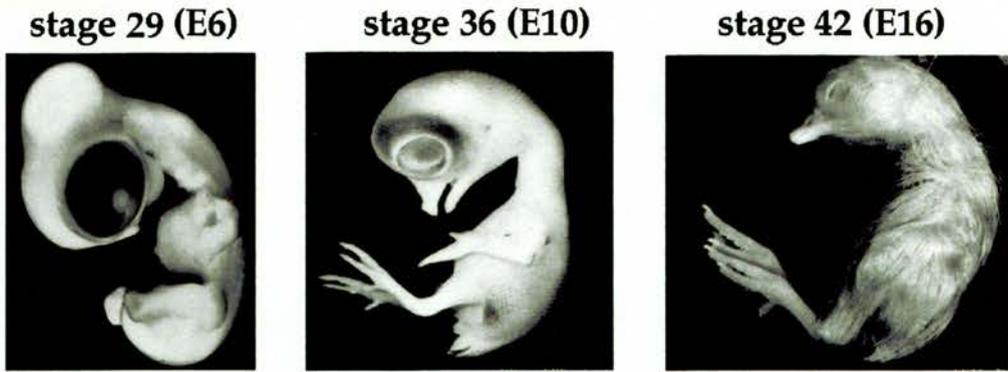


Figure 2.2. Photomontage showing selected stages in chicken development.

Features such as the visceral arches, eyelids, beak, limbs, feather germs and toes are used to accurately define the developmental stage (Hamburger and Hamilton, 1951) (not shown to scale).

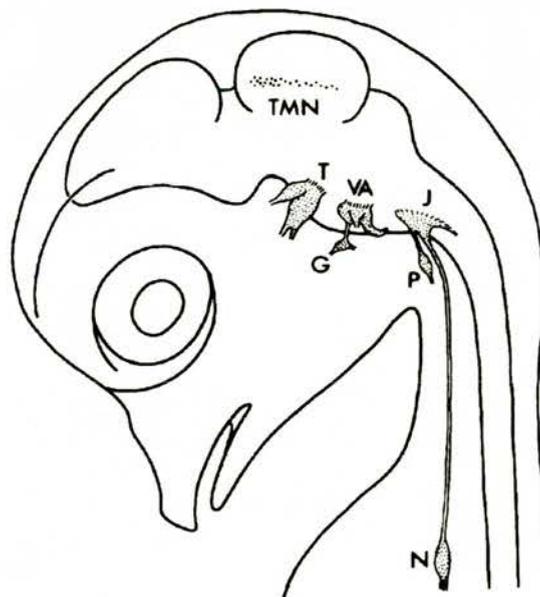


Figure 2.3. Location of populations of cranial sensory neurons in the chicken embryo.

Lateral aspect of the head and neck of an E10 chicken embryo showing the locations of the trigeminal mesencephalic nucleus (TMN) and the trigeminal (T), geniculate (G), vestibulo-acoustic (VA), petrosal (P), jugular (J) and nodose (N) ganglia. From Davies, 1988.

The trigeminal and vestibulo-acoustic ganglia were dissected after careful removal of the brain and subdissection of the cranial base into tissue blocks (Figure 2.4.). The superior cervical ganglion was also dissected from this region, being medially situated to the petrosal ganglion. After removal, the trigeminal ganglion was further sub-dissected into its neural crest-derived, NGF-dependent dorsomedial pole (dorsomedial trigeminal ganglion, DMTG) and placode-derived, BDNF-dependent ventrolateral pole (ventrolateral trigeminal ganglion, VLTG)(Figure 2.5.).

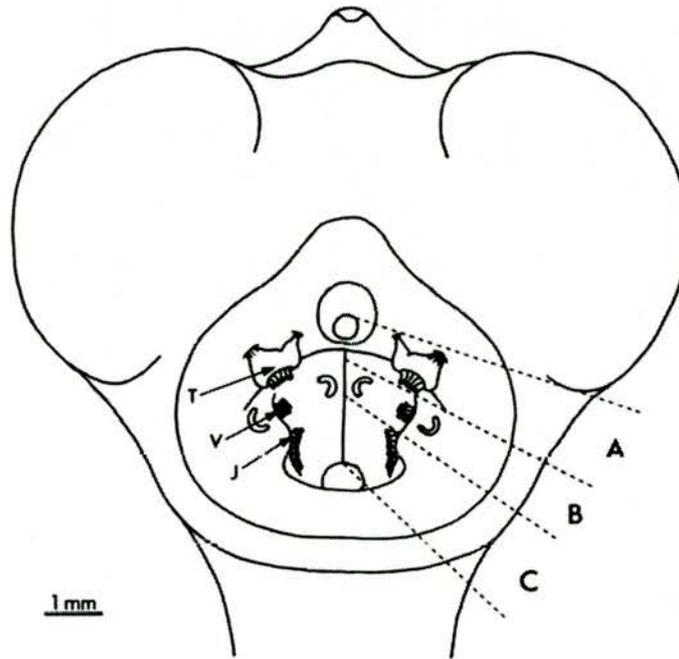


Figure 2.4. Dorsal aspect of the cranial base after removal of the brain.

Dotted lines indicate positions for sub-dissecting this tissue into blocks that contain the trigeminal ganglion (T, block A), vestibulo-acoustic and geniculate ganglia (block B) and the jugular and petrosal ganglia (block C). The roots of the vestibulo-acoustic (V) and jugular (J) ganglia are shown. From Davies, 1988.

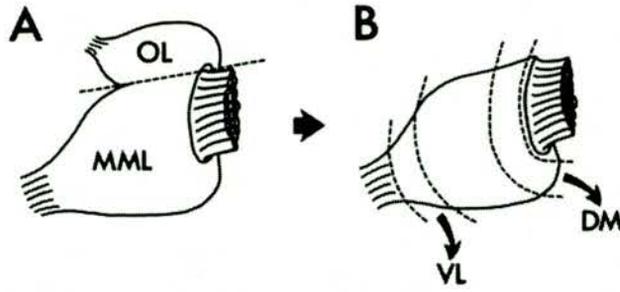


Figure 2.5. Subdissection of the trigeminal ganglion into dorsomedial (DM) and ventrolateral (VL) poles.

The ophthalmic lobe (OL) is separated from the maxillo-mandibular lobe (MML) along the interrupted line shown in A. The MML is subdissected along the lines shown in B to obtain the DM and VL poles. From Davies, 1988.

The ciliary ganglion was easily removed from its location adjacent to the exiting optic nerve on the back of the eye.

In the chicken, the nodose ganglia are located on either side of the midline in tissues lying in front of the vertebral column at the base of the neck. Forceps were used to expose the great vessels emerging from the heart and a block of tissue, containing both nodose ganglia, was removed. The ganglia were easily recognised in connective tissues surrounding the great vessels by their glistening white appearance, spindle shape and prominent attached vagus nerve (Figure 2.6.).

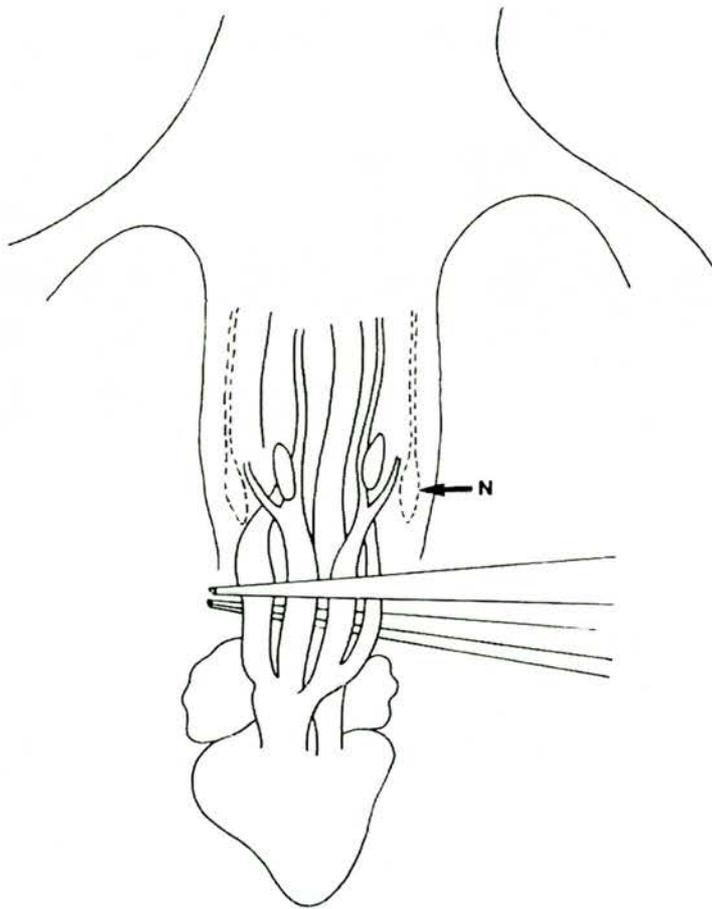


Figure 2.6. Location of the nodose ganglion in the chicken embryo.

Ventral aspect of the thoracic region of an E10 chicken embryo after removal of the skin and reflection of the ventral thoracic wall to either side of the midline, exposing the heart and great vessels. The connective tissues lying on either side of the great vessels contain the two nodose ganglia (N) attached to the vagus nerves (interrupted lines). From Davies, 1995.

The paravertebral sympathetic chain was dissected from the lumbar region of chicken embryos where it was found just lateral to the vertebral column, lying ventral to the dorsal root ganglia. The intact chain was readily peeled away with forceps with little adherent connective tissue (Figure 2.7.).

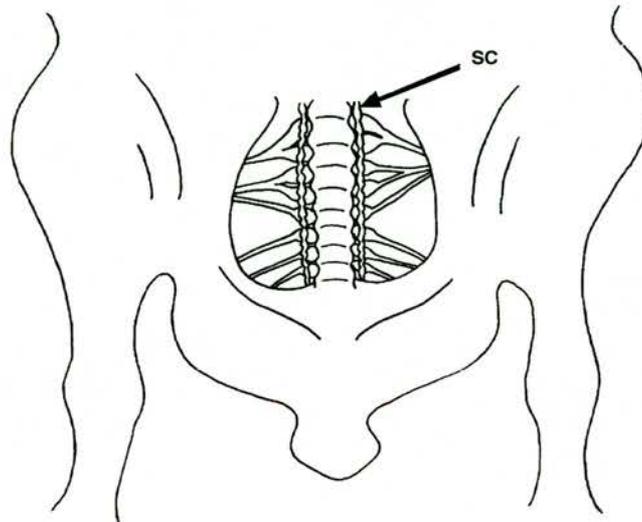


Figure 2.7. Location of the paravertebral sympathetic chain in the chicken embryo.

Ventral aspect of the lumbo-sacral region of an E10 chicken embryo after evisceration. The paravertebral sympathetic chain (SC) can be seen lateral to the vertebral column and ventral to the dorsal root ganglia. From Davies, 1988.

The dissected ganglia were collected in calcium-, magnesium-free Hank's balanced salt solution (CMF-HBSS) and incubated in 0.1 % trypsin, at 37 °C, for 8, 10, 15, 20, 22 and 25 minutes for E6, E8, E10, E12, E14 and E16 ganglia, respectively. With the exception of sympathetic chain, approximately 50 ganglia were required per experiment. Following trypsinisation, the ganglia were washed twice in Ham's F-12 medium supplemented with 10 % heat-

inactivated horse serum (HIHS) in order to arrest trypsin action. The ganglia were washed twice with HBSS to remove any residual serum, and then mechanically dissociated by gentle trituration using a fire-polished, siliconized Pasteur pipette to give a single cell suspension. The trituration was monitored by examining a drop of the dissociated cell suspension on a glass slide using an inverted phase-contrast microscope. Four to five passages of the cells through the tip of the Pasteur pipette were usually sufficient to dissociate trypsinised ganglia. If the trituration has been performed successfully, neurons should be isolated from surrounding cells but still have fairly long processes attached to their cell bodies.

Because this study utilised mid to late embryonic dissociated ganglionic tissue, it was necessary to purify neurons away from contaminating non-neuronal cells. Non-neuronal cells may contribute to unacceptably high levels of survival in control cultures, as they can synthesize and release neurotrophic factors. In addition, the presence of non-neuronal cells makes the interpretation of any effects of neurotrophic factors on neurons difficult, since neurotrophic factors may act on non-neuronal cells which in turn may produce factors which indirectly enhance neuronal survival. The method employed to remove satellite cells, Schwann cells and fibroblasts was differential gravity sedimentation (Davies, 1988). This technique is based on the fact that the neurons, being larger, sediment more quickly than non-neuronal cells. The main advantages of this method, compared to differential adhesion techniques, are that efficiency is high, at least 60 % of the starting neurons will be recovered, and neuronal viability is very high as the cells sediment through culture medium. An added benefit is that cellular debris is removed. Sedimentation was carried out in an autoclaved 100 ml cylindrical glass dropping funnel with a ground glass outlet tap. The funnel was filled to a height of 8-10 cm with F-14 medium plus 10 % HIHS and

placed in a stand, on an anti-vibration table, in a refrigerated incubator ( $2\pm 0.5$  °C). The funnel was allowed to equilibrate at this temperature overnight before use. The temperature was held constant to avoid convection currents being generated in the medium. The dissociated cell suspension, in a volume of 2 ml of HBSS, was very carefully layered onto the medium and left for one hour, after which 4-5 ml aliquots were collected (Figure 2.8.). Samples of these fractions were examined by phase-contrast microscopy to select those containing only neurons. Neuronal cells were clearly distinguishable from other cell types present due to their characteristic morphology, i.e. large, round, phase bright cell bodies as compared to the small, dark, irregular profiles of non-neuronal cells (Figure 2.9.). It should be noted that neuronal processes largely retract into the cell body during sedimentation. The neuronal fractions (>95 % pure) were pooled and plated onto 35 mm plastic tissue culture dishes (Nunc, Gibco) at a density of 500-2000 neurons per dish. The dishes had previously been prepared by coating with a substratum of poly-DL-ornithine (0.5 mg/ml in 0.15 M borate buffer, pH 8.4, overnight at room temperature) and laminin (20 µg/ml in F-14 medium, 4 hours at 37 °C). The cells were grown in 2 ml of F-14 medium supplemented with sodium bicarbonate (0.2 %) and 10 % HIHS, in a humidified 5.5 % CO<sub>2</sub> incubator, at 37 °C. Various purified recombinant neurotrophic factors were added to the cultures at the time of plating. Figure 2.10. shows the typical appearance of ciliary neurons cultured for 48 hours with 10 ng/ml of CNTF.

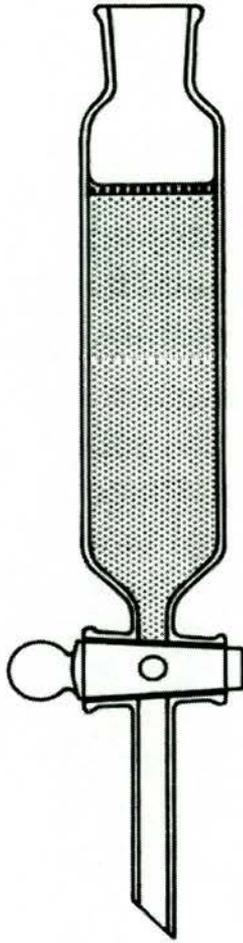


Figure 2.8. Neuron enrichment using differential sedimentation.

Dropping funnel containing a cell suspension (heavy stiple) layered on culture medium.

From Davies, 1995.

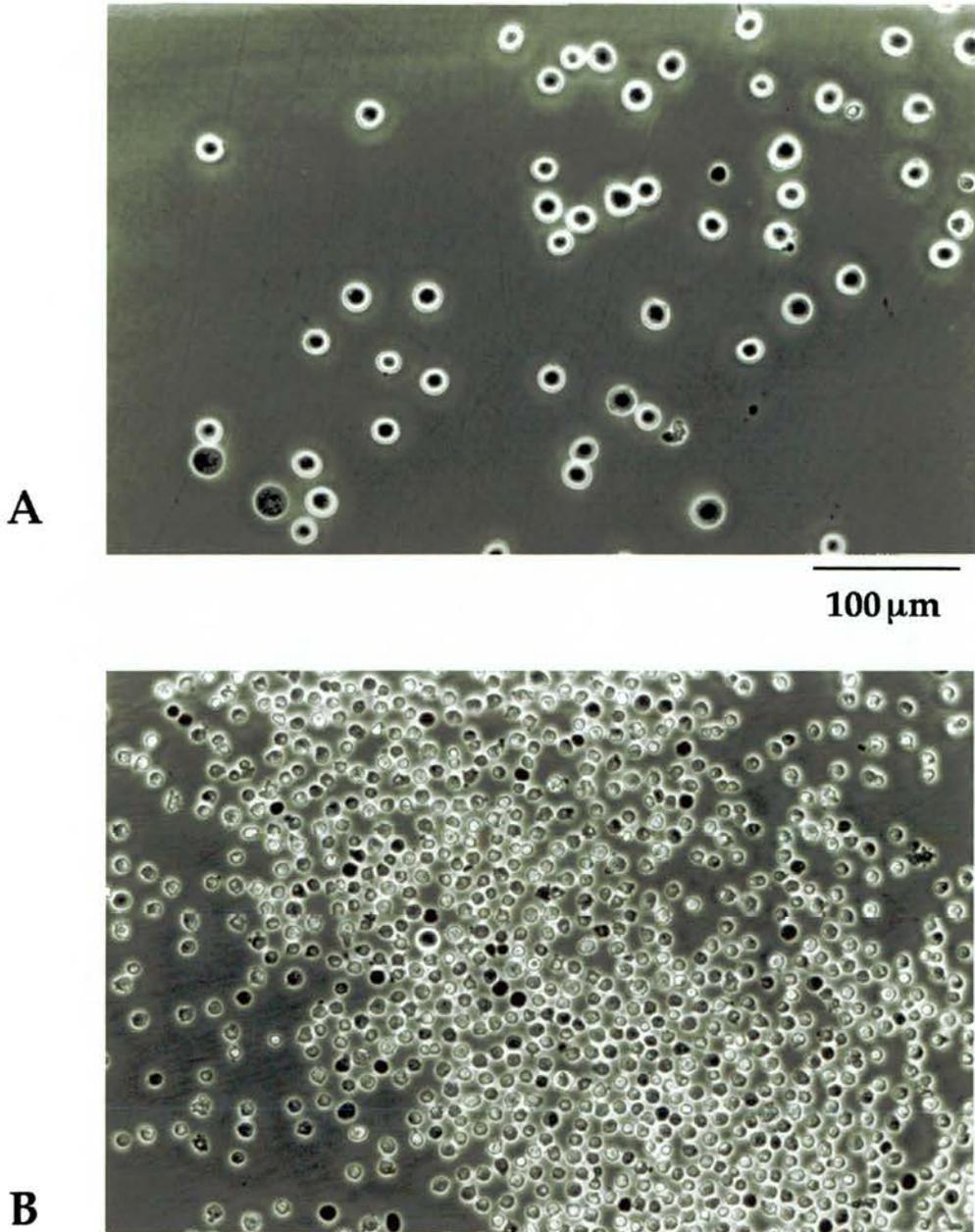
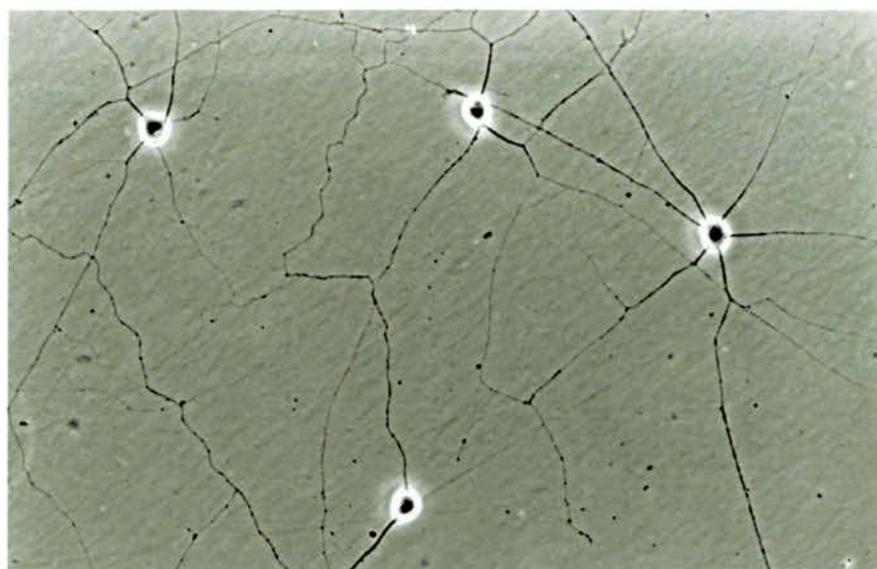


Figure 2.9. Phase-contrast photomicrographs showing the successive stages in the separation of TMN neurons from other cells by differential sedimentation.

- (A) An aliquot of neuronal cells taken from the dropping funnel after sedimentation.
- (B) An aliquot of non-neuronal cells taken from the dropping funnel after sedimentation.



100  $\mu$ m

Figure 2.10. Photomicrograph of E10 ciliary neurons cultured for 48 hours in the presence of 10 ng/ml CNTF.

Three to six hours after plating, the number of attached neurons in the centre of each dish was counted using a 12 x 12 mm standard graticule and an inverted phase-contrast microscope at 100x magnification. 48 hours after plating, the number of surviving phase-bright, process-bearing neurons in the same area was ascertained. By this time, almost all neurons had died in control cultures without neurotrophic factors. The survival response of neurons to a particular factor was expressed as percent survival, i.e. the number of surviving cells at 48 hours expressed as a quotient of the number present at 3 hours. Within each experiment, triplicate cultures were established for each condition and all experiments were repeated at least three times.

### **2.2.2. Measurement of receptor mRNA levels in purified neurons using competitive RT-PCR**

In this collaborative study, competitive RT-PCR was used to analyse the levels of expression of GDNF and neurturin receptors in purified neurons that responded to these factors in culture. Receptor mRNAs were detected and quantified by using Ret, GFR $\alpha$ 1 and GFR $\alpha$ 2 specific primers to PCR amplify reverse transcribed total RNA (RT-PCR), extracted from various populations of purified neurons at different stages of development. To facilitate comparison of the relative levels of the receptors in different samples, the levels of mRNA for the housekeeping protein L27 were also measured.

E8 and E12 ciliary and nodose ganglia and E10 and E16 lumbar sympathetic chain were dissected from White Leghorn chicken embryos. The ganglia were dissociated to a single cell suspension and neurons were separated from non-neuronal cells by differential sedimentation. 1 ml aliquots of purified neurons were collected, spun down and the cell pellet lysed to liberate RNA in 0.5 ml of solution D (4 M guanidinium thiocyanate, 25 mM tri-sodium citrate, pH7.0, 0.5 % N-lauroylsarcosine, 0.1 M 2-mercapto-ethanol).

All work after this stage was carried out by a colleague, Epaminondas Doxakis, and hence the methods used are described only briefly. Total RNA was extracted from the cells, purified and treated with DNAase. Total RNA was reverse transcribed using Superscript enzyme in the presence of dNTPs, random hexanucleotides, and specific competitor cRNAs. An aliquot of this reverse transcription reaction was amplified in a PCR reaction containing dNTPs, Taq polymerase and  $^{32}\text{P}$  5' end-labelled primers. Forward and reverse assay primers, specific for each cDNA of interest, were used and the

cycling conditions were adjusted as appropriate. The RT-PCR products were run on non-denaturing polyacrylamide gels that were subsequently dried and autoradiographed. The intensity of the autoradiograph bands resulting from RT-PCR amplification of mRNAs and competitor cRNAs was analysed using a laser densitometer, enabling the amount of GFR $\alpha$ 1, GFR $\alpha$ 2, Ret or L27 mRNAs in each total RNA sample to be calculated. Specific details of the competitors, primers and reaction conditions used can be found elsewhere (Forgie *et al.*, 1999).

## 2.3. Results

### 2.3.1. Survival responses of chicken parasympathetic neurons to the GDNF ligand family

The ciliary ganglion is comprised of parasympathetic neurons innervating the iris, ciliary body and choroid. CNTF (Barbin *et al.*, 1984), GPA (Buj-Bello and Davies, unpublished data) and GDNF (Buj-Bello *et al.*, 1995) promote the survival of these neurons in culture, however they are not supported, *in vitro*, by the neurotrophins (Allsopp *et al.*, 1993). The survival responses of these parasympathetic neurons to other members of the GDNF ligand family were investigated by establishing low-density, glial-free cultures of ciliary ganglion neurons from E6, E8, E10 and E12 chicken embryos. As expected, the majority of neurons survived for 48 hours in saturating concentrations of CNTF (10 ng/ml) whereas all neurons grown in the absence of neurotrophic factors (control) died. Neurturin, like GDNF, was found to support the survival of the majority of neurons when present at a concentration of 10 ng/ml. Both persephin and artemin, on the other hand, failed to support the survival of these neurons in culture, even at concentrations of up to 50 ng/ml. There were little or no additive effects of combining GDNF plus neurturin or either of these factors plus CNTF, indicating that the subsets of ciliary neurons responding to GDNF, neurturin and CNTF are largely overlapping. Figure 2.11. shows the results from E8 ciliary neurons. Similar results were demonstrated at E6, E10 and E12 (data not shown).

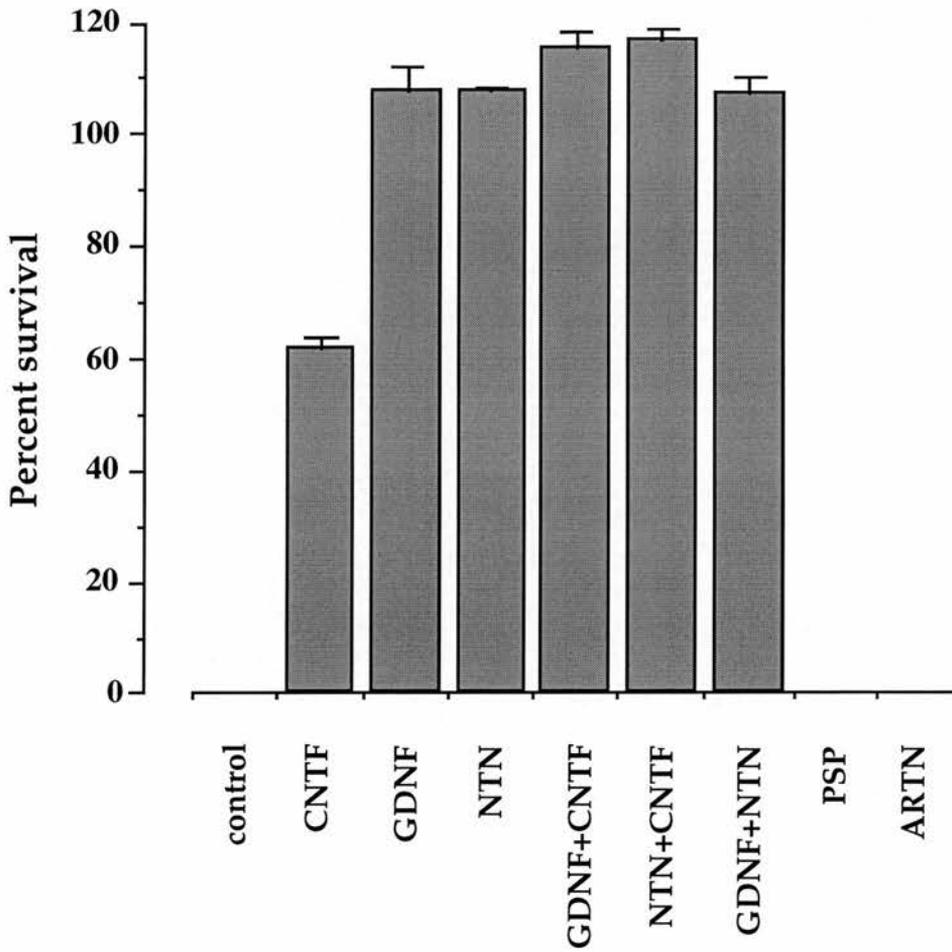
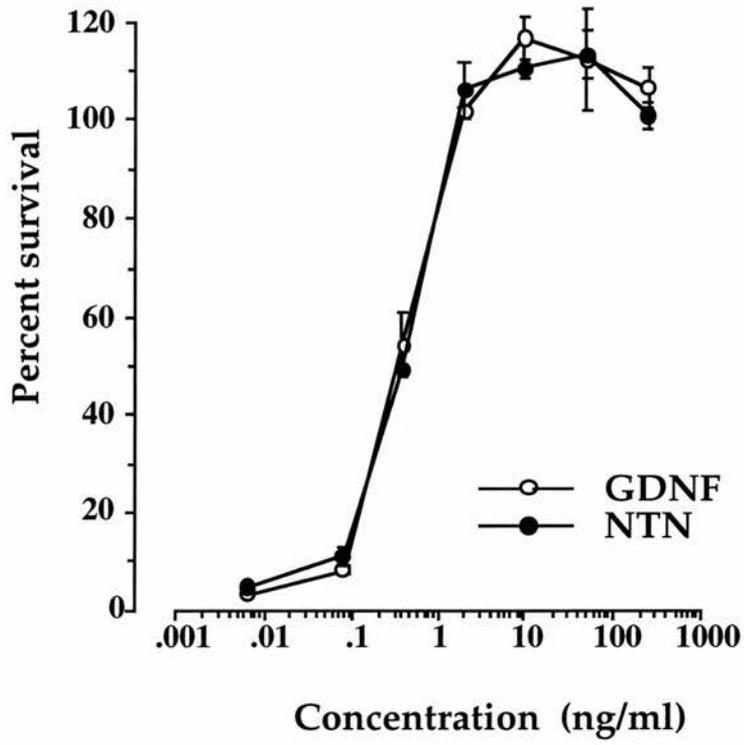


Figure 2.11. Bar chart showing percent survival of E8 ciliary neurons cultured for 48 hours with members of the GDNF ligand family.

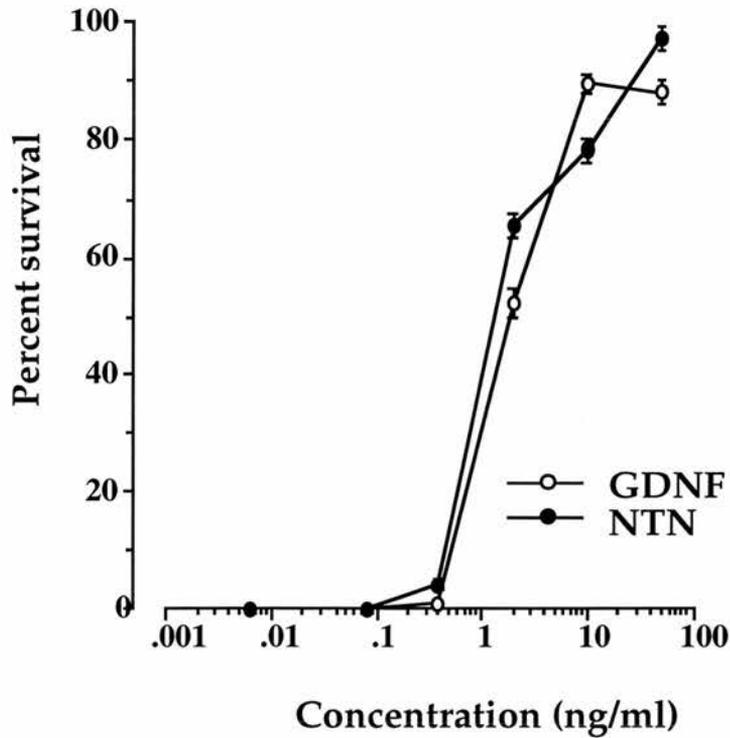
The number of neurons surviving after 48 hours incubation is expressed as a percentage of the number of attached neurons counted 3 hours after plating. CNTF was used at a saturating concentration of 10 ng/ml. GDNF, NTN and ARTN were also present at 10 ng/ml and PSP at 50 ng/ml. The mean  $\pm$  standard error is shown ( $n \geq 3$  for each condition).

To compare more fully the survival responses of parasympathetic neurons to GDNF and neurturin, low-density, glial-free cultures of ciliary ganglion neurons were established at E6, E8, E10 and E12 and a dose response assay performed with concentrations of each factor ranging from 0.0064 ng/ml to 250 ng/ml. Saturating concentrations of GDNF and neurturin promoted the survival of similar numbers of neurons at each stage of development studied, whereas virtually all the neurons died within 24 hours of plating in the absence of neurotrophic factors (Figure 2.12. A-D). Almost 100 % of E6, E8 and E10 ciliary neurons survived in saturating concentrations of GDNF and neurturin, however by E12, the maximal percent survival had fallen to between 30 and 40 % with each factor. Although saturating concentrations of both factors promoted the survival of similar numbers of neurons at each age, the dose response analysis revealed a marked difference in responsiveness to these two factors at later ages brought about by a developmental shift in the GDNF dose response to higher concentrations as the age of the neurons increased. This phenomenon is best demonstrated by calculating, by interpolation, the concentrations of each factor required to promote half maximal survival at each age, i.e. the EC<sub>50</sub> value (Figure 2.12. E). At E6 and E8, GDNF and neurturin were equally potent in promoting the survival of ciliary neurons. However, between E6 and E12 there was a 12-fold increase in the concentration of GDNF required to promote half maximal survival; EC<sub>50</sub> for GDNF was 1.25 ng/ml in E6 cultures and 15.41 ng/ml in E12 cultures ( $p=0.05$ , Student's t-test). The EC<sub>50</sub> for neurturin remained essentially unchanged throughout this period of development with an average value of 1.08 ng/ml. Thus, whereas ciliary neurons remained highly responsive to neurturin throughout this period of development, they rapidly lost responsiveness to GDNF.

**A: E6 ciliary ganglion neurons**

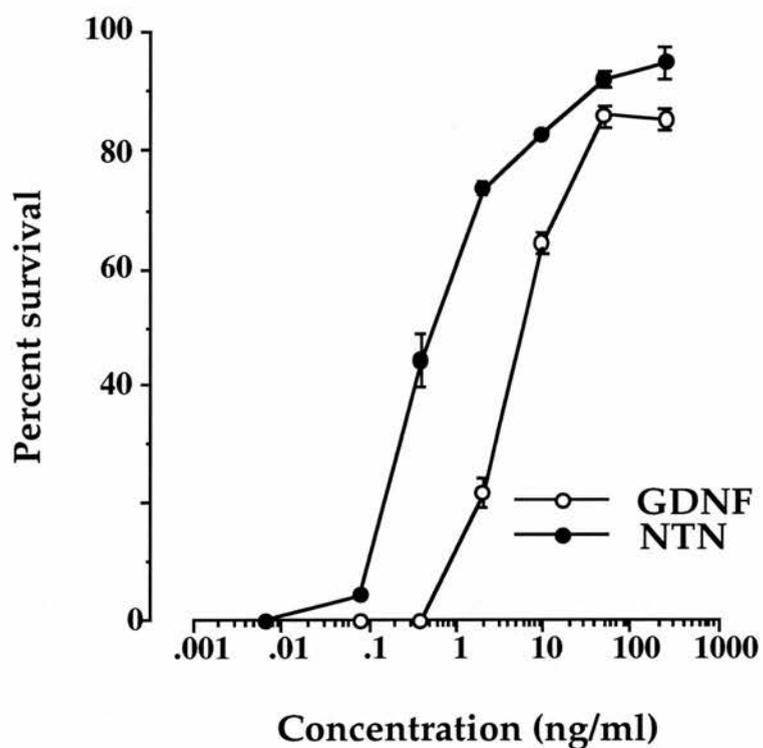


**B: E8 ciliary ganglion neurons**

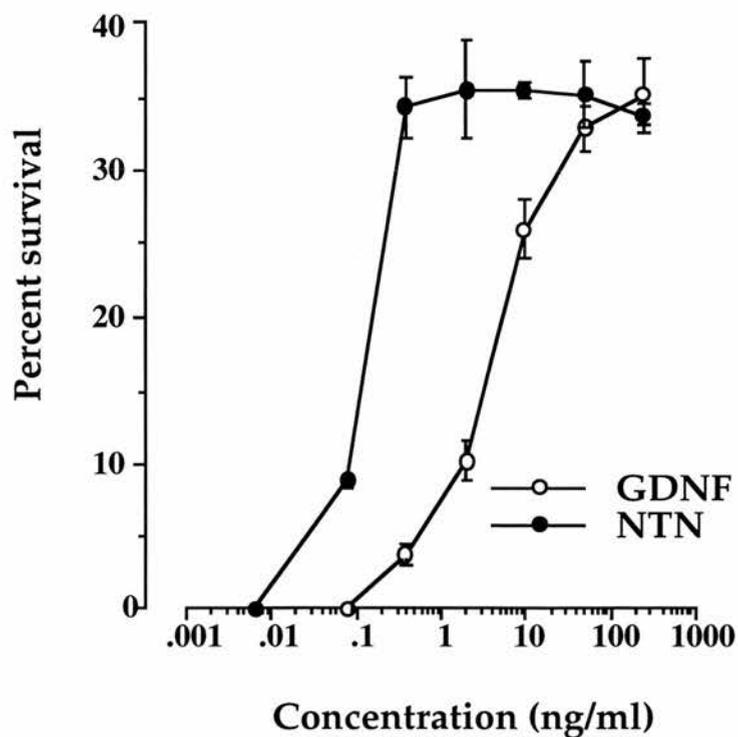


(Figure 2.12.)

C: E10 ciliary ganglion neurons



D: E12 ciliary ganglion neurons



(Figure 2.12.)

### E: EC<sub>50</sub> values

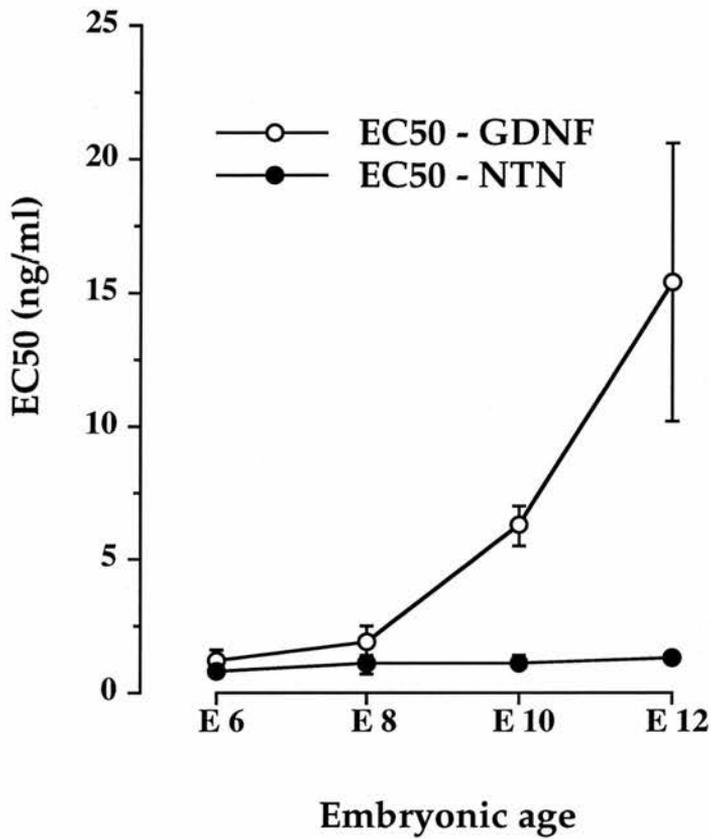


Figure 2.12. Graphs of the percent survival of E6 (A), E8 (B), E10 (C) and E12 (D) ciliary ganglion neurons cultured for 48 hours in a range of concentrations of GDNF and neurturin and their corresponding EC<sub>50</sub> values (E).

(A-D) The number of neurons surviving after 48 hours incubation is expressed as a percentage of the number of attached neurons counted 3 hours after plating. GDNF and NTN were used at concentrations ranging from 0.0064 ng/ml to 250 ng/ml. The dose responses shown are taken from representative experiments set up in triplicate at E6, E8, E10 and E12. The mean  $\pm$  standard error is shown. (E) The EC<sub>50</sub> values are derived from 3 or 4 separate experiments at each age (mean  $\pm$  standard error shown).

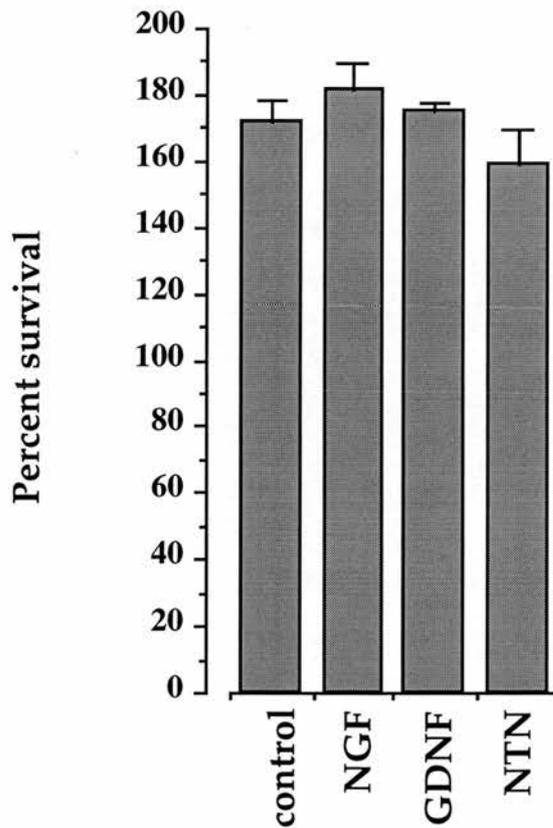
### 2.3.2. Survival responses of chicken sympathetic neurons to the GDNF ligand family

Sympathetic neurons of the embryonic paravertebral sympathetic chain are supported by NGF (Levi-Montalcini and Angeletti, 1968; Chun and Patterson, 1977), CNTF (Barbin *et al.*, 1984) and GDNF (Buj-Bello *et al.*, 1995) in culture. To determine if these neurons are also responsive to other members of the GDNF ligand family, low-density, glial-free cultures of neurons from the lumbar paravertebral chain of E8, E10, E12, E14 and E16 chicken embryos were established. The number of neurons surviving for 48 hours in E8 control cultures was high: 160 % survival in control dishes and dishes supplemented with either 10 ng/ml NGF or 50 ng/ml GDNF or neurturin (Figure 2.13. A). Even after 96 hours in culture, control survival was still at least 60 % (data not shown). The neurotrophin-independent survival observed at E8 is in agreement with previous findings (Ernsberger *et al.*, 1989). The increase in the total number of neurons in E8 cultures after 48 hours incubation reflects proliferation of early sympathetic neurons (Rohrer and Thoenen, 1987; Rothman *et al.*, 1978). By E10, and in older control cultures, the number of surviving neurons decreased markedly and the effects of neurotrophic factors on neuronal survival became more apparent. Hence, further experiments focussed on E10 and older embryos.

The majority of E10 sympathetic neurons survived for 48 hours in saturating concentrations of NGF (10 ng/ml). In agreement with previous studies, over 90 % of the neurons survived in the presence of GDNF (50 ng/ml) and a similar number were found to respond to neurturin at the same concentration. There were no additive effects of growing the neurons with GDNF or neurturin in combination with NGF. This suggests that the populations of sympathetic neurons responding to GDNF or neurturin and NGF are largely overlapping. Later in embryonic development, some

additive effects were apparent, indicating that by this stage (E16) these populations of neurons may be partially distinct (data not shown). When E10 sympathetic neurons were grown in the presence of GDNF and neurturin together, there was negligible additional survival, suggesting that the subsets of sympathetic neurons that respond to GDNF and neurturin are largely overlapping. Persephin and artemin did not promote survival of sympathetic neurons above control levels. Figure 2.13. B shows the responses of E10 sympathetic neurons to the GDNF family. Similar results were obtained at later stages in development (data not shown).

### A: E8 sympathetic neurons



(Figure 2.13.)

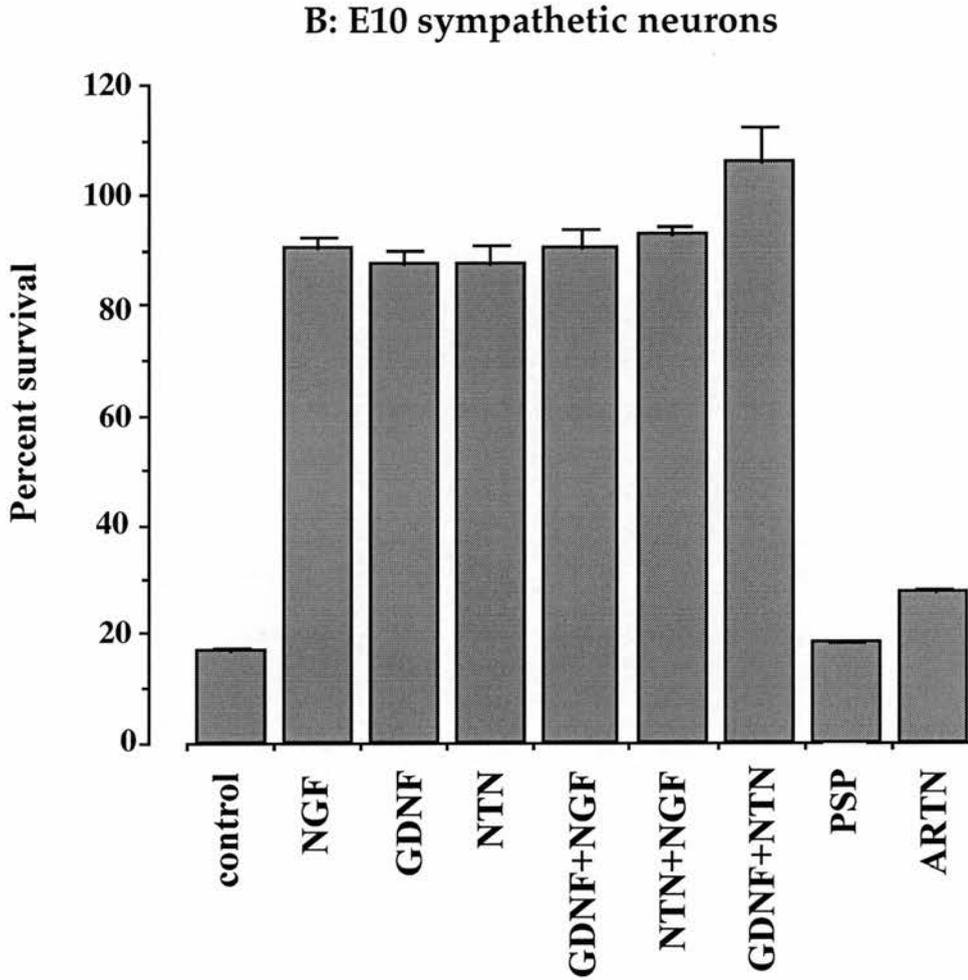


Figure 2.13. Bar charts showing percent survival of E8 (A) and E10 (B) paravertebral sympathetic neurons cultured for 48 hours with members of the GDNF ligand family.

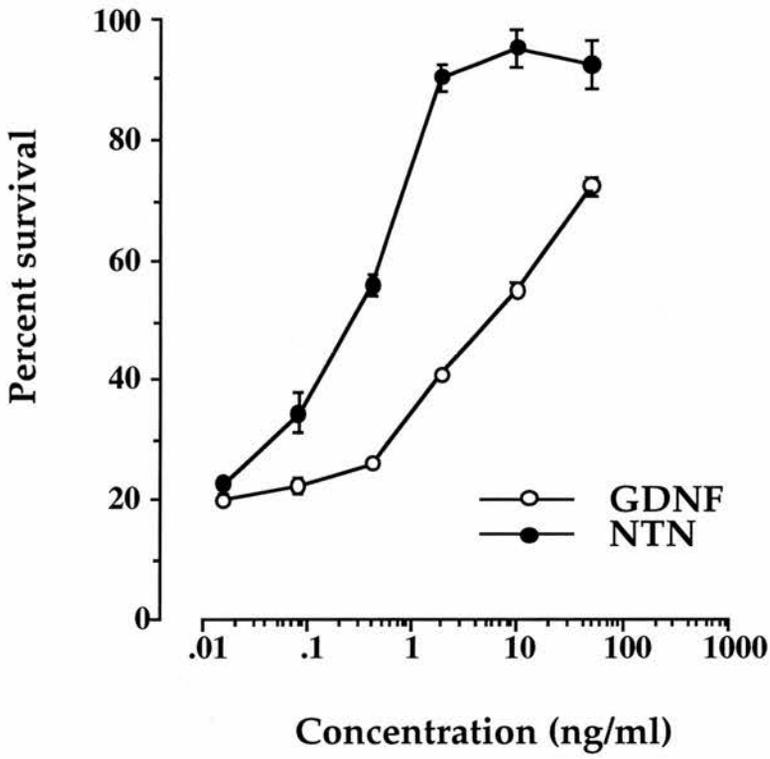
The number of neurons surviving after 48 hours incubation is expressed as a percentage of the number of attached neurons counted 3 hours after plating. NGF was used at a saturating concentration of 10 ng/ml. GDNF, NTN and PSP were present at 50 ng/ml and ARTN at 10 ng/ml. The mean  $\pm$  standard error is shown ( $n \geq 3$  for each condition).

To compare the survival responses of sympathetic neurons to GDNF and neurturin in more detail, low-density, glial-free cultures of neurons from the lumbar paravertebral sympathetic chain of E10 to E16 chicken embryos were established and a dose response assay performed with concentrations of each factor ranging from 0.016 ng/ml to 250 ng/ml. Throughout this period of development, the majority of neurons were supported by saturating concentrations of either GDNF or neurturin, although an appreciable number of neurons survived for 48 hours incubation without added neurotrophic factors (between 10 and 30 %) (Figure 2.14. A-D). Like ciliary neurons, sympathetic neurons were more sensitive to neurturin than GDNF during a particular stage of their development. At E10 and E12, sympathetic neurons were more responsive to neurturin than GDNF: the EC<sub>50</sub> for neurturin being 5- to 7- fold lower than that for GDNF. However, whereas ciliary neurons became less responsive to GDNF with age, sympathetic neurons became more responsive to GDNF with age: the EC<sub>50</sub> decreased 3.5-fold from E10 to E14 ( $p=0.005$ , Student's t-test). By E14 and at E16, sympathetic neurons were equally responsive to both factors: there was no significant difference in the EC<sub>50</sub> values for GDNF and neurturin (Figure 2.14. E).

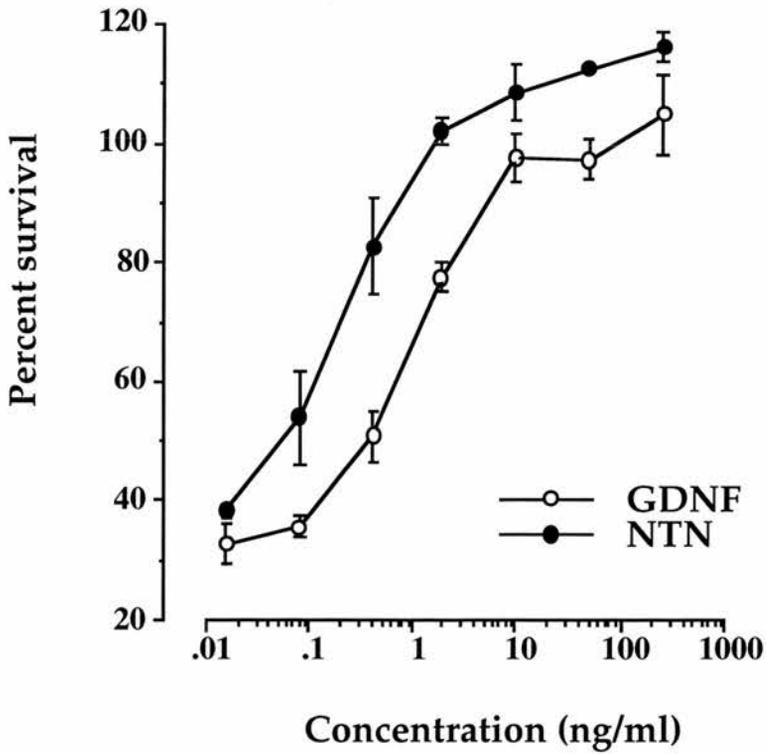
The survival responses of another population of sympathetic neurons, namely those of the superior cervical ganglion, to GDNF and neurturin were also examined in a limited number of experiments. SCG neurons are supported by NGF in culture (Levi-Montalcini and Angeletti, 1968; Chun and Patterson, 1977) and they provide postganglionic sympathetic innervation to cranial and cervical viscera, such as the iris and thyroid gland, as well as blood vessels and some of the upper airways. Saturating concentrations of NGF (10 ng/ml) supported the majority of E10 and E12 SCG neurons after 48 hours in culture whereas the survival in control cultures was minimal (<10 %). Like sympathetic neurons of the paravertebral chain, SCG neurons

responded to both GDNF and neurturin, but not to persephin, and like their more caudal counterparts were more sensitive to neurturin than GDNF at E10 (EC<sub>50</sub> for neurturin, 0.62 ng/ml and for GDNF, 8.25 ng/ml) and E12 (EC<sub>50</sub> for neurturin, 0.22 ng/ml and for GDNF, 6.62 ng/ml). Results from combinatorial experiments demonstrated that the subpopulations of SCG neurons responding to NGF, GDNF and neurturin were largely overlapping (data not shown).

**A: E10 sympathetic neurons**

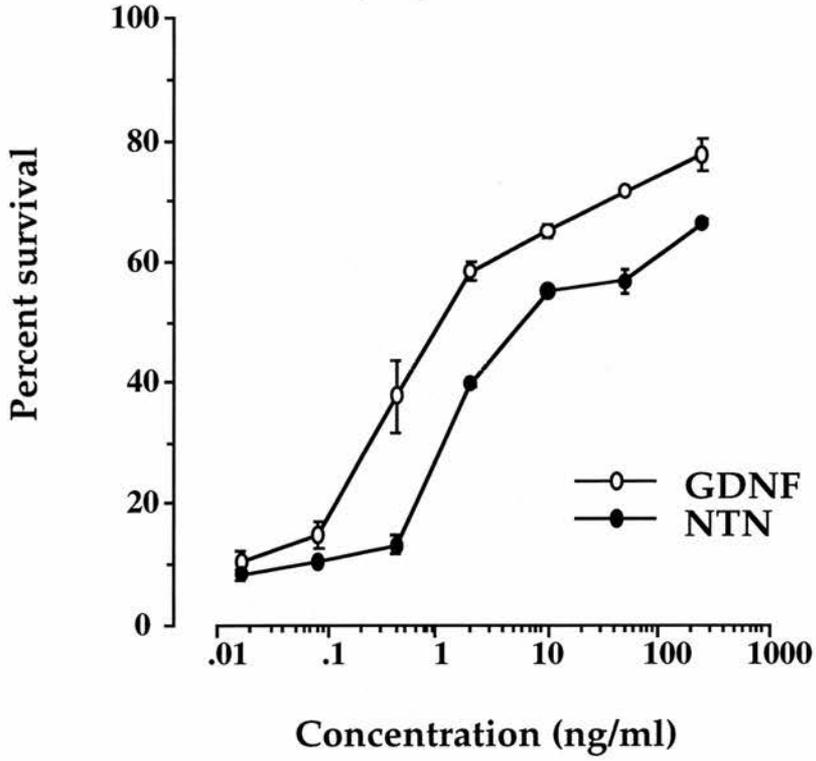


**B: E12 sympathetic neurons**

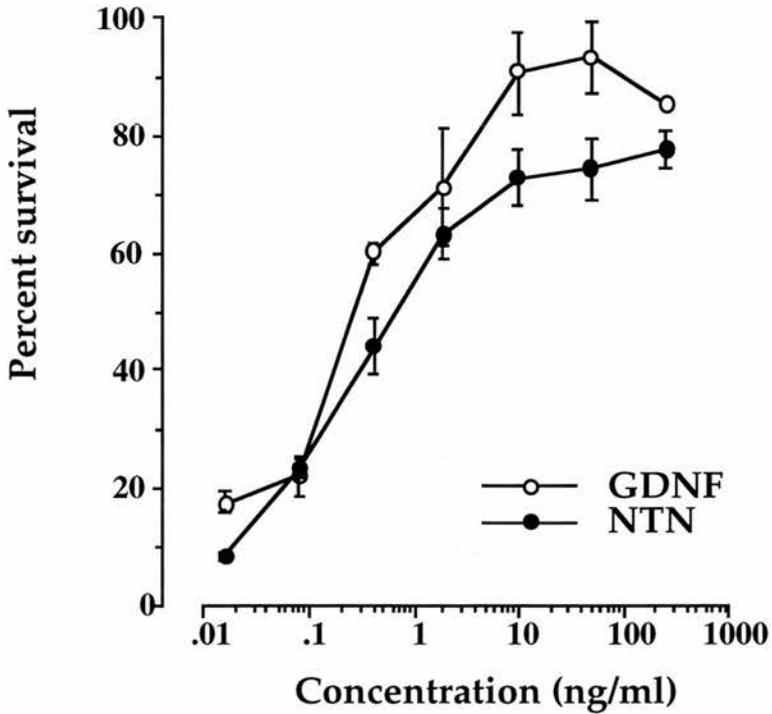


(Figure 2.14.)

C: E14 sympathetic neurons



D: E16 sympathetic neurons



(Figure 2.14.)

### E: EC<sub>50</sub> values

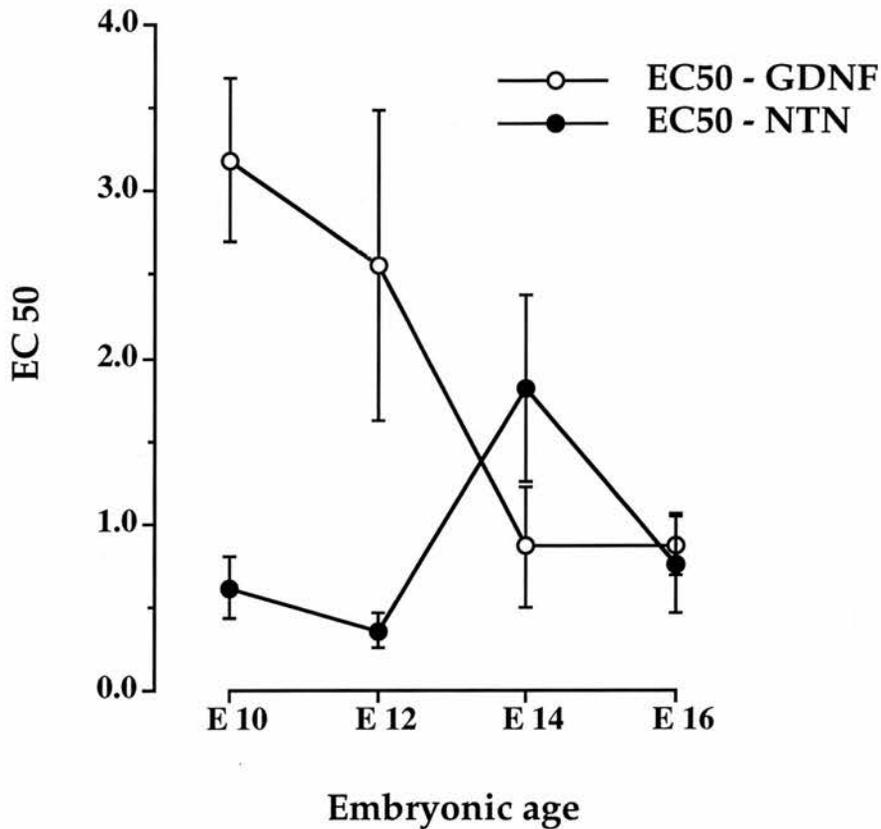


Figure 2.14. Graphs of the percent survival of E10 (A), E12 (B), E14 (C) and E16 (D) paravertebral sympathetic neurons cultured for 48 hours in a range of concentrations of GDNF and neurturin and their corresponding EC<sub>50</sub> values (E).

(A-D) The number of neurons surviving after 48 hours incubation is expressed as a percentage of the number of attached neurons counted 3 hours after plating. GDNF and NTN were used at concentrations ranging from 0.016 ng/ml to 250 ng/ml. The dose responses are taken from representative experiments set up in triplicate at E10, E12, E14 and E16. The mean  $\pm$  standard error is shown. (E) The EC<sub>50</sub> values are derived from 4 to 6 separate experiments at each age (mean  $\pm$  standard error shown).

### 2.3.3. Survival responses of chicken enteroceptive neurons to the GDNF ligand family

The enteroceptive neurons of the chicken nodose ganglion innervate thoracic and abdominal viscera. A large subset of these sensory neurons respond to BDNF in culture (Lindsay *et al.*, 1985; Davies *et al.*, 1986) and subsets of these neurons are known to respond to NT3 (Buj-Bello *et al.*, 1994), CNTF/GPA (Buj-Bello and Davies, unpublished data) and GDNF (Buj-Bello *et al.*, 1995). To determine if these neurons also respond to other members of the GDNF ligand family, low-density, glial-free cultures of nodose ganglion neurons were established from E8, E10 and E12 chicken embryos. The majority of E8 nodose neurons survived for 48 hours in saturating concentrations of BDNF (10 ng/ml), whilst in control cultures, there was negligible survival at this age. In agreement with previous studies, around 60 % of nodose ganglion neurons survived for 48 hours in the presence of GDNF (50 ng/ml) and a similar response was seen to 50 ng/ml neurturin. Additive effects of growing these neurons with GDNF and neurturin in combination were minimal, indicating that the subsets of nodose neurons that respond to GDNF and neurturin are largely overlapping. This was not the case when these neurons were exposed to combinations of either GDNF and BDNF or neurturin and BDNF. Rather, partial additive effects were evident, suggesting that the populations of neurons responding to GDNF and neurturin, and BDNF are partially distinct. Persephin and artemin had no survival-promoting effects on these enteroceptive neurons. Figure 2.15. shows the responses of E8 nodose neurons to the GDNF ligand family. Similar data was obtained at E10 and E12 (data not shown).

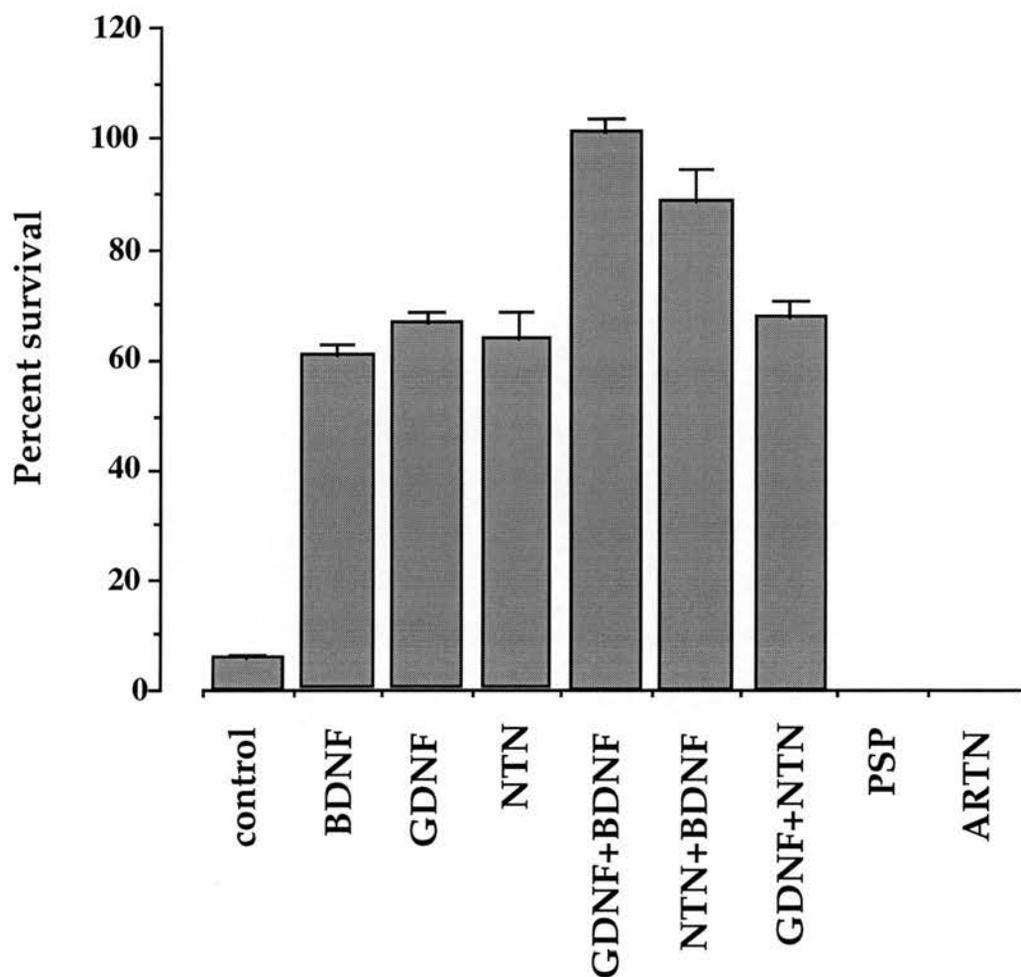
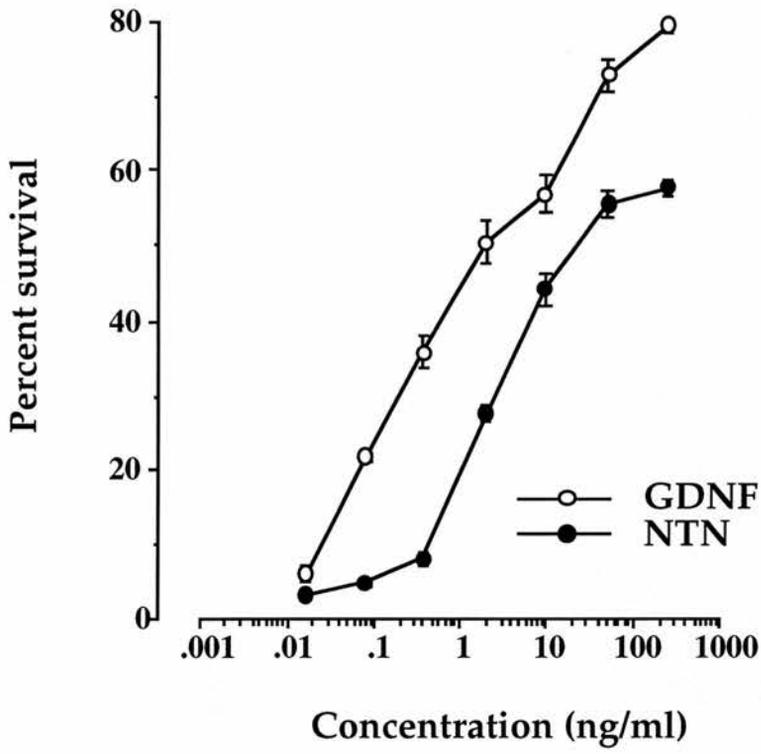


Figure 2.15. Bar chart showing percent survival of E8 nodose neurons cultured for 48 hours with members of the GDNF ligand family.

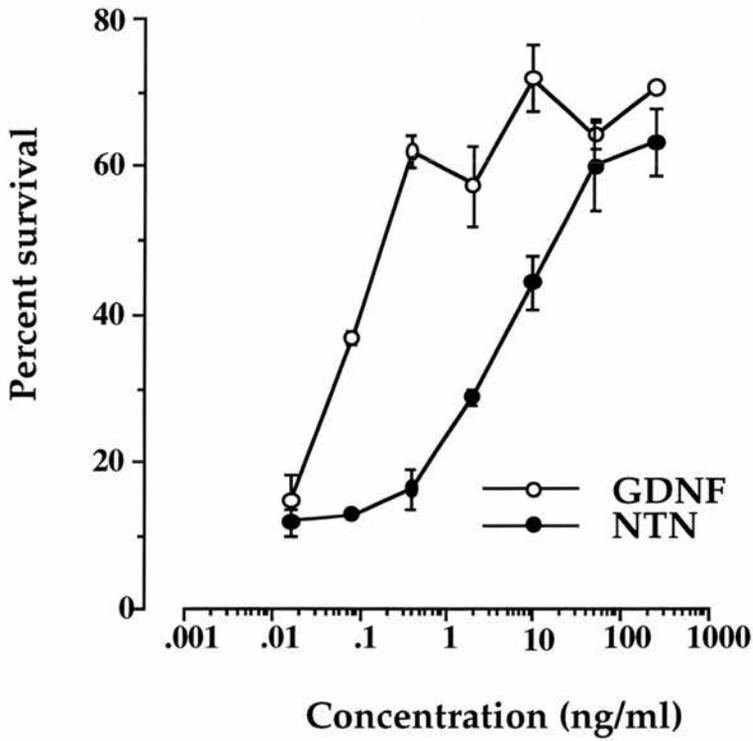
The number of neurons surviving after 48 hours incubation is expressed as a percentage of the number of attached neurons counted 3 hours after plating. BDNF was used at a saturating concentration of 10 ng/ml. GDNF, NTN and PSP were present at 50 ng/ml and ARTN at 10 ng/ml. The mean  $\pm$  standard error is shown ( $n \geq 3$  for each condition).

To compare the survival responses of enteroceptive neurons to GDNF and neurturin in more detail, low-density, glial-free cultures of nodose ganglion neurons were established and dose response assays were performed with concentrations of each factor ranging from 0.0064 ng/ml to 250 ng/ml. Although saturating concentrations of GDNF and neurturin promoted the survival of similar numbers of neurons at each stage of development, the dose response analysis revealed striking differences in the biological activities of these two factors (Figure 2.16. A-D). In contrast to parasympathetic and sympathetic neurons, nodose neurons were far more sensitive to GDNF than neurturin at all ages studied, and became increasingly responsive to GDNF from E8 to E12. After 48 hours incubation, the EC<sub>50</sub> for GDNF decreased 6-fold from E8 to E12, i.e. from 0.84 ng/ml at E8 to 0.07 ng/ml at E12. The EC<sub>50</sub> for neurturin was significantly greater than for GDNF at each age, being 8-fold and 60-fold higher than the EC<sub>50</sub> for GDNF at E8 and E12, respectively ( $p=0.0079$  and  $p=0.0041$  at E8 and E12, respectively, Student's t-test). This is depicted in Figure 2.16. E.

**A: E8 nodose ganglion neurons**

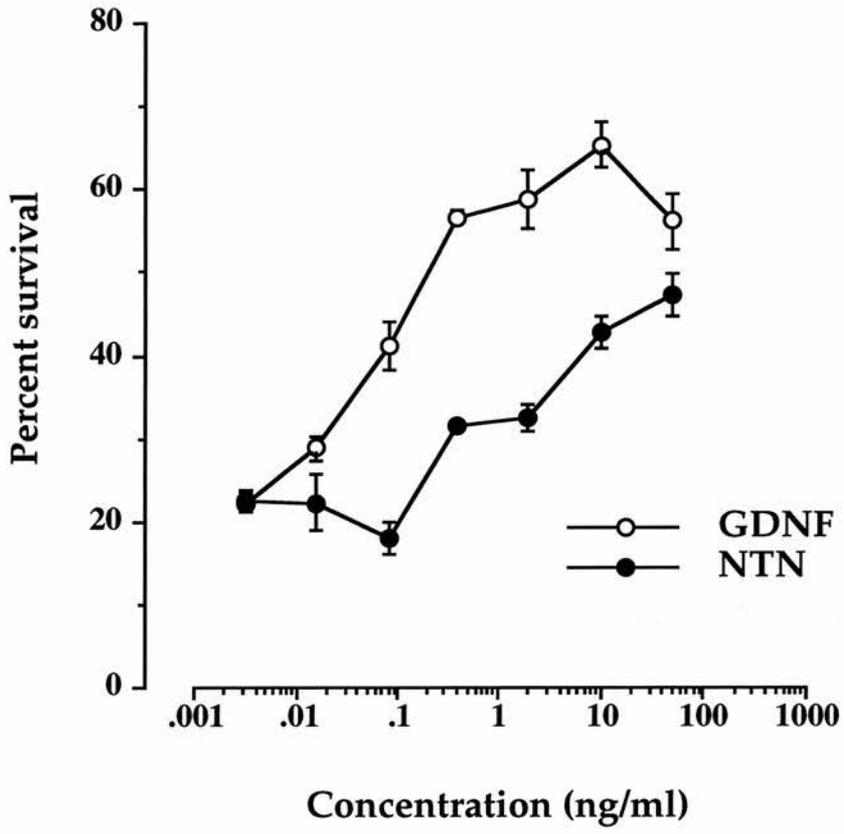


**B: E10 nodose ganglion neurons**



(Figure 2.16.)

C: E12 nodose ganglion neurons



(Figure 2.16.)

#### D: EC<sub>50</sub> values

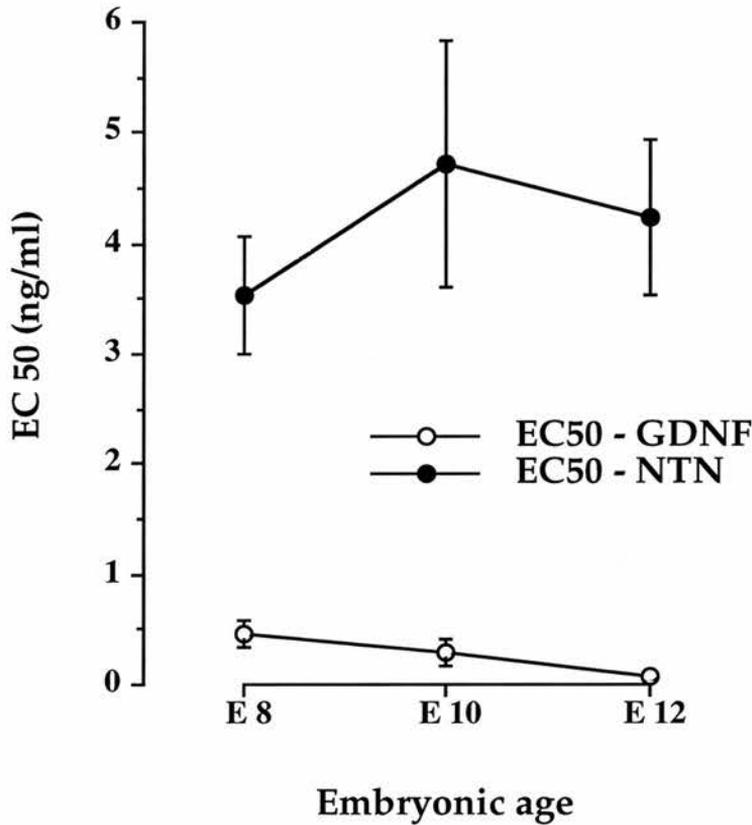


Figure 2.16. Graphs of the percent survival of E8 (A), E10 (B) and E12 (C) nodose ganglion neurons cultured for 48 hours in a range of concentrations of GDNF and neurturin and their corresponding EC<sub>50</sub> values (D).

(A-C) The number of neurons surviving after 48 hours incubation is expressed as a percentage of the number of attached neurons counted 3 hours after plating. GDNF and NTN were used at concentrations ranging from 0.0064 ng/ml to 250 ng/ml. The dose responses are taken from representative experiments set up in triplicate at E8, E10 and E12. The mean  $\pm$  standard error is shown. (D) The EC<sub>50</sub> values are derived from 3 to 5 separate experiments at each age (mean  $\pm$  standard error shown).

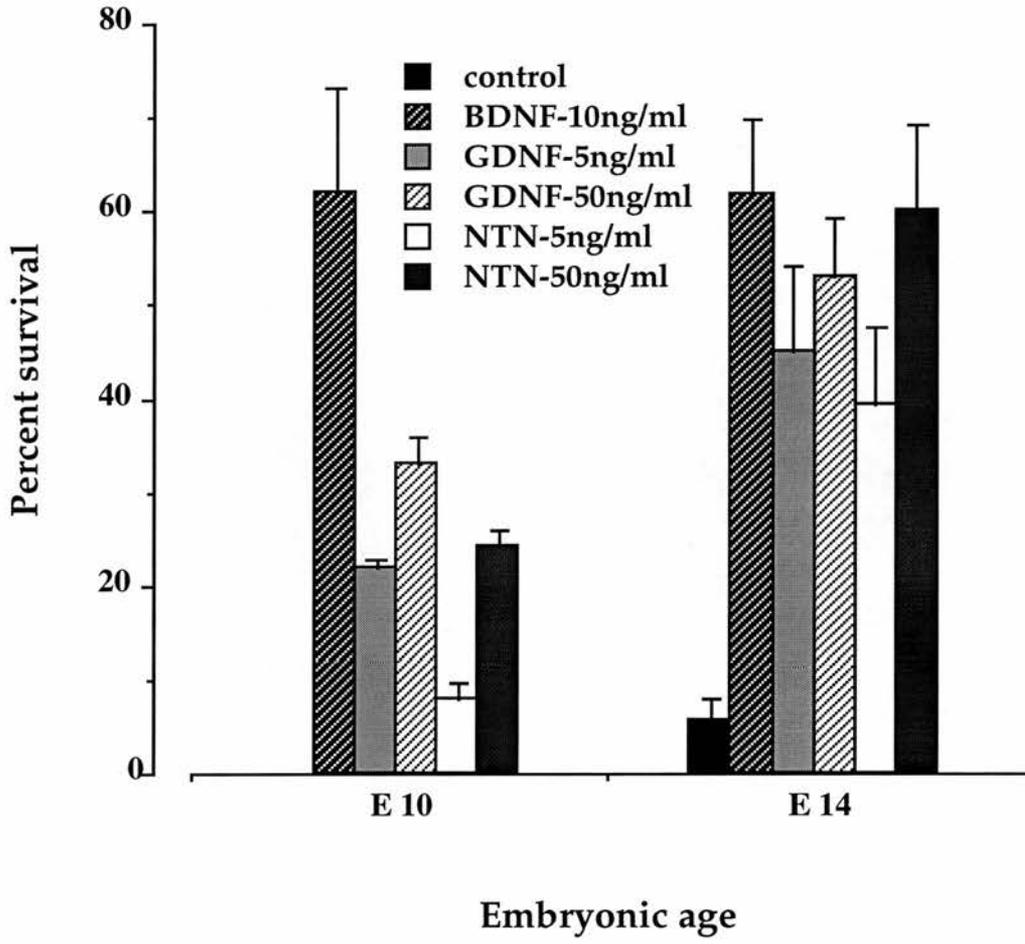
#### 2.3.4. Survival responses of chicken cutaneous sensory neurons to the GDNF ligand family

The embryonic chicken trigeminal ganglion comprises two anatomically segregated populations of cutaneous sensory neurons which innervate the facial region. Those in the ventrolateral portion of the ganglion (VLTG neurons) are placode-derived and respond to BDNF (Davies *et al.*, 1986), whereas those in the dorsomedial portion of the ganglion (DMTG neurons) are neural crest-derived and respond to NGF (Buj-Bello *et al.*, 1994). Both populations respond to GDNF, however, this is only evident at later stages in their development (Buj-Bello *et al.*, 1995). To determine if these neurons are also responsive to other members of the GDNF ligand family, low-density, glial-free cultures of VLTG and DMTG neurons were established from E10, E12 and E14 chicken embryos. After 48 hours incubation, virtually all the neurons in control cultures had died, whereas the majority of VLTG neurons were supported by BDNF and the majority of DMTG neurons by NGF at all ages studied. In agreement with previous studies, there was a marked increase in the number of VLTG and DMTG neurons responding to GDNF from E10 to E14. Such an increase was also evident in the responsiveness of these neuronal populations to neurturin such that by E14, similar numbers of VLTG neurons were supported by either neurturin or BDNF and similar numbers of DMTG neurons were supported by either neurturin or NGF (Figure 2.17. A&B). Hence, BDNF-responsive VLTG neurons and NGF-responsive DMTG neurons acquire GDNF and neurturin responsiveness as they mature. In VLTG neuronal cultures, there was negligible additional neuronal survival when the neurons were grown with either GDNF or neurturin in combination with BDNF (Figure 2.18. A). However, when DMTG neurons were grown in the presence of GDNF or neurturin with NGF, there was a considerable increase in survival compared to cultures containing the most effective neurotrophic factor alone (Figure 2.18. B). This

demonstrates that the populations of DMTG neurons responding to NGF and GDNF or neurturin are partially distinct. There were no additive effects of growing these neurons with GDNF and neurturin in combination, indicating that the subsets of neurons responding to GDNF and neurturin are largely overlapping (Figure 2.19., data for VLTG not shown). A detailed dose response investigation of the effects of GDNF and neurturin on chicken trigeminal neurons was not undertaken. However, from the analysis of the limited number of concentrations used, no striking differences in the potency of each factor were apparent. Persephin and artemin had no survival-promoting effects on these cutaneous sensory neurons (Figure 2.19.).

The survival responses of vestibular neurons to GDNF and neurturin were also examined in a limited number of experiments. Vestibular neurons innervate the sensory epithelium of the inner ear and are supported by BDNF and NT-3 (Davies *et al.*, 1986; Avila *et al.*, 1993; Pirvola *et al.*, 1994) in culture. Saturating concentrations of BDNF (10 ng/ml) supported the survival of the majority of vestibular neurons (E8-E12) after 48 hours in culture, whilst there were no cells remaining in control cultures. Vestibular neurons, like trigeminal sensory neurons, showed no responsiveness to GDNF or neurturin at E8 and E10. At E12, a very limited survival response to GDNF and neurturin (<10 %) was observed (data not shown). It is possible that these neurons may acquire greater responsiveness to GDNF and neurturin at later developmental stages, however, this was not investigated in this study.

A: VLTG neurons



(Figure 2.17.)

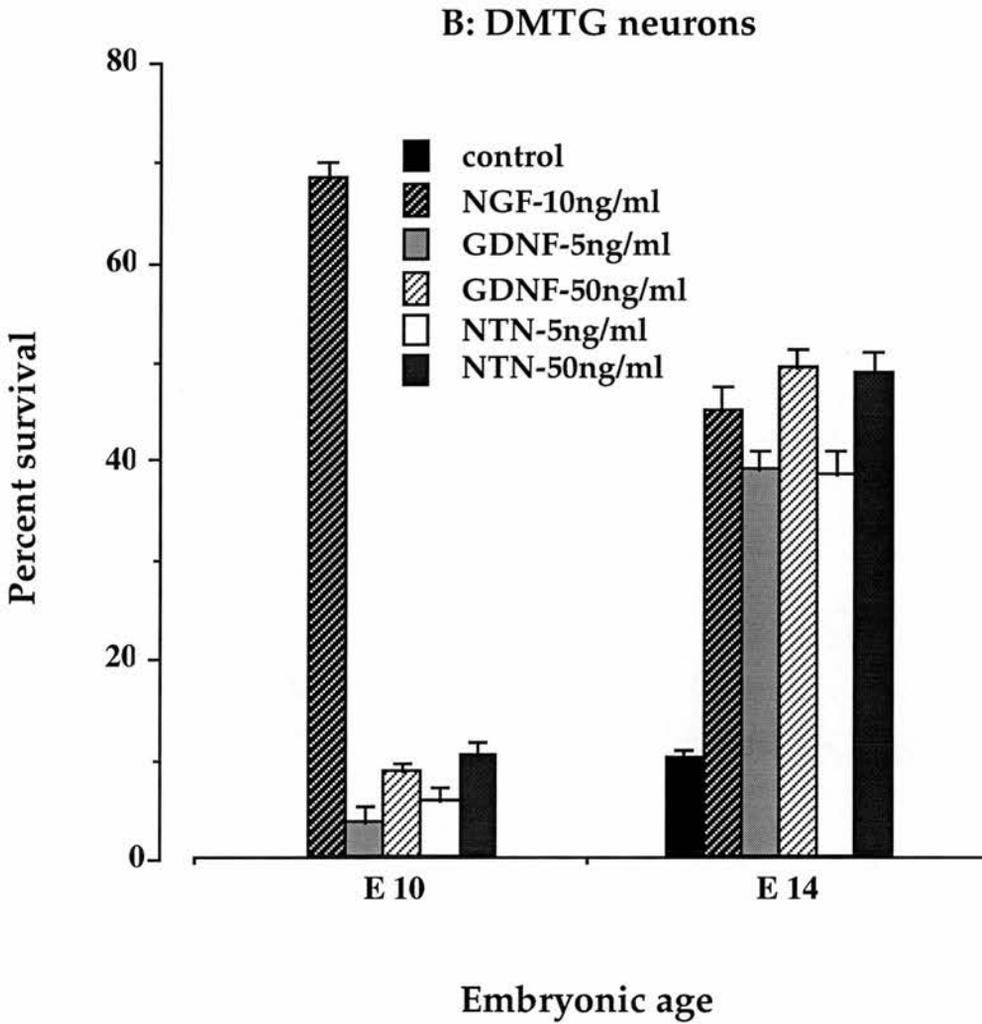


Figure 2.17. Bar chart comparing percent survival of E10 and E14 VLTG (A) and DMTG (B) neurons in control cultures and in cultures containing GDNF and neurturin.

The number of neurons surviving after 48 hours incubation is expressed as a percentage of the number of attached neurons counted 3 hours after plating. BDNF and NGF were used at a saturating concentration of 10 ng/ml. GDNF and NTN were present at either 5 or 50 ng/ml as indicated. The mean  $\pm$  standard error is shown ( $n \geq 3$  for each condition).

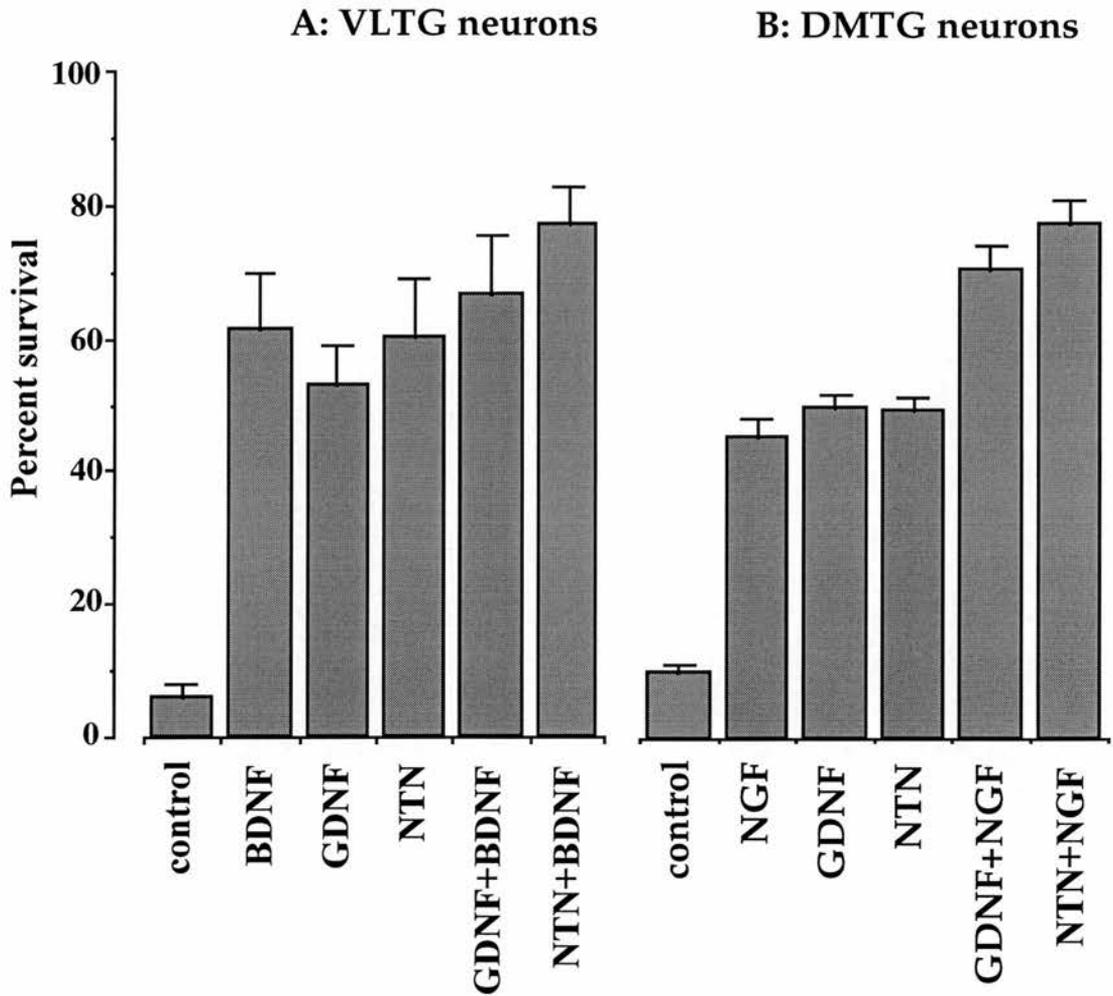


Figure 2.18. Bar charts comparing percent survival of E14 VLTG (A) and DMTG (B) neurons in control cultures and in cultures containing BDNF (A), NGF (B), GDNF and NTN and these factors in combination.

The number of neurons surviving after 48 hours incubation is expressed as a percentage of the number of attached neurons counted 3 hours after plating. BDNF and NGF was used at saturating concentrations of 10 ng/ml. GDNF and NTN were present at 50 ng/ml. The mean  $\pm$  standard error is shown ( $n \geq 3$  for each condition).

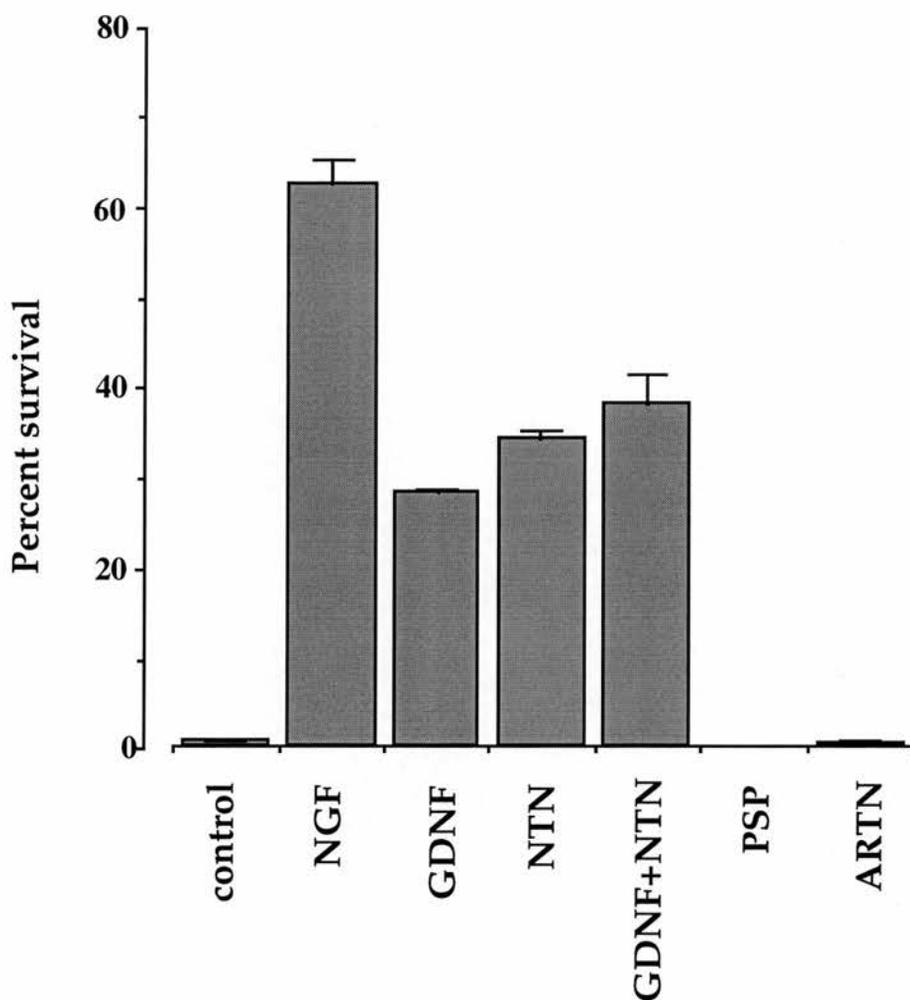
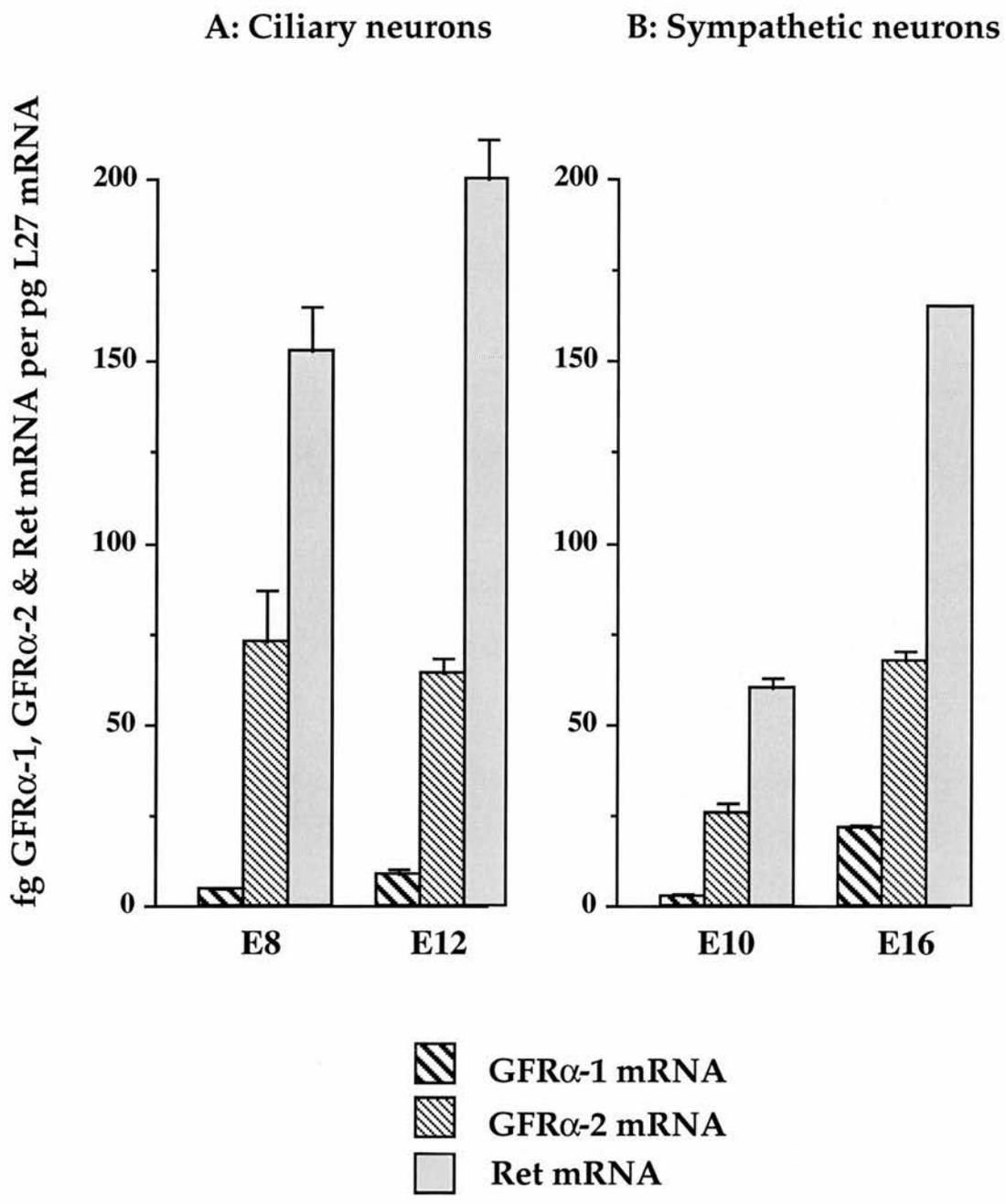


Figure 2.19. Bar chart comparing percent survival of E12 DMTG neurons in control cultures and cultures containing NGF and members of the GDNF ligand family.

The number of neurons surviving after 48 hours incubation is expressed as a percentage of the number of attached neurons counted 3 hours after plating. NGF was used at a saturating concentration of 10 ng/ml. GDNF, NTN and ARTN were also present at 10 ng/ml and PSP at 50 ng/ml. The mean  $\pm$  standard error is shown ( $n \geq 3$  for each condition).

### 2.3.5. Expression of Ret, GFR $\alpha$ 1 and GFR $\alpha$ 2 mRNA levels in purified neurons

To determine the relative levels of transcripts encoding components of the GDNF and neurturin receptor complexes in developing parasympathetic, sympathetic and sensory neurons, quantitative RT-PCR was used to measure the levels of Ret, GFR $\alpha$ 1 and GFR $\alpha$ 2 mRNAs in total RNA extracted from >95 % pure preparations of these neurons. To compare the relative levels of these transcripts in different RNA samples, quantitative RT-PCR was also used to measure the level of mRNA encoding the L27 ribosomal protein, a ubiquitous, constitutively expressed protein. Figure 2.20 (A-C) shows the levels of Ret, GFR $\alpha$ 1 and GFR $\alpha$ 2 mRNAs relative to L27 mRNA in these neurons at several different stages of development. At each age studied, all neurons expressed relatively high levels of Ret mRNA which is consistent with the demonstration that the Ret receptor tyrosine kinase is the common signalling receptor for both GDNF and neurturin. In contrast, there were marked differences in the relative levels of GFR $\alpha$ 1 and GFR $\alpha$ 2 mRNAs in these purified neuronal preparations. Whereas the levels of GFR $\alpha$ 2 mRNA were much higher than those of GFR $\alpha$ 1 mRNA in parasympathetic and sympathetic neurons, corresponding with the greater sensitivity of these neurons to neurturin, the levels of GFR $\alpha$ 1 mRNA were higher than GFR $\alpha$ 2 mRNA in nodose neurons, corresponding with the greater sensitivity of these neurons to GDNF. Nevertheless, the changes in expression of receptor mRNAs between early and late embryonic ages did not always reflect the changing responsiveness of the neurons to the respective ligands. Most notably, ciliary neurons lose responsiveness to GDNF with increasing age, however, the levels of GFR $\alpha$ 1 expression did not decrease from E8 to E12, in fact an increase in GFR $\alpha$ 1 mRNA expression was detected.



(Figure 2.20.)

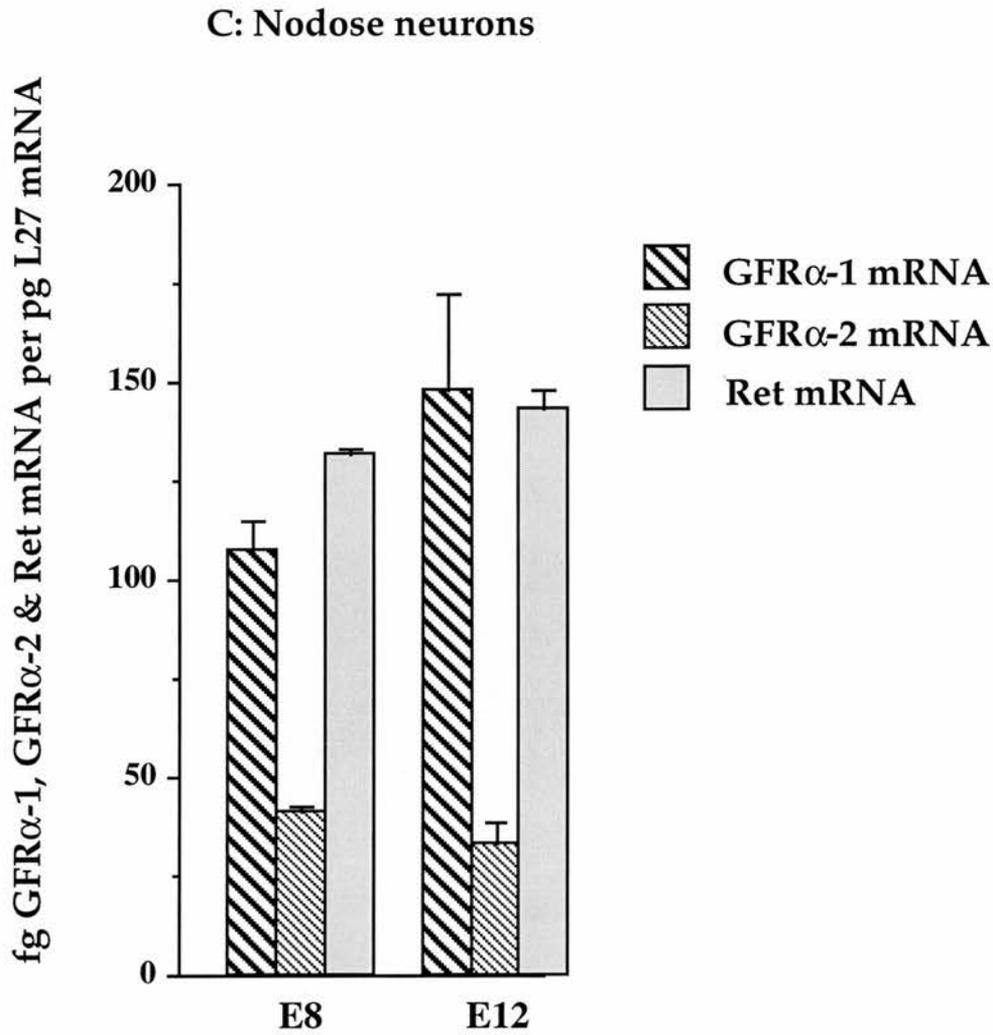


Figure 2.20. Bar charts indicating the levels of GFR $\alpha$ 1, GFR $\alpha$ 2 and Ret mRNAs relative to L27 mRNA in total RNA extracted from purified ciliary (A), sympathetic (B) and nodose (C) ganglion neurons.

The levels of GFR $\alpha$ 1, GFR $\alpha$ 2 and Ret mRNAs are expressed in femptograms per picogram of L27 mRNA in total RNA extracted from purified parasympathetic, sympathetic and enteroceptive neurons of chicken embryos at the ages indicated. The mean  $\pm$  standard error of 3 separate assays are represented by each bar.

## 2.4. Discussion

This study has investigated the ability of members of the GDNF ligand family to promote the *in vitro* survival of populations of embryonic chicken autonomic and sensory neurons. GDNF (as previously shown in studies by Buj-Bello *et al.*, 1995; Ebendal *et al.*, 1995; Trupp *et al.*, 1995) and neurturin promoted survival in chicken parasympathetic, sympathetic and cutaneous and enteroceptive sensory neurons whereas persephin and artemin were unable to promote the survival of any of these neuronal populations.

### 2.4.1. Survival responses to GDNF and neurturin

A comprehensive evaluation of the relative biological activities of GDNF and neurturin demonstrated marked differences and developmental changes in the responsiveness of parasympathetic, sympathetic and enteroceptive sensory neurons to these factors. Whereas parasympathetic neurons rapidly lose responsiveness to GDNF during development, sympathetic neurons and nodose ganglion neurons become more responsive to GDNF with age. In comparison, developmental changes in the response of these neurons to neurturin are less pronounced, but because of the marked changes in GDNF responsiveness, there are large changes in the relative sensitivity of these neurons to GDNF and neurturin during development. Whereas parasympathetic neurons are equally responsive to GDNF and neurturin early in development and far more responsive to neurturin at later stages, sympathetic neurons are considerably more responsive to neurturin early in development and equally responsive to GDNF and neurturin later on. Nodose neurons are much more responsive to GDNF throughout development, and by E12 are more sensitive to GDNF than autonomic neurons at any developmental stage studied. It is interesting to note that, with the exception of sympathetic neurons derived from E8 embryos, the

developmental changes in responsiveness to GDNF and neurturin observed in these cultures occur in post-mitotic neurons.

The changing responsiveness of chicken peripheral neurons to GDNF and neurturin observed here is not unique among neurotrophic factors. It is becoming increasingly recognised that many neuronal populations exhibit marked changes in their responsiveness to different neurotrophic factors during development (Davies, 1997). For example, many trigeminal ganglion neurons generated during the early stages of trigeminal ganglion formation switch survival dependence from NT-3 and BDNF to NGF (Buchman and Davies, 1993; Paul and Davies, 1995; Piñón *et al.*, 1996; Enokido *et al.*, 1999). Similarly, there is evidence that a subset of dorsal root ganglion neurons switch dependence from NT-3 to NGF at early developmental stages (Fariñas *et al.*, 1996). The opposite holds for a subset of sympathetic neurons which are dependent on NGF for survival early in development and subsequently acquire an additional requirement for NT-3 *in vivo* (Zhou and Rush, 1995; Wyatt *et al.*, 1997). The most striking developmental change in GDNF and neurturin responsiveness is seen in cutaneous sensory neurons of the chicken trigeminal ganglion. Such neurons are totally unresponsive to these factors at early developmental stages. However, several days after they become dependent on neurotrophins for survival, many of these neurons acquire a late survival response to GDNF and neurturin. Likewise, a subset of DRG neurons that depends on NGF for survival during embryonic development become responsive to GDNF in the postnatal period (Molliver *et al.*, 1997). Similarly, recent evidence has shown that chicken vestibular neurons are supported by BDNF and NT-3 from E8-E12, and thereafter completely lose responsiveness to these neurotrophins and become responsive to GDNF between E12 and E16 (Hashino *et al.*, 1999). Such a switch in the sensitivity of these neurons from BDNF to GDNF coincides with the timing of

completion of target innervation. Hence, the changes in vestibular neuron sensitivity may be regulated by changes in the available factors released from their peripheral targets, the inner ear epithelia.

The *in vitro* results obtained in this study suggest that GDNF and neurturin play a role in regulating the survival of various populations of PNS neurons at different stages of their development. However, as each of these populations is supported by at least one other neurotrophic factor and there is generally negligible additional survival in cultures containing this factor plus GDNF or neurturin, it is likely that GDNF and neurturin cooperate with various other neurotrophic factors in regulating neuronal survival. Furthermore, it has recently been suggested that TGF- $\beta$  is a necessary cofactor for the trophic effects of GDNF in a variety of neurons *in vitro* (Krieglstein *et al.*, 1998a) and *in vivo* (Schober *et al.*, 1999).

In agreement with the demonstration that GDNF promotes the *in vitro* survival of embryonic chicken sympathetic neurons, administration of GDNF to the chorio-allantoic membrane of chicken embryos results in significantly higher numbers of neurons in sympathetic ganglia as compared to control embryos (Oppenheim *et al.*, 1995). Surprisingly, in this experimental paradigm, GDNF is unable to rescue DRG, nodose or ciliary neurons after treatment during the main period of cell death in each of these ganglia. However, investigation of the phenotype of *GDNF* knockout mice has demonstrated that GDNF plays a critical role in promoting the survival of several developing neuronal populations *in vivo*, including those of the nodose ganglion, superior cervical ganglion and dorsal root ganglia (Moore *et al.*, 1996).

The physiological significance of the *in vitro* results presented in this chapter is further confirmed by the finding that a 0.8 kb GDNF transcript is expressed in all the tissues of the embryonic chicken that are innervated by neurons that respond to GDNF (Buj-Bello *et al.*, 1995). Moreover, it is observed that the developmental changes in the level of GDNF mRNA expression in some tissues seem to mirror the changes in the sensitivity of the innervating neurons to GDNF. For example, the level of GDNF mRNA in the heart and the response of nodose neurons to GDNF increase during development, whereas the level of GDNF mRNA in the choroid, ciliary body and iris and the response of ciliary neurons to GDNF decrease during development. Since the responsiveness of neurons to GDNF and neurturin may be regulated at the level of receptor expression, these results raise the question of whether GDNF itself regulates expression of its own receptor. This has recently been addressed, and GDNF was found not to influence GFR $\alpha$ 1, GFR $\alpha$ 2 or Ret mRNA expression in embryonic chicken autonomic and sensory neurons (Doxakis, personal communication).

The response of chicken sensory and autonomic neurons to neurturin is in accordance with the demonstration that E15 DRG, E18 nodose and E21 SCG neurons, of rat embryos, are supported *in vitro* by neurturin (Kotzbauer *et al.*, 1996). In the rat, these neurons are all more responsive to neurturin than GDNF in culture. This is in contrast to the finding that chicken enteroceptive neurons are more sensitive to GDNF than to neurturin throughout development. Such a discrepancy may be due to species differences in neuronal responsiveness to these factors. Definitive evidence of a physiological role for neurturin in the maintenance and survival of peripheral neurons comes from analysis of mice with a targeted null mutation in the neurturin gene (Heuckeroth *et al.*, 1999). Due to the similarities of the effects of GDNF and neurturin on cultured neurons and

their peripheral expression patterns, it seemed likely that in the PNS, neurturin knockout mice would display a similar phenotype to *GDNF* null mutant mice. However, this was not observed, demonstrating the unique roles of GDNF and neurturin in the development of the PNS. Mice homozygous for a null mutation in the neurturin gene demonstrate profound defects in their parasympathetic nervous system, which is seemingly unaffected in *GDNF* knockout mice. Neurturin knockout mice have a considerable reduction in parasympathetic innervation to the lacrimal gland and submandibular gland and a reduction in the total number of neurons in the newborn submandibular ganglion of 45 %. Ciliary ganglion neuronal profiles in adult mice are reduced by almost 50 % compared to wild-type littermates. These findings demonstrate that neurturin is an essential survival factor for certain populations of parasympathetic neurons *in vivo*. It is interesting to note that in this study, parasympathetic neurons of the ciliary ganglion were more sensitive to neurturin than GDNF throughout development. Similarly, the concentration of neurturin required to elicit half maximal effects on parasympathetic neurons was 4-fold less than that for sensory neurons. It would appear that there is considerable redundancy in the effects of GDNF and neurturin on many neuronal populations. However, in certain instances, these factors cannot substitute for one another, for example, GDNF and not neurturin is required for the maintenance of subpopulations of sympathetic and sensory neurons whereas neurturin and not GDNF is required to support subpopulations of parasympathetic neurons.

In neuronal populations where GDNF and neurturin are known to exert effects, it has been generally reported that they share very similar biological activities. However, as in this study of the effects of GDNF and neurturin on peripheral neurons, detailed comparative studies of the effects of these

factors on other kinds of neurons have revealed differences in efficacy and potency. For example, GDNF is twice as potent as neurturin in promoting the survival of embryonic rat spinal motoneurons *in vitro* (Klein *et al.*, 1997), whereas in E14 rat midbrain dopaminergic neurons in culture, the efficacy and potency of GDNF and neurturin in promoting survival and function are indistinguishable (Horger *et al.*, 1998). Differential effects of GDNF and neurturin are also apparent in postnatal substantia nigra dopaminergic neurons, where GDNF and neurturin promote comparable survival but only GDNF induces sprouting and hypertrophy of these neurons in culture (Akerud *et al.*, 1999). Similarly, GDNF and neurturin are equally potent in preventing 6-OHDA-induced cell death in adult dopaminergic neurons *in vivo*, but only GDNF induces tyrosine hydroxylase staining, sprouting and hypertrophy (Akerud *et al.*, 1999). Finally, a recent study has reported that in ageing sensory and motoneurons, there is an upregulation of GDNF mRNA, but not neurturin mRNA, in the target tissues, paralleled with an increase in the neuronal expression of the cognate receptors for GDNF (Ming *et al.*, 1999). While the resulting increase in GDNF signalling is thought to contribute in part to the characteristic phenotype of ageing neurons, the responsiveness of these neurons to neurturin is unaffected. Such differences in neuronal responsiveness to GDNF and neurturin underscore the unique roles of these two factors in the development and maintenance of the nervous system.

#### **2.4.2. Survival responses to persephin and artemin**

In this study, persephin did not support the survival of any of the chicken neuronal populations investigated, even when administered at relatively high concentrations ranging up to 100 ng/ml. These results are in agreement with data published after these experiments were undertaken, demonstrating that although persephin promotes the survival of ventral midbrain

dopaminergic neurons and motoneurons in culture, it does not, in contrast to GDNF and neurturin, promote survival of peripheral sensory or autonomic neurons (Milbrandt *et al.*, 1998). These results imply that persephin cannot utilise the same receptor complexes (i.e. Ret/GFR $\alpha$ 1 or Ret/GFR $\alpha$ 2) used by GDNF and neurturin. This hypothesis has been confirmed by fibroblast (Milbrandt *et al.*, 1998) and neuron overexpression studies, in which GFR $\alpha$ 4 has been shown to be the ligand binding subunit for persephin signalling (Enokido *et al.*, 1998). Quantitative RT-PCR has shown that GFR $\alpha$ 4 is expressed within purified neurons from chicken embryo peripheral ganglia, however, at very low levels compared to GFR $\alpha$ 1, GFR $\alpha$ 2 and Ret mRNAs (Doxakis, personal communication). Interestingly, there is also a substantial (5-fold) downregulation of GFR $\alpha$ 4 mRNA when these neurons have been in culture for 48 hours. Such a phenomenon of receptor downregulation as a result of being in culture was not apparent for GFR $\alpha$ 1 and GFR $\alpha$ 2 mRNA expression and may contribute to the lack of neuronal responsiveness to persephin *in vitro*. However, it is also possible that persephin may have effects, other than supporting survival, on responsive neurons, such as promotion of neurite outgrowth or modulation of neuronal phenotype. Nevertheless, studies of persephin mRNA expression have found only very low levels of expression within peripheral tissues (Milbrandt *et al.*, 1998), suggesting that persephin may not normally be available to peripheral neurons during development. It will be useful to investigate the phenotype of the persephin knockout mouse when it becomes available, to ascertain the role of this factor, if any, in the developing PNS *in vivo*.

Artemin, like persephin, was unable to promote the survival of developing chicken parasympathetic, sympathetic or sensory neurons. In the neonatal rat, artemin promotes the survival of peripheral sensory and sympathetic neurons *in vitro* (Baloh *et al.*, 1998). Indeed, its expression pattern suggests

that it may well have a role with respect to the maintenance of these populations *in vivo*. It is possible that this discrepancy in artemin responsiveness constitutes a species difference between mammals and birds. For example, CNTF has previously been shown to promote the survival of embryonic chicken sympathetic neurons but is ineffective on embryonic mouse sympathetic neurons (Buj-Bello and Davies, unpublished). Also, there may be substantial differences between human artemin, used in this study, and its homologue in chicken. Alternatively, artemin may require an additional cofactor in order to exert its biological activity which may have been present in the experiments by Baloh but absent in the culture system used here. For example, highly purified neuronal cultures were employed here, free from contaminating non-neuronal cells which can release a cocktail of potential trophic factors into the culture medium.

The lack of effect of artemin on these peripheral neurons was unexpected as GFR $\alpha$ 3, the proposed coreceptor of preference for artemin (Baloh *et al.*, 1998), is strongly expressed in peripheral ganglia (Worby *et al.*, 1998; Naveilhan *et al.*, 1998; Baloh *et al.*, 1998a; Widenfalk *et al.*, 1998; Trupp *et al.*, 1998). As observed for GFR $\alpha$ 4 mRNA expression, it is possible that a downregulation of receptor expression in culture could account for the lack of an artemin-mediated survival effect in chicken neurons. Artemin has been reported to signal via the GFR $\alpha$ 1/Ret complex in dopaminergic neurons (Baloh *et al.*, 1998). Although these receptor components are known to be present on the peripheral neurons under study, the functional significance of the GFR $\alpha$ 1-artermin interaction in the PNS is unclear.

### **2.4.3. Correlation between neuronal responsiveness to GDNF and neurturin and expression of receptor subunits**

The differences and developmental shifts in GDNF and neurturin responsiveness observed in this study may be due to differences in the expression of receptors for GDNF and neurturin or in their intracellular signal transduction pathways. The former was investigated by considering the expression of mRNAs encoding the receptor tyrosine kinase, Ret, and the GPI-linked coreceptors, GFR $\alpha$ 1 and GFR $\alpha$ 2.

Whereas all the populations of neurons studied expressed relatively high levels of Ret mRNA, there were differences in the relative levels of GFR $\alpha$ 1 and GFR $\alpha$ 2 mRNAs. Generally, neurons that were more responsive to GDNF expressed relatively higher levels of GFR $\alpha$ 1 mRNA, and neurons that were more responsive to neurturin expressed relatively higher levels of GFR $\alpha$ 2 mRNA. This is consistent with the idea that GFR $\alpha$ 1 is the preferred receptor for GDNF and GFR $\alpha$ 2 is the preferred receptor for neurturin (Baloh *et al.*, 1997; Buj-Bello *et al.*, 1997; Creedon *et al.*, 1997; Jing *et al.*, 1997; Klein *et al.*, 1997; Trupp *et al.*, 1998; Cacalano *et al.*, 1998; Rossi *et al.*, 1999) and suggests that differences in responsiveness of neurons to GDNF and neurturin are governed at least in part by the relative levels of expression of members of the GFR $\alpha$  family of GPI-linked receptors. However, developmental changes in responsiveness to these factors were not consistently paralleled by changes in the relative levels of GFR $\alpha$  transcripts. In particular, the marked decrease in the response of ciliary neurons to GDNF between E8 and E12 was not associated with a corresponding decrease in the level of GFR $\alpha$ 1 mRNA, indeed the ratio between GFR $\alpha$ 1 and GFR $\alpha$ 2 transcripts in ciliary neurons was very similar at both ages. Although it is possible that there are changes in the relative levels of GFR $\alpha$ 1 and GFR $\alpha$ 2 proteins in ciliary neurons during development that are not reflected

in the steady state levels of GFR $\alpha$  transcripts, these data raise the possibility that in addition to differences in the relative levels of GFR $\alpha$  receptors, other factors could affect the sensitivity of neurons to GDNF and neurturin during development. However, because of potential ligand/receptor cross talk, caution has to be exercised in the interpretation of these data. For example, in mice with a null mutation in the *GFR $\alpha$ 1* gene, the response of nodose neurons to GDNF is abolished but the response of submandibular neurons to GDNF is unaffected (Chapter 3; Cacalano *et al.*, 1998). This suggests that in some populations of neurons GDNF can act via GFR $\alpha$  receptors other than GFR $\alpha$  1. In contrast, neuronal responsiveness to NGF and BDNF clearly correlates with TrkA and TrkB mRNA expression due to the lack of promiscuity of these neurotrophins or their Trk receptors (Wyatt and Davies, 1993, 1995; Ninkina *et al.*, 1996; Robinson *et al.*, 1996).

In summary, the results presented in this chapter show that populations of embryonic PNS neurons have markedly different responses to GDNF and neurturin and that sympathetic and parasympathetic neurons switch responsiveness between these factors during development. The differences in responsiveness to these factors are due in part to differences in the expression of the GPI-linked receptors, GFR $\alpha$ 1 and GFR $\alpha$ 2. The survival of these neurons is not supported by persephin or artemin *in vitro*. These results further illustrate the growing complexity of trophic interactions governing PNS neurons at different stages of development.

## Chapter 3

### GFR $\alpha$ 1 is required for GDNF signalling in some, but not all, neuronal populations

#### 3.1. Introduction

Glial cell-line derived neurotrophic factor (GDNF) (Lin *et al.*, 1993), neurturin (Kotzbauer *et al.*, 1996), persephin (Milbrandt *et al.*, 1998) and artemin (Baloh *et al.*, 1998) constitute a class of secreted proteins that is structurally related to the transforming growth factor- $\beta$  family. Studies in primary neuronal cultures have demonstrated that GDNF is a survival factor for subpopulations of peripheral sensory, sympathetic and parasympathetic neurons (Buj-Bello *et al.*, 1995; Trupp *et al.*, 1995; Ebendal *et al.*, 1995; Forgie *et al.*, 1999). Likewise, neurturin (Kotzbauer *et al.*, 1996; Forgie *et al.*, 1999) and artemin (Baloh *et al.*, 1998) promote the survival of some sympathetic and sensory neurons, whereas persephin has been shown to have no survival-promoting activity with respect to peripheral neurons (Milbrandt *et al.*, 1998). Comprehensive survival studies of avian peripheral neurons throughout development demonstrate a widespread and sizeable response to GDNF and neurturin. Surprisingly, the few reports available considering the effects of these factors on cultured mammalian neurons show only a very limited survival response (Henderson *et al.*, 1994; Trupp *et al.*, 1995). For example, GDNF is unable to promote the survival of mouse trigeminal neurons (at E15 and E18) and sympathetic neurons (at E18) and supports a small subpopulation (40 %) of nodose neurons, at late embryonic stages only (Henderson *et al.*, 1994). Nevertheless, data from mice with null mutations in the *GDNF* or neurturin genes have demonstrated the essential physiological role of the GDNF ligand family in the embryonic development of subsets of peripheral sensory and autonomic neurons. Newborn mice with a null

mutation in the *GDNF* gene display deficits in the nodose-petrosal complex (40 %), dorsal root ganglia (23 %) and superior cervical sympathetic ganglion (35 %) (Moore *et al.*, 1996), whilst disruption of the neurturin gene results in profound reductions in parasympathetic neurons of the ciliary ganglion (48 %) and submandibular ganglion (45 %) (Heuckeroth *et al.*, 1999).

The biological actions of the GDNF ligand family are mediated via a common transmembrane receptor tyrosine kinase, Ret (Trupp *et al.*, 1996; Durbec *et al.*, 1996; Jing *et al.*, 1996; Treanor *et al.*, 1996; Vega *et al.*, 1996). Although GDNF has been shown in some studies to bind to Ret directly (Vega *et al.*, 1996; Trupp *et al.*, 1996), GDNF signalling requires another receptor, one of a family of GPI-linked cell surface proteins termed GFR $\alpha$ 's, of which, to date, there are four members: GFR $\alpha$ 1 (Jing *et al.*, 1996; Treanor *et al.*, 1996), GFR $\alpha$ 2 (Baloh *et al.*, 1997; Buj-Bello *et al.*, 1997; Creedon *et al.*, 1997; Jing *et al.*, 1997; Klein *et al.*, 1997; Sanicola *et al.*, 1997; Suvanto *et al.*, 1997), GFR $\alpha$ 3 (Baloh *et al.*, 1998a; Masure *et al.*, 1998; Naveilhan *et al.*, 1998; Nomoto *et al.*, 1998; Trupp *et al.*, 1998; Worby *et al.*, 1998) and GFR $\alpha$ 4 (Thompson *et al.*, 1998). The importance of these ligand-binding accessory proteins has been demonstrated in cell lines expressing Ret with or without a GFR $\alpha$  subunit. Ret alone is unable to associate with GDNF, whereas coexpression with a GFR $\alpha$  subunit leads to the formation of a tripartite membrane-bound complex that facilitates GDNF-mediated Ret activation (Jing *et al.*, 1996; Treanor *et al.*, 1996; Baloh *et al.*, 1997; Trupp *et al.*, 1998). Likewise, ectopic expression of Ret alone in cultured neurons does not confer a survival response to GDNF or neurturin, whereas coexpression of Ret with an appropriate GFR $\alpha$  subunit enables neurons to respond to GDNF or neurturin with enhanced survival (Buj-Bello *et al.*, 1997). This is reminiscent of the CNTF receptor, where a GPI-linked accessory protein, CNTFR $\alpha$ , is required

in addition to the transmembrane signalling proteins, gp130 and LIFR $\beta$ , to mediate a response to the ligand (Davis *et al.*, 1993).

The role of Ret in this multicomponent receptor is not restricted to transducing the GDNF signal across the cell membrane. Rather, it also influences the interactions between the various GDNF family ligands and GFR $\alpha$  receptors. When GFR $\alpha$ 1 was first isolated as a receptor for GDNF, neurturin, persephin and artemin had yet to be isolated. As more GDNF ligands and receptors are found the task of elucidating their signalling mechanisms becomes increasingly complex. Multiple biochemical and cell culture studies have addressed the issue of ligand specificity of the GFR $\alpha$  receptors with conflicting results. Equilibrium and competition binding studies of radiolabelled ligand to soluble recombinant receptor demonstrate a high affinity interaction between GFR $\alpha$ 1-GDNF ( $K_d=3$  pM) and GFR $\alpha$ 2-neurturin ( $K_d=10$  pM) (Klein *et al.*, 1997; Baloh *et al.*, 1998a; Jing *et al.*, 1997; Masure *et al.*, 1998). While some studies using this experimental approach report only low affinity interactions between GFR $\alpha$ 1-neurturin and GFR $\alpha$ 2-GDNF ( $K_d>1$  nM for each) (Klein *et al.*, 1997), it has been demonstrated that GFR $\alpha$ 2 can form a high affinity complex with GDNF (Sanicola *et al.*, 1997; Baloh *et al.*, 1998a) and GFR $\alpha$ 1 with neurturin (Masure *et al.*, 1998) in the presence of soluble Ret extracellular domain. Hence, in intact cells, expressing Ret and a GFR $\alpha$  subunit, the receptor-ligand interactions seen in cell free systems may be somewhat modified. Several studies in cell lines suggest that GFR $\alpha$ 1 can act equally well as a receptor for GDNF and neurturin (Creedon *et al.*, 1997; Baloh *et al.*, 1997, 1998a; Jing *et al.*, 1997; Naveilhan *et al.*, 1998) and GFR $\alpha$ 2 has been reported to be an equally effective receptor for both ligands (Sanicola *et al.*, 1997; Trupp *et al.*, 1998; Worby *et al.*, 1998). In contrast, other studies indicate that GFR $\alpha$ 2 is principally a receptor for neurturin, being 30-fold more sensitive to this

factor than to GDNF (Baloh *et al.*, 1997; Jing *et al.*, 1997; Naveilhan *et al.*, 1998). Further data, from cultured neurons micro-injected with expression plasmids encoding GFR $\alpha$ 1, GFR $\alpha$ 2 and Ret, may better reflect the ligand specificity of GFR $\alpha$  receptors in neuronal cells. In low concentrations of GDNF and neurturin (5 ng/ml), coexpression of GFR $\alpha$ 1 and Ret in cultured neurons confers a survival response to GDNF only, whereas neurons expressing GFR $\alpha$ 2 and Ret survive only in response to neurturin (Buj-Bello *et al.*, 1997). At higher concentrations (50 ng/ml), however, the fidelity of the GFR $\alpha$ 1-GDNF interaction is maintained whilst neurons coexpressing Ret and GFR $\alpha$ 2 now respond equally well to GDNF and neurturin. GFR $\alpha$ 1 and GFR $\alpha$ 2, whether as soluble recombinant protein or coexpressed with Ret in fibroblasts or cultured neurons, do not interact with persephin (Milbrandt *et al.*, 1998; Baloh *et al.*, 1998a; Enokido *et al.*, 1998). Similarly, GFR $\alpha$ 1 and GFR $\alpha$ 2 alone are incapable of binding artemin, however, artemin binds and initiates Ret tyrosine phosphorylation in fibroblasts coexpressing GFR $\alpha$ 1 and Ret (Baloh *et al.*, 1998).

Unlike the promiscuity of GFR $\alpha$ 1 and GFR $\alpha$ 2, the interactions of GFR $\alpha$ 3 and GFR $\alpha$ 4 are more straight forward as each receptor interacts with only one ligand. In a cell free system, direct high affinity binding of soluble GFR $\alpha$ 3 fusion protein with artemin, but not GDNF, neurturin or persephin, has been shown. Artemin also activates Ret tyrosine phosphorylation in fibroblasts transfected with Ret and GFR $\alpha$ 3 (Baloh *et al.*, 1998). Despite the fact that GDNF, neurturin or persephin cannot initiate Ret signalling in cells coexpressing Ret and GFR $\alpha$ 3 (Baloh *et al.*, 1998a; Masure *et al.*, 1998; Trupp *et al.*, 1998; Worby *et al.*, 1998), GFR $\alpha$ 3 can bind GDNF with low affinity in the presence of Ret, however, the functional significance of this interaction is unclear (Baloh *et al.*, 1998a; Trupp *et al.*, 1998). Again, this observation demonstrates the modulatory role of Ret in GFR $\alpha$  ligand binding.

Equilibrium and competition binding studies show that GFR $\alpha$ 4 specifically binds persephin but not other members of the GDNF family. Accordingly, ectopic expression of GFR $\alpha$ 4 and Ret in cultured neurons permits Ret activation and a subsequent survival response to persephin but not to GDNF and neurturin (Enokido *et al.*, 1998). The possible interactions between the GDNF ligands and their coreceptors are shown in Figure 3.1..

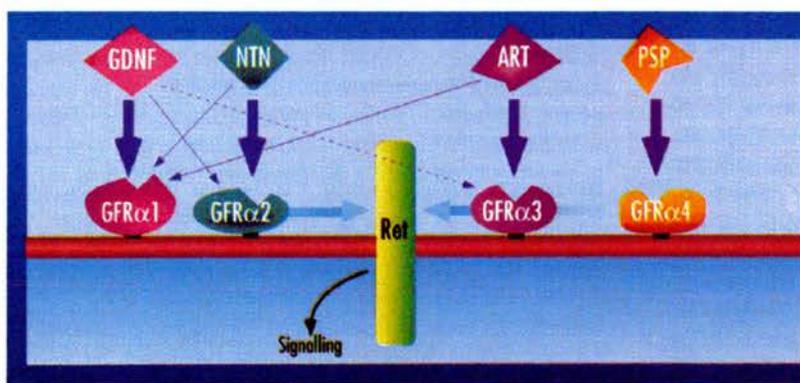


Figure 3.1. The interactions between the GDNF family and GFR $\alpha$  receptors.

Interactions that are demarcated by thin or discontinuous lines require the presence of Ret. The discontinuous line indicates that only binding, but not signalling, has been demonstrated. From Rosenthal, 1999.

Although *in vitro* systems are useful, caution must be exercised when interpreting cross talk data for ligand-receptor systems with multiple family members, especially in fibroblast-based models. Such models cannot recreate the neuronal environment *in vivo* where accessory factors or multiple forms of existing receptor components may alter specificity. For example, BDNF, NT-3 and NT-4/5 all initiate phosphorylation of TrkB expressed in fibroblasts with equivalent dose response relationships.

However, in PC12 cells, BDNF and NT-4/5 activate TrkB at a 100-fold lower concentration compared to NT-3, possibly due to an interaction with p75 which is present in PC12 cells and neurons, but not in fibroblasts (Ip *et al.*, 1993). To fully understand the interactions between ligands and their receptors *in vivo*, it is necessary to undertake a detailed analysis of ligand and receptor expression and to assess multiple biological responses in normal and null mutant mice. Such an approach has been employed to determine the preferred ligand/receptor interactions of members of the neurotrophin family. The recent generation of mice with null mutations in the *GDNF* (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996), *GFR $\alpha$ 1* (Cacalano *et al.*, 1998; Enomoto *et al.*, 1998), neurturin (Heuckeroth *et al.*, 1999), and *GFR $\alpha$ 2* (Rossi *et al.*, 1999) genes has already yielded valuable data regarding ligand/receptor interactions amongst GDNF family members. Mice lacking *GFR $\alpha$ 1* display deficits in the kidneys and enteric nervous system that are strikingly similar to those previously observed in *GDNF*- (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996) and *Ret*- (Schuchardt *et al.*, 1994) deficient mice. However, some peripheral ganglia, severely affected in *GDNF*- and *Ret*- deficient mice are only minimally affected, if at all, in *GFR $\alpha$ 1*<sup>-/-</sup> animals. These results therefore suggest a stringent pairing of *GFR $\alpha$ 1* and *GDNF* in the kidney and enteric nervous system during development, whilst indicating significant cross talk between *GDNF* and other *GFR $\alpha$*  coreceptors in certain neuronal populations. The phenotypes of neurturin- and *GFR $\alpha$ 2*-deficient mice display considerable similarity especially with respect to the profound deficits in the parasympathetic nervous system observed in these mutants. This indicates that *GFR $\alpha$ 2* is a physiological neurturin receptor, essential for the development of specific postganglionic parasympathetic neurons. These data will be discussed fully in section 3.4. of this chapter.

The aim of the study presented in this chapter was to use *GFR $\alpha$ 1* null mutant mice to first determine whether *GFR $\alpha$ 1* is essential to mediate all GDNF signalling and second, to investigate the extent to which neurturin can signal through the *GFR $\alpha$ 1* receptor. Before such experiments could be undertaken using *GFR $\alpha$ 1*-deficient mice, a thorough investigation of the responsiveness of peripheral sensory and autonomic neurons, derived from wild-type mice, to GDNF and neurturin was required. This study was later extended to include artemin, however, persephin was not investigated as this factor is known not to influence the survival of developing peripheral neurons (Chapter 2; Milbrandt *et al.*, 1998). The results of this preliminary study further demonstrated the differences in responsiveness of chicken and mouse peripheral neurons to these factors. Only a limited number of mammalian neuronal populations, at restricted developmental stages, responded to GDNF and neurturin unlike the widespread responsiveness of avian neurons to these factors. Conversely, artemin which has no neurotrophic activity with respect to chicken neurons supported the survival of selected mammalian neuronal populations. Due to time constraints, the study of *GFR $\alpha$ 1*<sup>-/-</sup> neurons has not been extended to include artemin.

The *in vitro* results from *GFR $\alpha$ 1*<sup>-/-</sup> mice presented in this chapter represent part of a collaborative study detailing multiple effects of a targeted disruption of the *GFR $\alpha$ 1* gene (Cacalano *et al.*, 1998). In this, the first study to consider the effects of knocking out a *GFR $\alpha$*  receptor, histological analyses of the kidneys, enteric nervous system and several neuronal populations of the PNS and CNS were undertaken as were *in vitro* experiments considering GDNF and neurturin-mediated cell survival in central and peripheral (this study) neurons in culture. *GFR $\alpha$ 1*-deficient sensory ganglia neurons no longer responded to GDNF or to neurturin, whereas *GFR $\alpha$ 1*-deficient parasympathetic neurons retained a normal response to these two factors.

These *in vitro* results, in conjunction with the findings of the other branches of this study, support the idea that GFR $\alpha$ 1 is a necessary receptor component for GDNF in certain neuronal populations, however, GDNF and neurturin can mediate some of their activities through a second receptor.

## 3.2. Methods

### 3.2.1 Neuronal cultures

Although the focus of this study was to use *GFR $\alpha$ 1* null mutant mice to investigate the role of GFR $\alpha$ 1 in mediating GDNF signalling, it was necessary to first conduct a pilot study to examine the responsiveness of wild-type mouse sensory neurons to members of the GDNF ligand family. This was achieved by monitoring neuronal survival in low-density, dissociated cultures supplemented with GDNF ligand family members.

Embryos (E12-E19) and postnatal mice (P3-P4) were obtained from overnight matings of CD-1 mice. The date of identification of a vaginal plug was designated to be embryonic day 1. Pregnant females were killed by cervical dislocation at the appropriate stage of gestation and embryos were removed and accurately staged according to the criteria of Theiler (1972). All dissections and subsequent preparations of neuronal cultures were carried out in a laminar flow hood using standard sterile technique. Dissections of peripheral ganglia were carried out in L-15 medium as described in Chapter 2, section 2.2.1.. Details of the dissections of several mouse peripheral ganglia are described below.

To isolate trigeminal ganglia from E12 embryos, two coronal incisions were made through the head using tungsten needles, one just above the eye and the other between the maxillary and mandibular processes of the first branchial arch (Figure 3.2.). The trigeminal ganglia can be seen as two opaque structures in the tissue slice obtained. Tungsten needles were used to remove the ganglia from the tissue slice and to free any adherent mesenchymal tissue. Dissection of the trigeminal ganglia from older

embryos was similar however, fine scissors were required in the initial stages of the dissection to cut through cartilage or bone of the developing head.

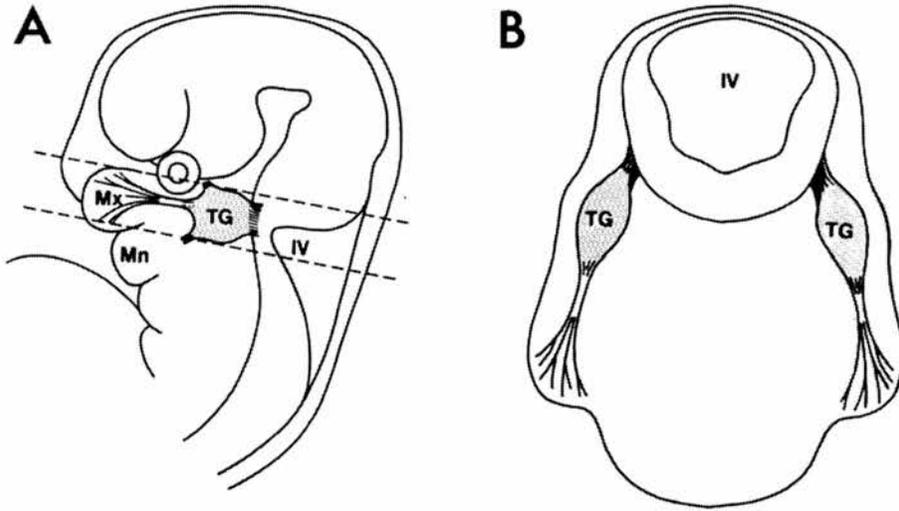


Figure 3.2. Dissection of the mouse trigeminal ganglion.

(A) Lateral aspect of an E11 head showing the location of the transverse incisions (interrupted lines) for obtaining a slice of tissue that contains both trigeminal ganglia.

(B) Rostral aspect of this slice showing the location of the trigeminal ganglia. TG, trigeminal ganglion; Mx, maxillary process; Mn, mandibular process; IV, fourth ventricle. From Davies, 1995.

In contrast to avian embryos, the mouse nodose ganglion (also termed the inferior vagal ganglion) remains situated close to the base of the skull. The dissection was similar at all ages except that, as for trigeminal ganglia, fine scissors, instead of tungsten needles, were required in the initial stages of the dissection in E13 and older embryos. Using the same plane of dissection as for the first incision in the trigeminal dissection, the top of the skull and underlying forebrain was removed. The embryo was decapitated just above the shoulders and the head bisected in the sagittal plane. After removal of the hindbrain, the jugular foramen was opened as shown in Figure 3.3., to reveal the nodose ganglion lying at its base. At this point in the dissection, in

E13 and older embryos, the superior cervical sympathetic ganglion could also be seen. The nodose ganglion is a spherical structure with the prominent vagus nerve attached to its distal aspect. This is clearly distinguished from the superior cervical ganglion which is an elongated structure that is attached caudally to the sympathetic chain which is much thinner than the vagus nerve.

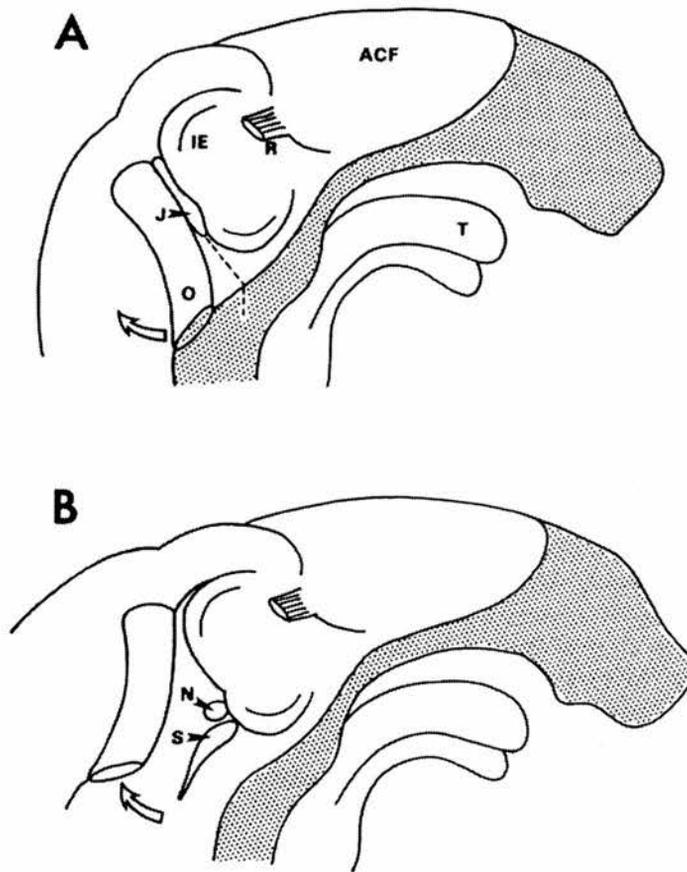


Figure 3.3. Medial aspect of the left half of an E14 mouse embryo head showing successive stages in the dissection of the nodose ganglion.

The bisected midline structures lying along and in front of the cranial base are stippled. (A) The incision passing from the jugular foramen (J) to the midline is shown by the interrupted line. The direction in which the large ossified part of occipital bone (O) should be reflected to open up the jugular foramen after making the previous incision is shown by the large curved arrow. T, tongue; ACF, anterior cranial fossa; R, root of trigeminal nerve; IE, inner ear. (B) The nodose ganglion (N) and superior cervical ganglion (S) are revealed after extending the jugular foramen to the midline and reflecting the large ossified part of the occipital bone backwards. From Davies, 1995.

The paravertebral sympathetic chain was dissected from the thoracic region of mouse embryos, where it was found just lateral to the vertebral column. After removal of the thoracic viscera, the sympathetic chain was carefully peeled away using fine forceps.

Dorsal root ganglia were removed from the lumbar region of mouse embryos. At E12, tungsten needles were used to dissect the DRG, seen from the dorsal aspect of the embryo as opaque structures lying in sequence alongside the developing spinal cord. At later ages, a tissue block containing the lumbar vertebral column and adjacent tissues was dissected using scissors. After removal of the abdominal viscera from the ventral surface and skin from the dorsal aspect, one blade of the scissors was inserted into the vertebral column along its long axis. The developing vertebral bodies were cut laterally and removed displaying the spinal cord and prominent dorsal root ganglia lying between adjacent pedicles. The DRG were simply plucked out using fine forceps to grasp the nerve roots, after which any contaminating connective tissues were carefully removed using tungsten needles.

The submandibular ganglion is located within the submandibular salivary gland. The submandibular glands were removed from the ventral aspect of the neck and mandible, after which the ganglion tissue was dissected away from surrounding mesenchymal and epithelial tissues using tungsten needles.

The dissected ganglia were collected in CMF-HBSS and incubated in 0.05 % trypsin, at 37 °C, for between 7 and 30 minutes for embryonic tissue. Postnatal ganglia were incubated in 0.1 % trypsin for 30 minutes. The subsequent dissociation procedure, differential sedimentation (required for

E18 and postnatal preparations only) and preparation of poly-DL-ornithine/laminin substratum on tissue culture grade dishes were as previously described (Chapter 2, section 2.2.1.).

A notable difference in the preparation of mouse versus chicken neuronal cultures was that chicken neurons were cultured in F-14 medium supplemented with 10 % horse serum, whereas mouse neurons were grown in F-14 medium supplemented with 2 mM L-glutamine and SATO (for chemical composition, see Appendix I). This defined medium has been found to give the best results for the cultivation of embryonic mouse neurons and has the advantage of suppressing the growth and proliferation of any contaminating non-neuronal cells which would otherwise thrive in a serum-containing environment. Neurons were plated onto 35 mm plastic tissue culture dishes (Nunc, Gibco) at a density of 500-2000 neurons per dish and grown in 2 ml of F-14 medium with glutamine and SATO supplement, in a humidified 5.5 % CO<sub>2</sub> atmosphere at 37 °C. Purified recombinant neurotrophic factors were added to the cultures at the time of plating. Three to six hours after plating, the number of attached neurons in the centre of each dish was counted using a 12 x 12 mm standard graticule and an inverted phase contrast microscope at 100x magnification. 24 and 48 hours after plating the number of surviving, phase-bright, process-bearing neurons in the same area was ascertained and the survival response of the neurons was expressed as a percentage of the number present at three hours. Within each experiment, triplicate cultures were established for each condition and all experiments were repeated at least three times.

### **3.2.2. Neuronal cultures derived from *GFR $\alpha$ 1* null mutant mice**

A small number of male and female mice that were heterozygous for disruption of the *GFR $\alpha$ 1* gene were received as a gift from Arnon Rosenthal,

Genentech Inc. USA. Because these mice were of a mixed background strain (129 x CD1, F2), breeding pairs were initially established with CD1 mice to produce third generation CD1 offspring. At four weeks of age, these progeny were anaesthetised with halothane, tail-tipped for genotyping and identified by a numbered ear tag in accordance with Home Office regulations governing the use of animals in scientific procedures. Wild-type animals were eliminated from the colony at a later date. Mice homozygous for a null mutation in the *GFR $\alpha$ 1* gene died 1-1.5 days after birth. Although *GFR $\alpha$ 1*<sup>-/-</sup> animals were the same size as wild-type littermates at birth and demonstrated normal limb and body movements and were able to suckle, bilateral kidney agenesis or severe dysgenesis resulted in rapid death (Cacalano *et al.*, 1998). Digestive abnormalities were also apparent in *GFR $\alpha$ 1*<sup>-/-</sup> neonates, reflecting severe deficits in the enteric nervous system. The genetic distribution of newborns derived from heterozygous intercrosses was approximately 25 % wild-type, 50 % heterozygote and 25 % knockout, demonstrating no significant increase in embryonic lethality in the absence of functional *GFR $\alpha$ 1* gene. Heterozygous mice were viable, normal in size, fertile and displayed no gross morphological or behavioural abnormalities (*GFR $\alpha$ 1*<sup>+/-</sup> animals all have two normal kidneys). For experimental purposes, heterozygous crosses were set up in order to generate offspring of all three possible genotypes, i.e. wild-type, heterozygote and knockout.

Pregnant females were killed by cervical dislocation at the appropriate stage of gestation and embryos were removed and collected in individually labelled sterile dishes of L-15 medium. At the time of establishing the cultures, the genotype of each embryo was unknown, hence the ganglia from each embryo had to be dissected, dissociated and plated separately. Nodose ganglia were dissected from E12 embryos and submandibular ganglia from E17 embryos, as detailed in section 3.2.1.. The remains of the embryo (at E12)

or just the legs and tail (at E17) were placed in correspondingly labelled 1.5 ml microfuge tubes and used for DNA extraction and genotyping (see section 3.2.3.). Pairs of ganglia were placed in individual tubes with CMF-HBSS and incubated in 0.02 % trypsin for 4 minutes for E12 tissue and 10 minutes at E17. Thereafter, 10 ml F-12 plus 10 % HIHS was added to each tube to inactivate the trypsin. The tubes were centrifuged at 2000 g for 2 minutes to sediment the ganglia, the F-12 was removed and 1 ml F-14 plus SATO was added to each tube. To standardise trituration between samples, the same flame-polished siliconised pipette was used to triturate the ganglia in each tube. The pipette was flushed through with HBSS between each sample to avoid cross-contamination. 10  $\mu$ l aliquots of each cell suspension were taken to monitor the trituration. The cells from each embryo were plated at a density of 500-2000 cells per dish in 2 ml of F-14 medium supplemented with 2 mM L-glutamine and SATO in 35 mm plastic tissue culture dishes (prepared as previously described, Chapter 2, section 2.2.1.), and placed in a 5.5 % CO<sub>2</sub> incubator at 37 °C. Neuronal survival was assessed as in section 3.2.1.. The genotypes were not determined until the end of the tissue culture experiment to avoid observer bias.

### **3.2.3. Genotyping of *GFR $\alpha$ 1* null mutant mice by PCR**

All stages in the preparation of DNA and subsequent PCR reactions were carried out in a plasmid DNA-free environment using standard procedures to prevent DNA contamination. DNA was extracted using a high salt method. Tissue from E12 and E17 mouse embryos was digested overnight in 500  $\mu$ l proteinase K solution at 55 °C, after which 210  $\mu$ l 5 M NaCl was added and mixed. After centrifugation at 13000 rpm for 10 minutes, 400  $\mu$ l of supernatant was removed and placed in fresh microfuge tubes, already containing an equal volume of 100 % ethanol. On mixing, DNA aggregates were clearly visible. The tubes were centrifuged again to pellet the DNA, the

ethanol was removed and the DNA pellet was washed with 500  $\mu$ l 70 % ethanol to remove any salts present in the pellet. After further centrifugation, the supernatant was discarded and the DNA pellet allowed to air-dry before being resuspended in an appropriate volume of water (usually 20-100  $\mu$ l dependent on pellet size). This DNA solution was ready for amplification by PCR.

In the generation of *GFR $\alpha$ 1* null mutant mice, a segment of the *GFR $\alpha$ 1* gene was replaced with a neomycin gene by homologous recombination in embryonic stem cells (Cacalano *et al.*, 1998). Each embryo can therefore carry one of three possible combinations of the two different *GFR $\alpha$ 1* alleles (i.e. wild-type or neo cassette). The presence of wild-type and mutated alleles was detected using a three primer PCR reaction as follows: A common primer (AU) was located in the *GFR $\alpha$ 1* coding sequence and could detect both alleles. A second primer (WT) was located in the region of the wild-type *GFR $\alpha$ 1* coding sequence that was replaced in the mutant, and therefore detected the wild-type allele only. The mutant allele was detected with a third primer (NEO) corresponding to part of the *neo* gene sequence. In the presence of these three primers, single PCR products of different sizes, 342 and 422 bp's, were amplified in wild-type and knockout animals respectively. Both PCR products were amplified in heterozygotes.

A reaction master mix was prepared using the appropriate volumes of reagents for the number of tubes required. Appropriate positive (known +/- DNA) and negative (no DNA) control PCR reactions were performed and all sample PCR reactions were set up in duplicate. Each reaction was carried out in a 20  $\mu$ l reaction volume in a 500  $\mu$ l microfuge tube as below:

2 $\mu$ l	10x reaction buffer
3 $\mu$ l	25 mM magnesium chloride
1 $\mu$ l	5 mM dNTPs
0.16 $\mu$ l	WT
0.16 $\mu$ l	AU
0.16 $\mu$ l	NEO
0.10 $\mu$ l	Taq thermostable DNA polymerase
1 $\mu$ l	template DNA
8.62 $\mu$ l	ddH <sub>2</sub> O
3.8 $\mu$ l	5x Q solution

The primer sequences were as follows:

WT	-5' CAGCTTCCTACCTAATCTG 3'
AU	-5' GTTGTAGAGAGACTTCTGC 3'
NEO	-5' GGAGCAAAGCTGCTATTGG 3'

Two drops of mineral oil were layered on top of the PCR reagents to prevent evaporation during thermocycling. After an initial denaturation step of 95 °C for 15 minutes, the PCR program comprised 33 cycles of the following conditions: 95 °C for 1 minute (denaturation), 55 °C for 1 minute (primer annealing) and 72 °C for 1 minute 30 seconds (elongation). This was followed by a final elongation stage of 72 °C for 10 minutes and completed with a refrigeration step at 4 °C.

The products of the PCR reaction were prepared in gel loading buffer and separated on 2 % agarose gels cast with TAE buffer and containing 1  $\mu$ g/ml ethidium bromide. The wild-type lower band and knockout upper band were visualised under ultra-violet light as shown in Figure 3.4..

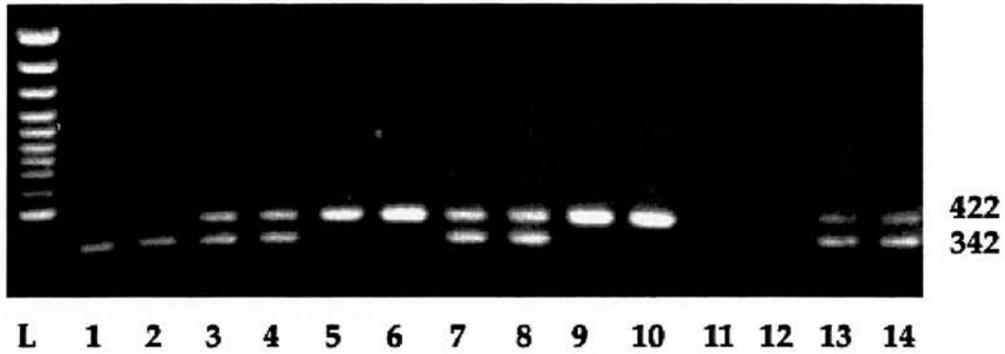


Figure 3.4. Gel showing the three possible genotypes of embryos from *GFRα1*<sup>+/-</sup> crosses.

Lanes 1 and 2 show the PCR products from wild-type DNA, lanes 3, 4, 7 and 8 show the PCR products from heterozygote DNA and lanes 5, 6, 9 and 10 show the PCR products from homozygote null mutant DNA. The PCR products of appropriate negative and positive control reactions are shown in lanes 11 and 12 (no DNA present) and 13 and 14 (known +/- DNA) respectively. The sizes of the products are shown with respect to a 1 kb reference ladder (L).

### 3.3. Results

#### 3.3.1. Survival responses of mouse trigeminal neurons to the GDNF ligand family

The mouse trigeminal ganglion is comprised of cutaneous sensory neurons innervating the facial region. These neurons are supported by BDNF and NT-3 at early stages of gangliogenesis (E10-11), and at later stages many switch survival dependence to NGF (E12 onwards) (Buchman and Davies, 1993; Paul and Davies, 1995; Piñón *et al.*, 1996; Enokido *et al.*, 1999). This is reflected in a 70 % loss of trigeminal neurons in *NGF*<sup>-/-</sup> (Crowley *et al.*, 1994) or *TrkA*<sup>-/-</sup> (Smeyne *et al.*, 1994) mice, a 60 % loss in *NT-3*-deficient mice (Ernfors *et al.*, 1994a; Fariñas *et al.*, 1994) and a 40 % deficit in *BDNF*<sup>-/-</sup> mice (Ernfors *et al.*, 1994; Jones *et al.*, 1994).

To determine if mouse trigeminal neurons are responsive to GDNF, neurturin or artemin, low-density, glial-free cultures of trigeminal ganglion neurons were established from E12, E15 and E18 mouse embryos and P3 neonates. The cultures were supplemented with NGF, GDNF, neurturin or artemin (each present at 10 ng/ml), alone or in combination. As expected, the majority of neurons at each age survived for 48 hours in saturating concentrations of NGF, whereas virtually all neurons grown in the absence of neurotrophic factors (control) died. Figure 3.5. demonstrates that at E12 and E15 there was a negligible survival response to GDNF, neurturin or artemin. However, this increased to a relatively good survival response at E18 and P3. At both ages, trigeminal neurons were equally sensitive to GDNF and artemin, but less sensitive to neurturin at the single concentration used. At E18 (data not shown) and P3, there were no additive effects of combining GDNF and neurturin, indicating that the subsets of neurons responding to these factors are overlapping. Additive effects were observed, however,

when E18 or P3 trigeminal neurons were exposed to combinations of either GDNF or neurturin, together with artemin, suggesting that the subpopulation of neurons responding to GDNF and neurturin is at least partially distinct from that responding to artemin.

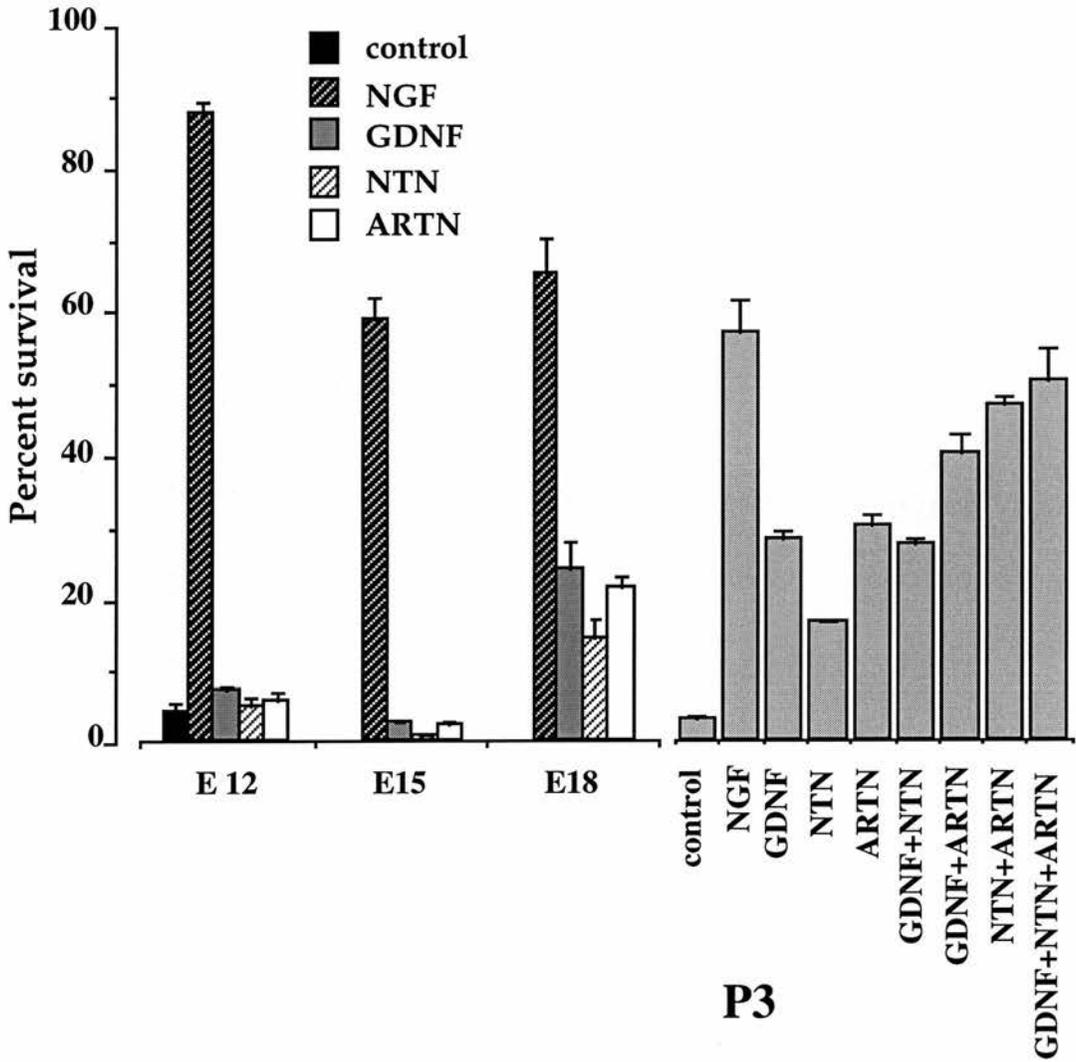


Figure 3.5. Bar chart showing percent survival of E12, E15, E18 and P3 trigeminal neurons cultured with members of the GDNF ligand family.

The number of neurons surviving after 48 hours is expressed as a percentage of the number of attached neurons counted 3 hours after plating. NGF, GDNF, NTN and ARTN were present at 10 ng/ml. The mean  $\pm$  standard error is shown ( $n \geq 3$  for each condition).

### 3.3.2. Survival responses of mouse DRG neurons to the GDNF ligand family

Dorsal root ganglia comprise a functionally heterogeneous population of sensory neurons that convey touch, pressure, pain and temperature sensations. Subpopulations of DRG neurons are supported by either NGF, BDNF or NT-3 in culture (Lindsay *et al.*, 1985). The relevance of these *in vitro* results has been demonstrated by deficits in the number of DRG neurons in mice with null mutations in either the *NGF* (Crowley *et al.*, 1994)/*TrkA* (Smeyne *et al.*, 1994) genes (70 % deficit in each mutation), *BDNF* (Ernfors *et al.*, 1994; Jones *et al.*, 1994)/*TrkB* (Klein *et al.*, 1993) genes (30 % deficit in each mutation) or NT-3 (Ernfors *et al.*, 1994a; Fariñas *et al.*, 1994)/ *TrkC* (Klein *et al.*, 1994) genes (65 % and 20 % losses respectively).

To examine the response of these neurons to GDNF, neurturin and artemin, low-density, glial-free cultures of lumbar DRG neurons were established from E12 and E19 embryos and P4 postnatal mice. The cultures were supplemented with NGF, GDNF, neurturin or artemin at 10 ng/ml, either alone or in combination. At each age studied, the majority of DRG neurons survived for 48 hours in saturating concentrations of NGF, and with the exception of P4 neurons, there was negligible survival in the absence of neurotrophic factors. Neither GDNF, neurturin or artemin could promote survival in E12 neurons, however, at late fetal and postnatal stages an increasing response to each of these factors was observed (Figure 3.6.). At the single concentration tested, DRG neurons appeared to be most sensitive to artemin and least sensitive to neurturin. When E19 (data not shown) and P4 neurons were grown in the presence of GDNF and neurturin together, some additional survival was apparent suggesting that the subpopulations of DRG neurons responding to GDNF and neurturin are at least partially distinct. Conversely, when E19 and P4 DRG neurons were exposed to

combinations of either GDNF or neurturin together with artemin, there were no additive effects. This indicates that all DRG neurons that respond to artemin also respond to GDNF and /or neurturin.

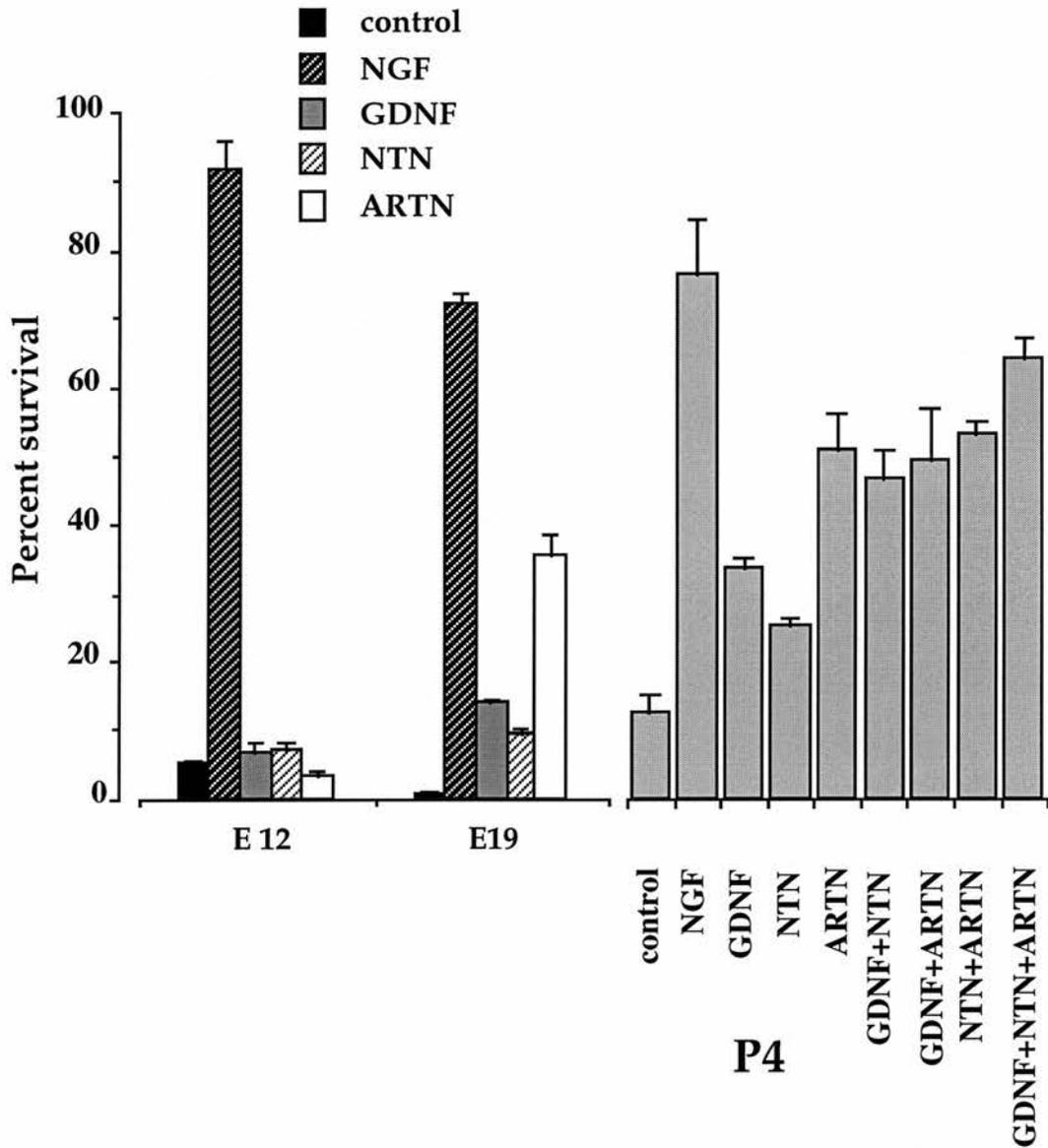


Figure 3.6. Bar chart showing percent survival of E12, E19 and P4 DRG neurons cultured with members of the GDNF ligand family.

The number of neurons surviving after 48 hours is expressed as a percentage of the number of attached neurons counted 3 hours after plating. NGF, GDNF, NTN and ARTN were present at 10 ng/ml. The mean  $\pm$  standard error is shown ( $n \geq 3$  for each condition).

### 3.3.3. Survival responses of mouse sympathetic neurons to the GDNF ligand family

Sympathetic neurons from the mouse superior cervical sympathetic ganglion and the thoracic paravertebral sympathetic chain were investigated. These neurons provide sympathetic innervation, via their postganglionic fibres, to involuntary effectors, such as smooth muscle and glands, of the cranial, cervical and thoracic region. The majority of sympathetic neurons are supported by NGF *in vitro* from E15 onwards (Wyatt and Davies, 1995), as demonstrated by the dramatic reduction in the number of SCG neurons in *NGF*<sup>-/-</sup> (Crowley *et al.*, 1994) and *TrkA*<sup>-/-</sup> (Smeyne *et al.*, 1994) mice (95 % deficit in each mutation by birth). A subset of sympathetic neurons acquires an additional requirement for NT-3 during the late embryonic/early postnatal period (Zhou and Rush, 1995; Wyatt *et al.*, 1997), accounting for a 50 % loss of SCG neurons in *NT-3* null mutant mice (Errfors *et al.*, 1994a; Fariñas *et al.*, 1994).

To determine if mouse sympathetic neurons are also responsive to members of the GDNF ligand family, low-density, glial-free cultures were established from E14 (SCG only) and E17 mouse embryos. Figure 3.7. demonstrates the response of SCG neurons to GDNF, neurturin and artemin, alone and in combination, each present at 10 ng/ml. As expected, the majority of E14 and E17 SCG neurons survived for 48 hours in the presence of 10 ng/ml NGF. The number of neurons surviving for 48 hours in E14 control cultures was high, demonstrating neurotrophin-independent survival of early sympathetic neurons (Ernsberger *et al.*, 1989; Wyatt and Davies, 1995). At E17, survival in control cultures was negligible. At both E14 and E17, GDNF and neurturin failed to elicit a survival response in SCG neurons. This was also the case for sympathetic neurons of the thoracic paravertebral chain (data not shown). Similarly, artemin was unable to promote a survival

response in E17 SCG neurons, however, in E14 SCG neurons, artemin promoted a survival response of similar magnitude to NGF. When artemin was present in combination with GDNF or neurturin at E14, synergism was apparent. This suggests that although GDNF and neurturin have no neurotrophic action on sympathetic neurons alone, they may facilitate artemin to exert its full neurotrophic potential. Alternatively, the presence of artemin, perhaps by acting as a cofactor or by increasing receptor expression, may allow GDNF and neurturin to elicit neurotrophic activity.

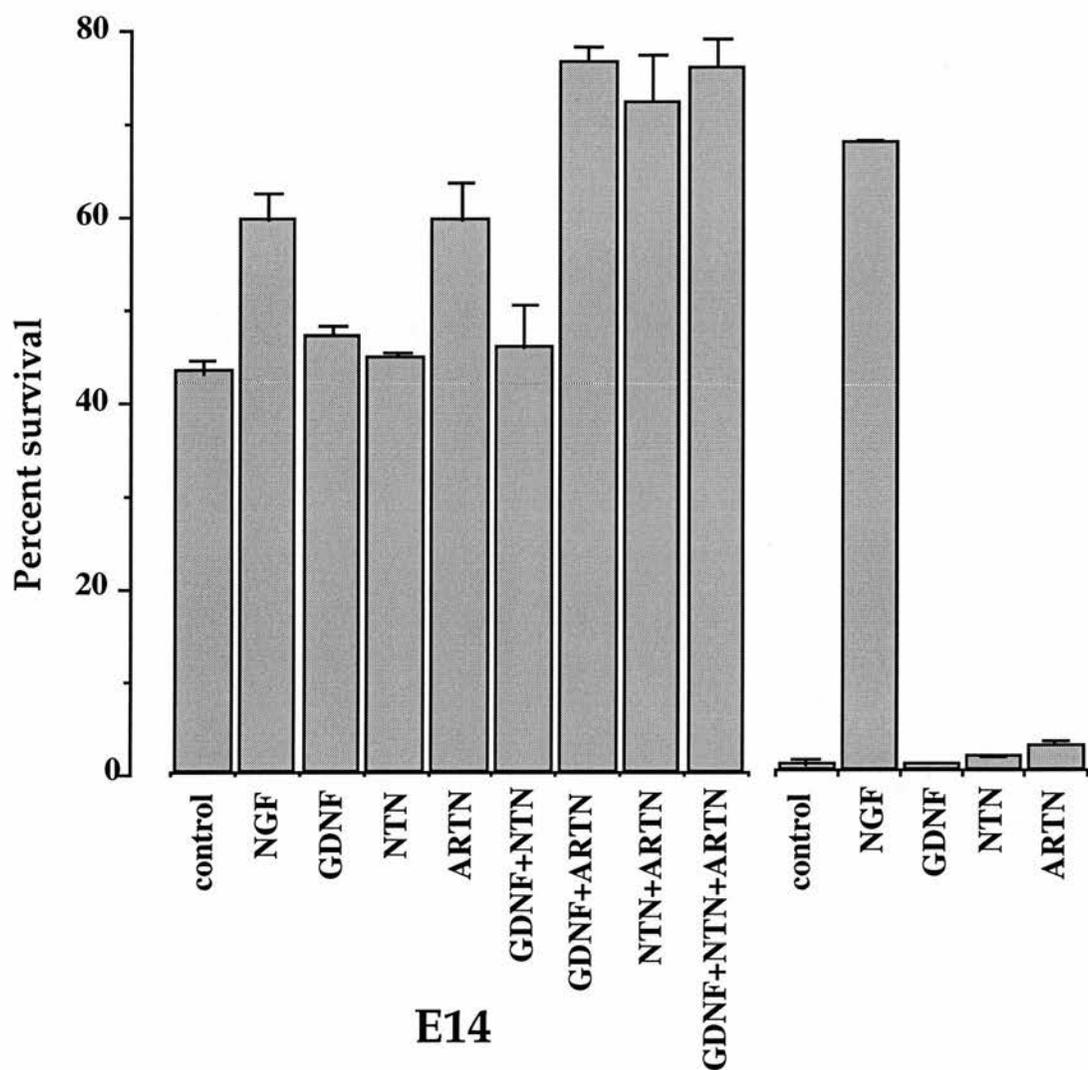


Figure 3.7. Bar chart showing percent survival of E14 and E17 sympathetic neurons cultured with members of the GDNF ligand family.

The number of neurons surviving after 48 hours is expressed as a percentage of the number of attached neurons counted 3 hours after plating. NGF, GDNF, NTN and ARTN were present at 10 ng/ml. The mean  $\pm$  standard error is shown ( $n \geq 3$  for each condition).

### 3.3.4. Survival responses of mouse nodose neurons to the GDNF ligand family

Neurons of the mouse nodose ganglion provide sensory innervation to the viscera of the thorax and abdomen. These sensory neurons are BDNF- and NT-4-responsive *in vitro* (Lindsay *et al.*, 1985; Davies *et al.*, 1993a), and hence, are markedly depleted in *BDNF*<sup>-/-</sup> (60%) (Ernfors *et al.*, 1994; Jones *et al.*, 1994), *NT-4*<sup>-/-</sup> (60%) (Liu *et al.*, 1995; Conover *et al.*, 1995) and *TrkB*<sup>-/-</sup> (90%) (Klein *et al.*, 1993) mice. NT-3 also plays a vital role in nodose gangliogenesis, reflected in the 40% loss of these neurons in NT-3-deficient mice (Ernfors *et al.*, 1994a; Fariñas *et al.*, 1994).

To ascertain the response of mouse nodose neurons to GDNF, neurturin and artemin, low-density, glial-free cultures, supplemented with these factors (each present at 10 ng/ml) were established. As shown in Figure 3.8., the majority of neurons at each age studied, survived for 48 hours in saturating concentrations of BDNF. With the exception of E12 nodose neurons, reflecting the relatively long period of neurotrophin-independent survival of early nodose neurons (Vogel and Davies, 1991), survival in cultures without added neurotrophic factors was negligible. GDNF and neurturin promoted survival of nodose neurons at each age, with the greatest response to both factors at E12. Artemin elicited only a very limited response which like that of GDNF and neurturin was greatest on younger nodose neurons. Experiments were carried out at E12, E15 and E18 to determine the effects of GDNF, neurturin and artemin in combination. No additive effects were detected (data not shown), indicating that the subpopulations of nodose neurons responding to these factors are largely overlapping.

To study the role of GFR $\alpha$ 1 in GDNF and neurturin signalling using *GFR $\alpha$ 1* null mutant mice, it was necessary to find a population of neurons in the

embryo that has a sizeable survival response to GDNF and neurturin. Due to the early death of *GFR $\alpha$ 1*<sup>-/-</sup> neonates, such experiments could not be carried out at postnatal stages, when several populations of peripheral neurons become increasingly responsive to GDNF and neurturin. For this reason, E12 nodose neurons were chosen for this study. Although, at the single concentration of GDNF and neurturin used in preliminary experiments, the sensitivity of nodose neurons to each of these factors was approximately the same, it was necessary to carry out a detailed dose response analysis before embarking on experiments in the *GFR $\alpha$ 1*<sup>-/-</sup> mouse. The dose response analysis, using concentrations of GDNF and neurturin ranging from 0.0064 ng/ml to 250 ng/ml, revealed a difference in the potency of these two factors. Figure 3.9. shows that although saturating concentrations of GDNF and neurturin promoted the survival of similar numbers of E12 nodose neurons, the neurons were more sensitive to GDNF than neurturin at lower concentrations. This is reflected in the EC<sub>50</sub> values, i.e., the concentration at which each factor exerts its half-maximal effect. The EC<sub>50</sub> for GDNF was 0.04 ng/ml and for neurturin was 1.32 ng/ml, indicating that GDNF is approximately 30-fold more potent at promoting nodose neuron survival than neurturin.

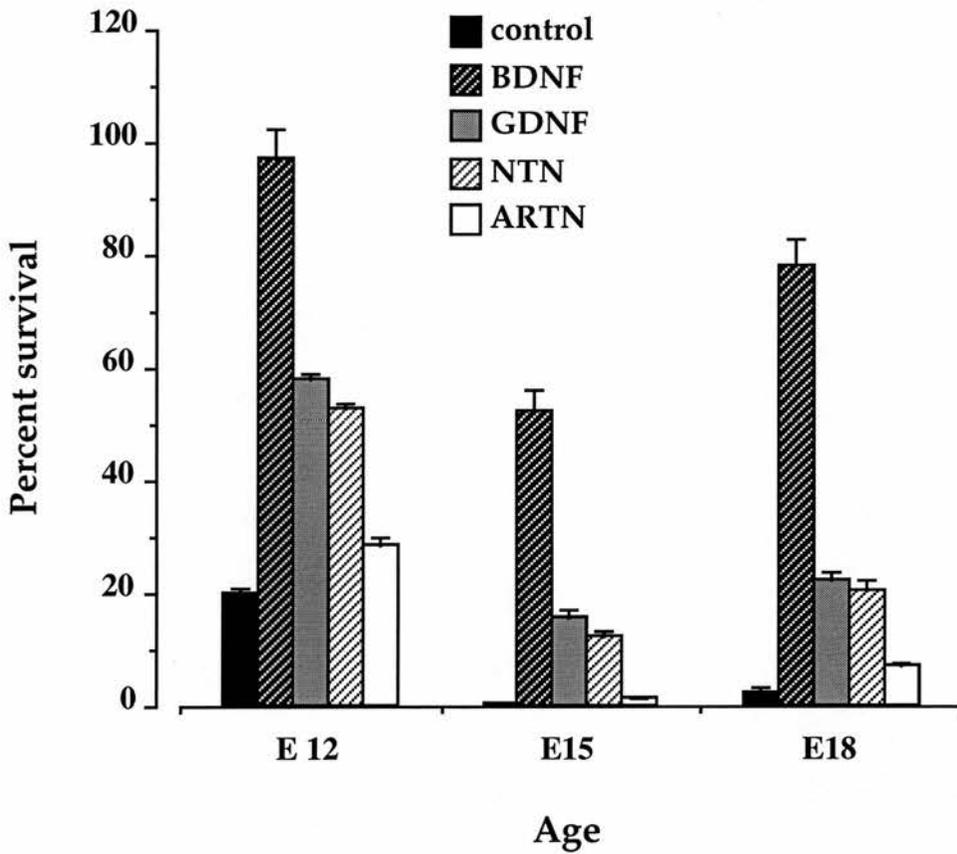


Figure 3.8. Bar chart showing percent survival of E12, E15 and E18 nodose neurons cultured with members of the GDNF ligand family.

The number of neurons surviving after 48 hours is expressed as a percentage of the number of attached neurons counted 3 hours after plating. BDNF, GDNF, NTN and ARTN were present at 10 ng/ml. The mean  $\pm$  standard error is shown ( $n \geq 3$  for each condition).

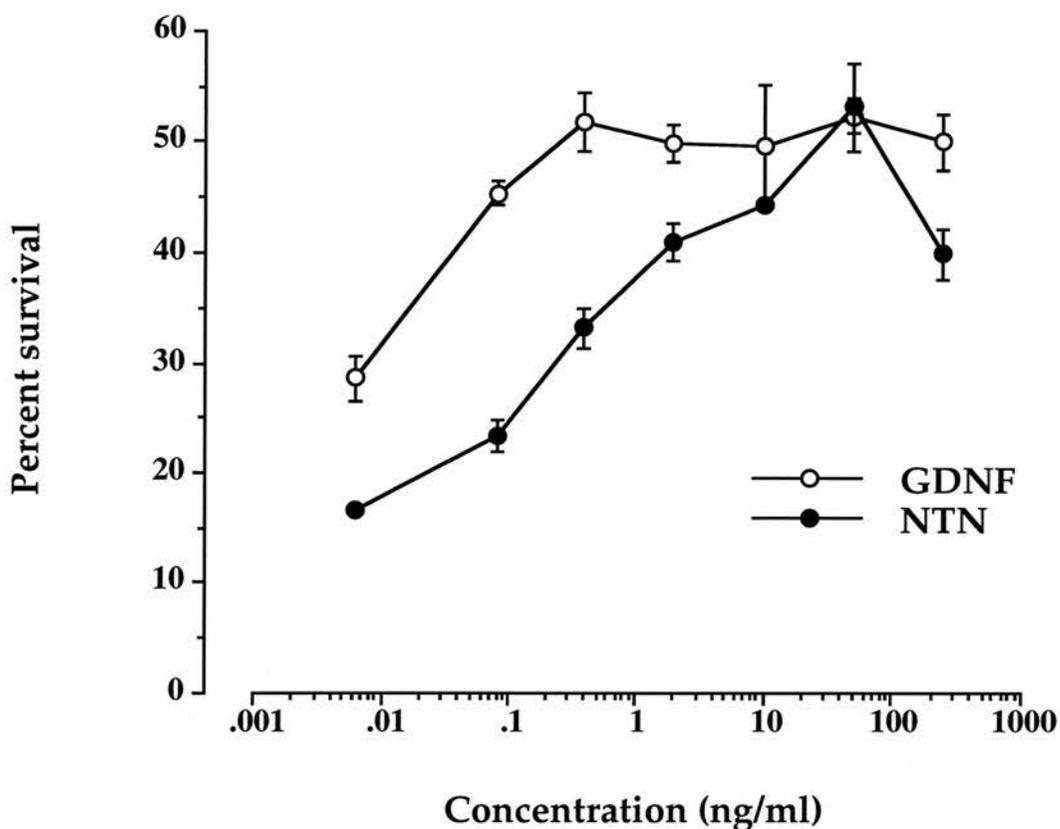


Figure 3.9. Graph showing the percent survival of E12 nodose neurons cultured with different concentrations of GDNF and neurturin.

The number of neurons surviving after 48 hours is expressed as a percentage of the number of attached neurons counted 3 hours after plating. GDNF and NTN were present at concentrations ranging from 0.0064 ng/ml to 250 ng/ml. In control cultures, survival was relatively high at 15 %, reflecting the ability of young nodose neurons to survive in the absence of neurotrophic factors. The mean  $\pm$  standard error is shown with the dose response taken from representative experiments set up in triplicate.

### 3.3.5. Survival responses of mouse parasympathetic neurons to the GDNF ligand family

The submandibular ganglion comprises parasympathetic neurons which provide innervation to the submandibular salivary glands and sublingual glands. The neurotrophic requirements of these neurons have not been extensively studied *in vitro*, however, CNTF was observed to promote the survival of the majority of these neurons in culture for 48 hours. To determine if submandibular neurons are responsive to members of the GDNF ligand family, low-density, glial-free cultures were established from E17 mouse embryos. After 48 hours incubation, neuronal survival in cultures without neurotrophins was negligible, whereas around 100 % of those supplemented with GDNF or neurturin (both present at 10 ng/ml) survived. Artemin (10 ng/ml) failed to elicit any survival response (data not shown). Such a dramatic survival response to GDNF and neurturin made submandibular neurons an ideal population for further study in the *GFR $\alpha$ 1* knockout mouse. First, it was necessary to analyse the response of these neurons to different concentrations of GDNF and neurturin (0.016 ng/ml to 250 ng/ml), as shown in Figure 3.10.. Although saturating concentrations of both factors promoted the survival of similar numbers of neurons, parasympathetic neurons of the submandibular ganglion were more sensitive to neurturin than GDNF, as reflected in the EC<sub>50</sub> values of 0.14 ng/ml for neurturin and 0.36 ng/ml for GDNF. Experiments in which submandibular neurons were grown with GDNF and neurturin in combination showed no additive effects (data not shown), indicating that the populations of neurons responding to each of these factors are overlapping.

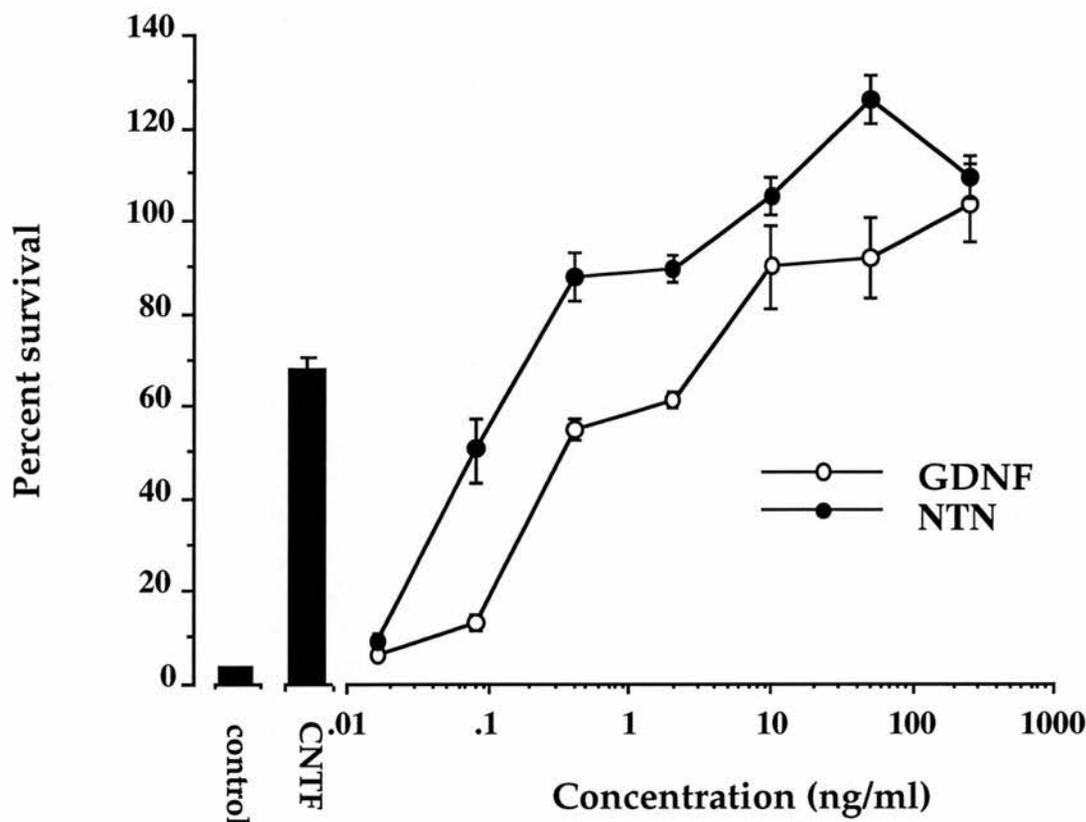


Figure 3.10. Graph showing the percent survival of E17 submandibular neurons cultured with different concentrations of GDNF and neurturin.

The number of neurons surviving after 48 hours is expressed as a percentage of the number of attached neurons counted 3 hours after plating. GDNF and NTN were present at concentrations ranging from 0.016 ng/ml to 250 ng/ml. Survival in 10 ng/ml CNTF is also shown. The mean  $\pm$  standard error is shown with the dose response taken from representative experiments set up in triplicate.

### 3.3.6. Survival responses of *GFR $\alpha$ 1* null neurons from the nodose ganglion to GDNF and neurturin

Having established that E12 nodose neurons show a good survival response to GDNF and to a lesser extent to neurturin, this study examined the response of nodose neurons, from *GFR $\alpha$ 1*-deficient mice to these factors. Low-density, glial-free cultures of nodose neurons from E12 wild-type, *GFR $\alpha$ 1*<sup>+/-</sup> and *GFR $\alpha$ 1*<sup>-/-</sup> embryos were supplemented with GDNF or neurturin, (2 or 50 ng/ml). Survival in BDNF (10 ng/ml) was also monitored. After 48 hours incubation, all neurons in control cultures were dead. Similar numbers of wild-type, *GFR $\alpha$ 1*<sup>+/-</sup> and *GFR $\alpha$ 1*<sup>-/-</sup> nodose neurons survived in the presence of BDNF, which mediates its signal through the TrkB tyrosine kinase receptor (Klein *et al.*, 1991a; Soppet *et al.*, 1991), indicating that the mutation had no effects on general cell viability. Absolute values for percent survival were reduced in these experiments compared to those carried out on neurons from CD1 mouse embryos. This may reflect differences in the genetic background of the mice. Alternatively, since these cultures were established from individual embryos and not from pooled material, the time taken to establish these cultures was considerably greater, and this may have affected cell viability. Figure 3.11. shows nodose neuron survival from each of the three genotypes after 48 hours in culture. GDNF promoted the survival of a subset of nodose neurons derived from wild-type embryos. However, consistent with the idea that *GFR $\alpha$ 1* is an essential GDNF receptor component, GDNF failed to rescue the majority of *GFR $\alpha$ 1*-deficient neurons, even when applied at high concentrations ( $p=0.0002$ , Student's t-test). Surprisingly, nodose ganglion neurons from the *GFR $\alpha$ 1*<sup>-/-</sup> lost responsiveness to both the low ( $p=0.002$ ) and high ( $p=0.0001$ , Student's t-test) concentrations of neurturin. Additionally, the survival of nodose neurons derived from *GFR $\alpha$ 1*<sup>+/-</sup> embryos was significantly reduced in the presence of GDNF and neurturin at both low and high concentrations

( $p < 0.05$ ) indicating a possible gene dosage effect. Thus, it appears that GFR $\alpha$ 1 is necessary for the survival response of nodose ganglion neurons to both GDNF and neurturin. In GFR $\alpha$ 1 $^{-/-}$  mice, the residual survival response to each of these factors is likely to be mediated by another member of the GFR $\alpha$  family, most probably GFR $\alpha$ 2.

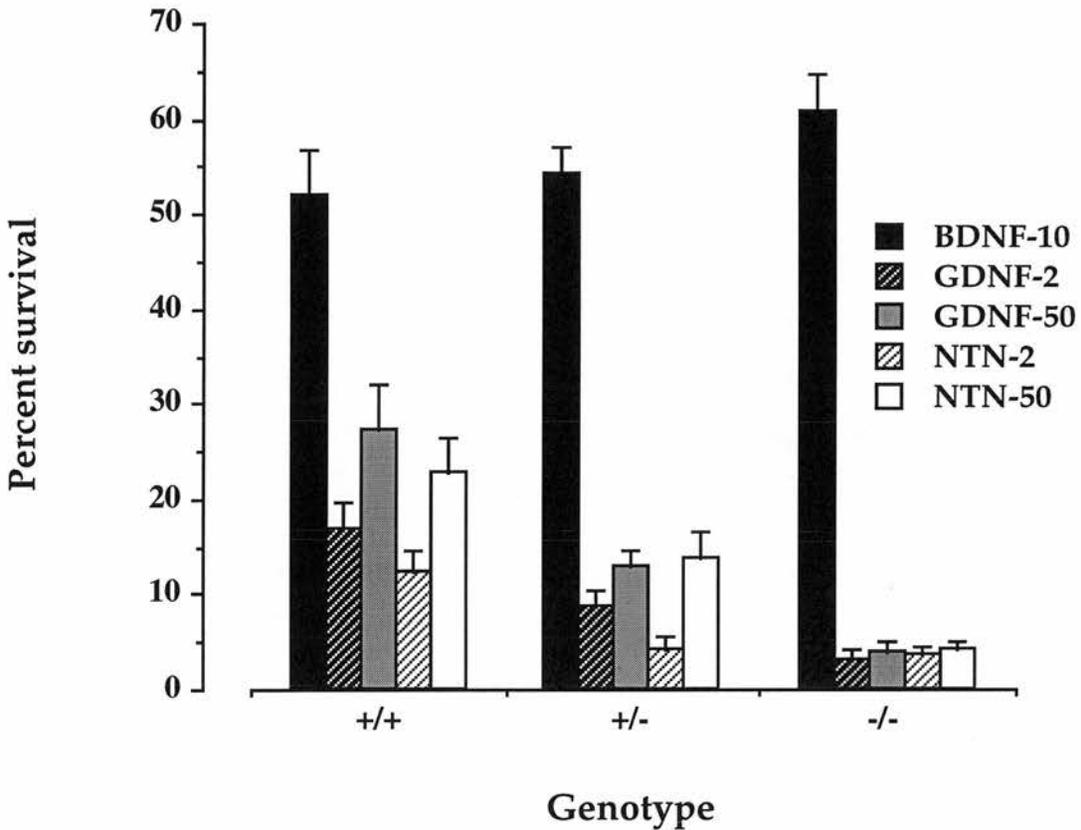


Figure 3.11. Bar chart showing percent survival of E12 nodose neurons from wild-type, GFR $\alpha$ 1 $^{+/-}$  and GFR $\alpha$ 1 $^{-/-}$  mice in the presence of GDNF and neurturin.

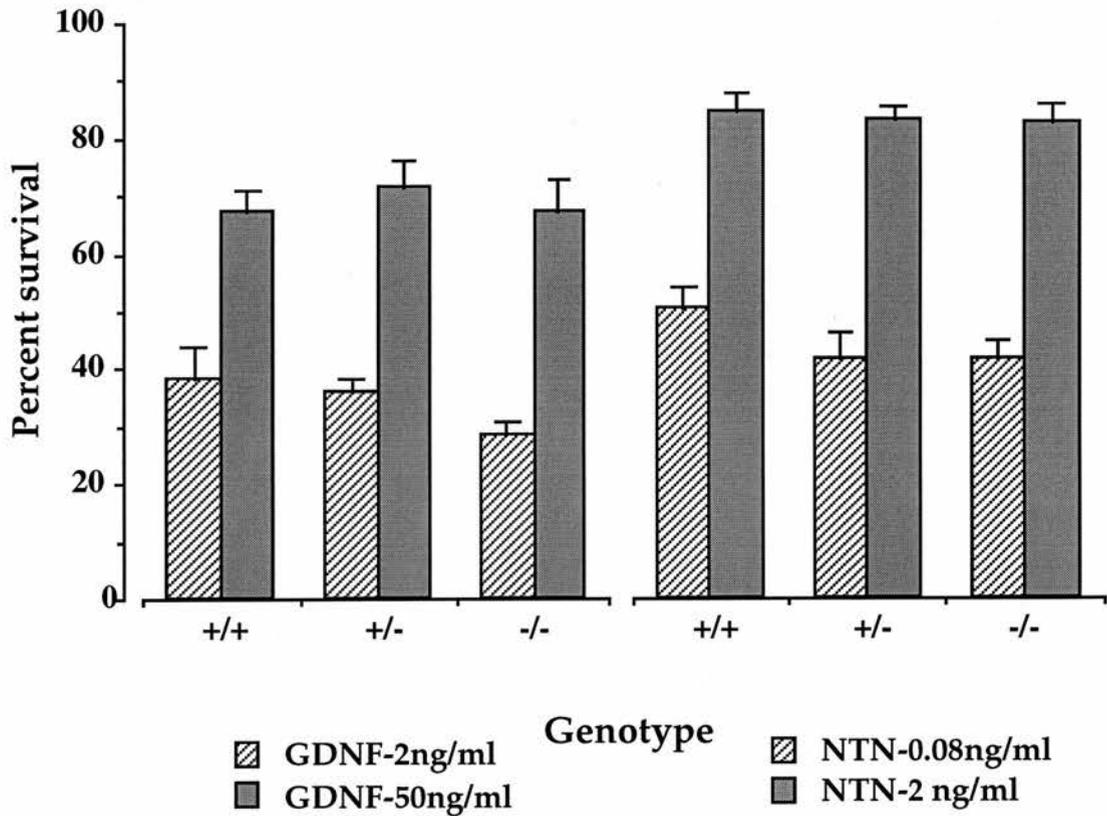
The number of neurons surviving after 48 hours is expressed as a percentage of the number of attached neurons counted 3 hours after plating. GDNF and NTN were present at concentrations of either 2 or 50 ng/ml. BDNF was present at 10 ng/ml. The mean  $\pm$  standard error are shown. Data were taken from cultures set up from 13 wild-type, 10 GFR $\alpha$ 1 $^{+/-}$  and 14 GFR $\alpha$ 1 $^{-/-}$  embryos. After 48 hours incubation, there were no surviving neurons in control cultures from each of the three genotypes.

### 3.3.7. Survival responses of *GFR $\alpha$ 1* null neurons from the submandibular ganglion to GDNF and neurturin

The role of the *GFR $\alpha$ 1* receptor in GDNF and neurturin signalling was also investigated in the parasympathetic neurons of the submandibular ganglion, the population of neurons that was found to be the most sensitive to neurturin. Low-density cultures of submandibular neurons were established from wild-type, *GFR $\alpha$ 1*<sup>+/-</sup> and *GFR $\alpha$ 1*<sup>-/-</sup> embryos and their survival in the presence of GDNF and neurturin was examined. Cultures were supplemented with two concentrations of GDNF (2 and 50 ng/ml) and neurturin (0.08 and 2 ng/ml) approximating to the half maximal and saturating concentration for each of these factors respectively. As shown in Figure 3.12. A, GDNF, at each of the concentrations used, promoted the survival of the majority of wild-type and *GFR $\alpha$ 1*<sup>+/-</sup> submandibular neurons. Surprisingly, although the response to GDNF was completely dependent on *GFR $\alpha$ 1* in nodose neurons, *GFR $\alpha$ 1*<sup>-/-</sup> submandibular neurons survived in the presence of GDNF as well as their wild-type counterparts (no significant differences in wild-type and knockout survival were found  $p > 0.05$ , Student's-test). Hence, it appears that in this neuronal population, GDNF can act via an alternative receptor, possibly *GFR $\alpha$ 2*. Figure 3.12. B demonstrates that neurturin also promoted the survival of the majority of wild-type and *GFR $\alpha$ 1*<sup>+/-</sup> submandibular neurons cultured for 48 hours. The survival response of submandibular neurons from *GFR $\alpha$ 1*<sup>-/-</sup> embryos to neurturin was also not compromised ( $p > 0.05$ , Student's t-test). After 48 hours incubation, all neurons in control cultures of all genotypes were dead.

In a limited number of experiments, a detailed dose response analysis was undertaken to compare the responsiveness of E17 submandibular neurons derived from wild-type and *GFR $\alpha$ 1* knockout embryos to a range of concentrations of GDNF and neurturin (0.016-50 ng/ml). As shown in

Figure 3.13. A, the response of wild-type and *GFR $\alpha$ 1*<sup>-/-</sup> submandibular neurons to GDNF was indistinguishable. Similarly, as shown in Figure 3.13. B, wild-type and *GFR $\alpha$ 1*<sup>-/-</sup> submandibular neurons exhibited a comparable survival response to neurturin over the range of concentrations tested. Thus, unlike nodose neurons, the response of submandibular neurons to GDNF and neurturin does not require GFR $\alpha$ 1 and must be mediated by a different receptor, most likely another member of the GFR $\alpha$  family.



**A**

**B**

Figure 3.12. Bar chart showing percent survival of E17 submandibular neurons from wild-type,  $GFR\alpha1^{+/-}$  and  $GFR\alpha1^{-/-}$  mice in the presence of GDNF (A) and neurturin (B).

The number of neurons surviving after 48 hours is expressed as a percentage of the number of attached neurons counted 3 hours after plating. GDNF and NTN were present at concentrations of 2 and 50 ng/ml and 0.08 and 2 ng/ml respectively. The mean  $\pm$  standard error are shown. Data were taken from cultures set up from 8 wild-type, 10  $GFR\alpha1^{+/-}$  and 12  $GFR\alpha1^{-/-}$  embryos.

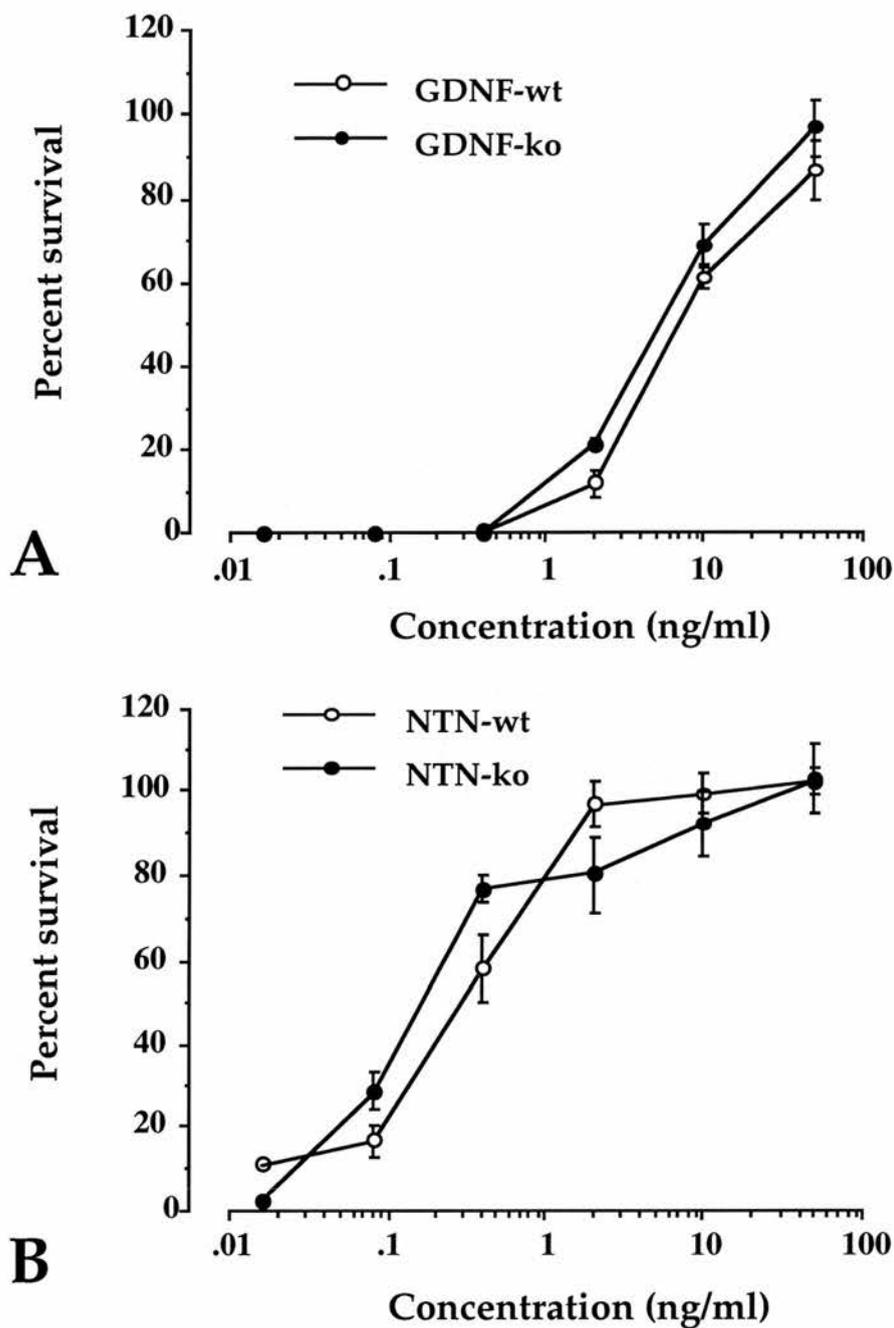


Figure 3.13. Graphs showing the percent survival of E17 submandibular neurons derived from wild-type and  $GFR\alpha1^{-/-}$  mice with different concentrations of GDNF (A) and neurturin (B).

The number of neurons surviving after 48 hours is expressed as a percentage of the number of attached neurons counted 3 hours after plating. GDNF and NTN were present at concentrations ranging from 0.016 ng/ml to 50 ng/ml. The mean  $\pm$  standard error is shown with the dose response taken from representative experiments set up in triplicate.

### **3.4. Discussion**

#### **3.4.1. The response of mouse sensory and autonomic neurons to members of the GDNF ligand family**

This preliminary study investigated the ability of members of the GDNF ligand family to promote the survival of mouse parasympathetic, sympathetic and sensory neurons at different stages of development. Unlike the considerable survival promoting effects of GDNF and neurturin on peripheral neurons derived from chicken embryos, the effects of these factors on mouse neurons were restricted to particular populations of neurons at specific stages of development. Similarly, artemin had only a limited effect on the survival of embryonic mouse PNS neurons.

Mouse trigeminal neurons are not responsive to GDNF and neurturin until late foetal stages, with the response increasing in the postnatal period. Given the fact that rodent development is more protracted than in avians, this can be likened to the situation in chicken, where trigeminal neurons acquire responsiveness to these factors during the mid-embryonic period. A similar late foetal/postnatal response to GDNF and neurturin was observed in neurons of the dorsal root ganglia. In contrast to trigeminal and DRG neurons, GDNF and neurturin supported nodose neurons most effectively in the early stages of gangliogenesis. As in the chicken, nodose neurons were found to be more sensitive to GDNF than neurturin in the mouse. While neither GDNF nor neurturin could support the survival of sympathetic neurons from the superior cervical ganglion in culture, parasympathetic neurons of the submandibular ganglion were highly responsive to both these factors, and like parasympathetic neurons of the chicken ciliary ganglion, were more sensitive to neurturin than GDNF.

The peripheral expression patterns of GDNF and neurturin suggest that both these factors may be available as target-derived neurotrophic factors for the PNS neuronal populations that respond to them *in vitro*. GDNF (Suter-Crazzolaro and Unsicker, 1994; Trupp *et al.*, 1995; Suvanto *et al.*, 1996) and neurturin (Kotzbauer *et al.*, 1996; Widenfalk *et al.*, 1997) transcripts are widely distributed in numerous peripheral tissues that receive sympathetic and sensory innervation. GDNF is expressed in skeletal muscle of the developing limb bud, consistent with a target-derived action on proprioceptive neurons of the DRG (Trupp *et al.*, 1995; Wright and Snider, 1996). GDNF (Trupp *et al.*, 1995) and neurturin (Widenfalk *et al.*, 1997; Luukko *et al.*, 1998) are both expressed in the embryonic whisker pad reinforcing a role for these factors in promoting the survival of a subpopulation of trigeminal neurons. High levels of neurturin mRNA are also expressed in the salivary glands (Widenfalk *et al.*, 1997) which receive innervation from parasympathetic neurons shown to be highly responsive to neurturin in culture. It is interesting to note that endogenous production of GDNF mRNA is detected in newborn DRG and SCG of the rat suggesting that, in addition to its role as a target-derived neurotrophic factor, GDNF may have a paracrine or autocrine mechanism of action in these ganglia (Trupp *et al.*, 1995).

Data from *GDNF* and neurturin null mutant mice have clarified the physiological significance of these factors in the development of the peripheral nervous system, identifying GDNF as an essential neurotrophic factor for subpopulations of sensory neurons of the nodose and dorsal root ganglia and sympathetic neurons of the superior cervical ganglion (Moore *et al.*, 1996). Additionally, the depletion of GFR $\alpha$ 1-expressing trigeminal neurons in the *GDNF*<sup>-/-</sup> mouse indicates a physiological role for GDNF in supporting this subpopulation of trigeminal neurons (Naveilhan *et al.*, 1998). With the exception of sympathetic neurons, the deficits observed in these

null mutant animals largely correlate with the responsiveness of these neurons to GDNF *in vitro*. Although the majority of GDNF-responsive neurons also respond to neurturin *in vitro*, neurturin is unable to rescue these neurons *in vivo* in the absence of GDNF. In accordance with *in vitro* data, the profound deficits, in submandibular and ciliary neuronal populations as well as GFR $\alpha$ 2-expressing neurons of the trigeminal (68 % loss) and dorsal root (45 % loss) ganglia in the neurturin null mutant, demonstrate a role for neurturin as an essential neurotrophic factor for parasympathetic neurons and some sensory neurons (Heuckeroth *et al.*, 1999). Interestingly, no neurons are lost in the nodose ganglion of *NTN* knockout mice, although these neurons respond in culture to this factor. Thus, the *in vitro* survival response of nodose neurons to neurturin is not physiologically relevant for the *in vivo* survival of these neurons.

The limited responsiveness of mammalian sensory and sympathetic neurons to GDNF *in vitro* observed in this study agrees with previous reports (Henderson *et al.*, 1994; Trupp *et al.*, 1995), whereas the observed responsiveness to neurturin is less than previously reported. For example, it has been shown that E18 rat nodose neurons are supported *in vitro* by neurturin to a greater extent than by BDNF and that neurturin can promote the survival of E21 rat sympathetic neurons almost as well as NGF (Kotzbauer *et al.*, 1996). In addition, E12 mouse trigeminal neurons have been reported to respond to neurturin, however, this study considered only explant cultures (Luukko *et al.*, 1998).

Recent data has suggested that GDNF requires the presence of TGF- $\beta$  to exert its full neurotrophic potential on peripheral neurons *in vitro* (Krieglstein *et al.*, 1998a). Immunoneutralisation of TGF- $\beta$ , present in the serum supplement of the culture medium, has been reported to abolish the

neurotrophic effect of GDNF on chicken peripheral neurons. Hence, it has been suggested that the reason that GDNF does not support the survival of as many mammalian peripheral neurons in low-density dissociated culture is that mammalian neurons are cultured in a chemically defined medium, whereas chicken neurons are cultured in serum-containing medium. In support of this hypothesis, it has been shown that GDNF and TGF- $\beta$  receptors are colocalised on GDNF-responsive neuronal populations *in vivo*. Despite these findings, and the demonstration that GDNF rescues target-deprived sympathetic spinal cord neurons only in the presence of TGF- $\beta$  *in vivo* (Schober *et al.*, 1999), this seems an unlikely explanation for the apparent discrepancy in sensitivity of embryonic mammalian and chicken peripheral neurons to GDNF, as the administration of exogenous TGF- $\beta$  (each of the three isoforms) to mammalian peripheral neurons cultured in defined medium does not confer a survival response to GDNF (Doxakis and Andres, personal communication).

Sensory neurons of the trigeminal and dorsal root ganglia acquired responsiveness to artemin at late foetal stages, and this response increased in the postnatal period. Artemin was unable to support the survival of nodose sensory neurons and parasympathetic submandibular neurons *in vitro*, whereas a survival response was observed in early, but not late embryonic, sympathetic neurons. These results are in agreement with a previous study demonstrating a survival response of P1 rat trigeminal and dorsal root ganglia neurons *in vitro* to artemin. Contrary to the results presented in this chapter, artemin was also shown to support newborn nodose sensory and sympathetic neurons in this study (Baloh *et al.*, 1998). Expression of artemin mRNA is predominantly outwith the CNS, with high levels of expression found in peripheral tissues, nerves and ganglia. This expression pattern suggests a role for artemin as a paracrine and/or target-derived factor for

developing peripheral neurons (Baloh *et al.*, 1998). Consistent with this idea is the finding that GFR $\alpha$ 3, the preferred coreceptor for artemin signalling, is strongly expressed in particular peripheral ganglia throughout development. For example, neuronal subpopulations of the trigeminal ganglion, dorsal root ganglia and sympathetic ganglia all express high levels of GFR $\alpha$ 3 (Worby *et al.*, 1998; Naveilhan *et al.*, 1998; Baloh *et al.*, 1998a; Widenfalk *et al.*, 1998; Trupp *et al.*, 1998), substantiating the *in vitro* findings detailed above. Nevertheless, it should be noted that expression of GFR $\alpha$ 3 mRNA in these ganglia is also found at stages of development other than those at which the neurons respond to artemin *in vitro*. Experiments assessing the survival-promoting effects of various combinations of GDNF, neurturin and artemin in trigeminal neurons demonstrated that the subpopulations responding to GDNF and artemin are partially distinct, whilst the neurturin and artemin-responsive subsets are almost entirely distinct. This *in vitro* finding is consistent with the pattern of GFR $\alpha$  expression within distinct subpopulations of trigeminal neurons. Whereas some neurons coexpress GFR $\alpha$ 1 and GFR $\alpha$ 3, no neurons coexpress GFR $\alpha$ 2 and GFR $\alpha$ 3 (Naveilhan *et al.*, 1998). In contrast to these additive effects, trophic synergism was apparent in cultures of E14 superior cervical sympathetic neurons treated with combinations of artemin with GDNF or neurturin. This is particularly interesting given the complete loss of the SCG in *Ret*<sup>-/-</sup> mice (Schuchardt *et al.*, 1994; Durbec *et al.*, 1996), 35 % loss of SCG neurons in *GDNF*<sup>-/-</sup> mice (Moore *et al.*, 1996) and no loss in *NTN*<sup>-/-</sup> mice (Heuckeroth *et al.*, 1999), suggesting that another member of the GDNF ligand family is important in supporting these neurons at an early stage in their development. Artemin, possibly acting in concert with GDNF or neurturin, represented a potential candidate. This assertion has very recently been substantiated by analysis of mice with a null mutation in the *GFR $\alpha$ 3* gene (Nishino *et al.*, 1999). At birth, *GFR $\alpha$ 3*<sup>-/-</sup> mice exhibit severe defects in the SCG, whereas other ganglia

appear normal. A detailed study of the number and location of SCG precursors in *GFRα3*<sup>-/-</sup> embryos has demonstrated that the rostral migration of SCG precursor cells is impaired. After birth, the few remaining SCG neurons undergo progressive cell death indicating that GFRα3-mediated signalling is also required for the survival of mature SCG neurons. Furthermore, the synergistic effects of GDNF and artemin may indicate why there is a substantial deficit in SCG neurons of newborn *GDNF* null mutant mice, whilst no survival-promoting effects of GDNF on embryonic sympathetic neurons *in vitro* can be found.

#### **3.4.2. The role of GFRα1 in GDNF and neurturin signalling**

The lack of a survival response of nodose neurons to GDNF, in the absence of GFRα1, demonstrates the essential role of this receptor in mediating the GDNF signal within this neuronal population. In similar experiments, primary dopaminergic neurons from *GFRα1*<sup>-/-</sup> embryos were also no longer able to respond to GDNF at any concentration tested, unlike their wild-type counterparts (Cacalano *et al.*, 1998). Neuronal survival, in the presence of GDNF was, however, restored following addition of exogenous, soluble, recombinant GFRα1 to neurons from *GFRα1*<sup>-/-</sup> mice, supporting the idea that these neurons failed to survive in the presence of GDNF solely due to the absence of GFRα1. These data confirm previous findings from fibroblast and neuron overexpression studies demonstrating that Ret alone is insufficient to mediate GDNF signalling (Jing *et al.*, 1996; Treanor *et al.*, 1996; Baloh *et al.*, 1997; Buj-Bello *et al.*, 1997; Trupp *et al.*, 1998).

The physiological relevance of the GFRα1 receptor *in vivo* is also confirmed by the analysis of the phenotype of *GFRα1*<sup>-/-</sup> mice. These mice have a deficit of lumbar (24 %) and trigeminal (22 %) motoneurons and nodose ganglion sensory neurons (15 %) (Cacalano *et al.*, 1998). In addition, they display a

complete absence of the enteric nervous system distal to the stomach and exhibit a bilateral renal agenesis (Cacalano *et al.*, 1998; Enomoto *et al.*, 1998). The striking similarities in the phenotypes of  $GFR\alpha1^{-/-}$  (Cacalano *et al.*, 1998; Enomoto *et al.*, 1998),  $Ret^{-/-}$  (Schuchardt *et al.*, 1994) and  $GDNF^{-/-}$  (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996) mice, particularly with respect to deficits in the kidneys and enteric nervous system, strongly supports the proposal that these three molecules are components of the same signalling cascade, and that Ret and  $GFR\alpha1$  serve as coreceptors for GDNF.

Biochemical and cell culture studies have generated conflicting results regarding the specificity of the  $GFR\alpha$  receptors. This study has provided new data on the ligand specificity of  $GFR\alpha1$  *in vivo*. The results presented in this chapter show that GDNF can, in certain cell types, mediate its response through an alternative receptor, as illustrated by the ability of GDNF to promote the survival of  $GFR\alpha1^{-/-}$  submandibular neurons. Furthermore, examination of  $GFR\alpha1^{-/-}$  neurons for their response to neurturin has demonstrated that  $GFR\alpha1$  can serve as a coreceptor for neurturin on nodose ganglion neurons, while submandibular neurons respond to this factor via another receptor, most likely  $GFR\alpha2$ . Similar experiments have demonstrated that while wild-type dopaminergic neurons are responsive to both GDNF and neurturin *in vitro*, neither GDNF nor neurturin can promote the survival of these neurons from  $GFR\alpha1^{-/-}$  embryos. Thus, as for nodose neurons,  $GFR\alpha1$  functions as a receptor for both GDNF and neurturin in developing dopaminergic neurons *in vitro*. Although these findings are consistent with the proposal that GDNF and neurturin can activate multiple receptors, it is important to note that these receptors are activated with different potencies, as shown by dose response survival assays. In nodose neurons,  $GFR\alpha1$  appears to be preferentially activated by GDNF, whereas

the receptor present on submandibular neurons is preferentially activated by neurturin.

It appears that the receptor-ligand interactions demonstrated in fibroblast overexpression models (i.e. that GDNF and neurturin can each signal via GFR $\alpha$ 1 or GFR $\alpha$ 2, albeit with different potencies of interaction) (Sanicola *et al.*, 1997; Trupp *et al.*, 1998; Worby *et al.*, 1998; Creedon *et al.*, 1997; Baloh *et al.*, 1997, 1998a; Jing *et al.*, 1997; Naveilhan *et al.*, 1998) are borne out by studies of GFR $\alpha$ 1-deficient neurons *in vitro*. However, such promiscuous receptor-ligand interactions may not predominate *in vivo*. For example, the striking similarities in the phenotype of GDNF (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996) and GFR $\alpha$ 1<sup>-/-</sup> (Cacalano *et al.*, 1998; Enomoto *et al.*, 1998) mice with respect to deficits in the kidneys, enteric nervous system and trigeminal and spinal lumbar motoneurons suggests a very tight pairing of the GFR $\alpha$ 1-GDNF interaction in these tissues *in vivo*. In addition, *in situ* hybridisation experiments have revealed a reduction in GFR $\alpha$ 1-expressing neurons, but not GFR $\alpha$ 2- or GFR $\alpha$ 3-expressing neurons in the trigeminal ganglion of GDNF<sup>-/-</sup> mice, indicating that GFR $\alpha$ 1 is the preferred receptor for GDNF in the trigeminal ganglion *in vivo* (Naveilhan *et al.*, 1998). Nevertheless, despite the overall similarity in the phenotype of GDNF and GFR $\alpha$ 1 null mutant mice, some notable differences exist. The most striking of these is the 35 % loss of SCG neurons in GDNF<sup>-/-</sup> mice (Moore *et al.*, 1996) and a normal complement of SCG neurons in GFR $\alpha$ 1<sup>-/-</sup> mice (Cacalano *et al.*, 1998; Enomoto *et al.*, 1998). Likewise, GDNF null mutants display deficits of 40 % and 23 % respectively, in the number of nodose and DRG neurons (Moore *et al.*, 1996), whilst GFR $\alpha$ 1 null mutants display only a small deficit of 15 % in the number of nodose neurons and have no losses in DRG neurons (Cacalano *et al.*, 1998). The fact that GDNF<sup>-/-</sup> mice display more extensive neuronal deficits compared to GFR $\alpha$ 1<sup>-/-</sup> animals suggests that GDNF can

indeed mediate its signals through another GDNF family receptor, most probably GFR $\alpha$ 2, as demonstrated in parasympathetic submandibular neurons *in vitro*. Surprisingly, the comparison between *GDNF*<sup>-/-</sup> and *GFR $\alpha$ 1*<sup>-/-</sup> mice suggests that GDNF can rescue a subset of nodose neurons independently of GFR $\alpha$ 1 *in vivo*, whereas *in vitro*, GDNF could not promote the survival of nodose neurons derived from *GFR $\alpha$ 1*<sup>-/-</sup> embryos. Thus, it is possible that the effects of GDNF on this neuronal population *in vivo* are indirect or that GDNF influences the development of a subpopulation of nodose neurons at a stage other than that at which they were cultured.

New information about the specificity of GFR $\alpha$ 2 has recently been gleaned from studies of *NTN*<sup>-/-</sup> mice (Heuckeroth *et al.*, 1999). Neurturin-deficient mice display defects in the enteric nervous system and parasympathetic innervation to the lacrimal and salivary glands, not unlike the deficits observed in *GFR $\alpha$ 2*<sup>-/-</sup> animals, suggesting a tight pairing of GFR $\alpha$ 2-neurturin *in vivo*. This idea is reinforced by the depletion of GFR $\alpha$ 2-expressing trigeminal and dorsal root ganglion neurons from neurturin null mutant animals (Heuckeroth *et al.*, 1999). Similarly, trigeminal neurons derived from *GFR $\alpha$ 2*<sup>-/-</sup> embryos, in dissociated or explant culture, lose responsiveness to neurturin, whilst retaining a response to GDNF comparable with neurons derived from wild-type littermates (Rossi *et al.*, 1999). However, close inspection of the losses of parasympathetic neurons in neurturin and *GFR $\alpha$ 2* null mutants reveals some interesting differences. The numbers of parasympathetic ciliary and submandibular neurons are reduced by 48 % and 45 %, respectively, in *NTN*<sup>-/-</sup> mice (Heuckeroth *et al.*, 1999) and by 0 % and 81 %, respectively, in *GFR $\alpha$ 2*<sup>-/-</sup> mice (Rossi *et al.*, 1999). Hence, in ciliary neurons, neurturin can mediate its signal via an alternative GFR $\alpha$  receptor, whereas the increased cell loss in the submandibular ganglion of *GFR $\alpha$ 2*<sup>-/-</sup> mice relative to the neurturin knockout signifies that GFR $\alpha$ 2 is an essential

neurturin receptor for this population and also that additional GDNF ligands can utilise this receptor in the absence of neurturin to promote the survival of submandibular neurons in development. Finally *GFR $\alpha$ 2*<sup>-/-</sup> mice exhibit a severe growth retardation which is not evident in *NTN*<sup>-/-</sup> mice (Rossi *et al.*, 1999). Although the reason for this growth retardation is unclear, this significant difference between these two phenotypes suggests neurturin-independent signalling of *GFR $\alpha$ 2* *in vivo*.

Receptor-ligand cross talk *in vivo* may be governed by the temporal and spatial expression patterns of receptors and ligands. The limited ability of *GFR $\alpha$ 2* to compensate for loss of *GFR $\alpha$ 1* *in vivo* is consistent with the tissue distribution of these two receptors. For example, high levels of *GFR $\alpha$ 1* mRNA, but not *GFR $\alpha$ 2* mRNA, are found in nodose sensory ganglia neurons (Buj-Bello *et al.*, 1997) and on spinal motor neurons (Klein *et al.*, 1997; Widenfalk *et al.*, 1997), both of which show deficits in *GFR $\alpha$ 1*<sup>-/-</sup> mice. Likewise, developing dopaminergic neurons, which do not respond to GDNF *in vitro* in *GFR $\alpha$ 1*<sup>-/-</sup> mice, normally only express *GFR $\alpha$ 1* (Horger *et al.*, 1998). Similarly, the developing kidney, an organ that fails to develop in *GFR $\alpha$ 1*<sup>-/-</sup> mice, normally only expresses *GFR $\alpha$ 1* transcripts (Baloh *et al.*, 1997). In contrast, submandibular neurons, which retain responsiveness to both GDNF and neurturin in the absence of *GFR $\alpha$ 1*, express multiple *GFR $\alpha$* 's (Wyatt, personal communication). Likewise, DRG and SCG neurons which are unaffected in *GFR $\alpha$ 1*<sup>-/-</sup> mice, express both *GFR $\alpha$ 1* and *GFR $\alpha$ 2* during development (Baloh *et al.*, 1997; Nosrat *et al.*, 1997; Widenfalk *et al.*, 1998). Since the potencies with which GDNF and neurturin can each activate *GFR $\alpha$ 1* and *GFR $\alpha$ 2* are different, the successful activation of receptors *in vivo* may critically depend on the levels of expression of these ligands and receptors at different times. Additionally, the prevalence of receptor-ligand

cross talk *in vivo* may be influenced by the expression of different receptor splice variants which may respond differently to the GDNF ligands.

This study, together with other data from the analysis of *GFR $\alpha$ 1*<sup>-/-</sup> mice, has clarified the physiological significance of GFR $\alpha$ 1 for mediating responses to members of the GDNF ligand family in different populations of developing neurons. Despite the high degree of promiscuity in ligand-receptor interactions *in vitro*, these interactions are generally quite specific *in vivo* with GDNF predominantly utilising GFR $\alpha$ 1 and neurturin mainly activating GFR $\alpha$ 2, although notable exceptions do exist. Although GDNF and neurturin may be able to activate more than one GFR $\alpha$  receptor *in vivo*, their distinct functions and ligand/receptor specificity may be controlled, to a large extent, by the tissue distribution and levels of expression of both ligands and receptors and the receptor splice variants expressed by neurons. Generation and analysis of persephin, artemin and *GFR $\alpha$ 4* null mutant mice will further clarify these issues. The availability of double GFR $\alpha$  receptor knockouts and conditional knockouts would enable new functions of these signalling components to be elucidated, both in development and in the mature nervous system.

# Chapter 4

## Production of polyclonal antibodies to chicken GFR $\alpha$ 2 and GFR $\alpha$ 4

### 4.1. Introduction

The newly emerging family of GDNF ligands is responsible for the development and maintenance of various populations of sensory and autonomic neurons. GDNF, neurturin, persephin and artemin mediate their cellular responses via a shared transmembrane receptor tyrosine kinase, Ret, and one of a family of structurally-related accessory proteins, termed the GDNF family receptor alpha (GFR $\alpha$ ) receptors, which are thought to confer ligand specificity (reviewed in Airaksinen *et al.*, 1999; Saarma and Sariola, 1999).

To date, four members of the GFR $\alpha$  family have been identified: GFR $\alpha$ 1 (Durbec *et al.*, 1996; Jing *et al.*, 1996; Treanor *et al.*, 1996; Trupp *et al.*, 1996; Vega *et al.*, 1996), GFR $\alpha$ 2 (Baloh *et al.*, 1997; Buj-Bello *et al.*, 1997; Jing *et al.*, 1997; Klein *et al.*, 1997; Sanicola *et al.*, 1997; Suvanto *et al.*, 1997), GFR $\alpha$ 3 (Baloh *et al.*, 1998a; Masure *et al.*, 1998; Naveilhan *et al.*, 1998; Nomoto *et al.*, 1998; Trupp *et al.*, 1998; Worby *et al.*, 1998) and GFR $\alpha$ 4 (Thompson *et al.*, 1998). Amino acid sequence alignment demonstrates 49 % identity between chicken GFR $\alpha$ 1 and GFR $\alpha$ 2 and 40 % identity between these receptors and GFR $\alpha$ 4. GFR $\alpha$ 3 is the most divergent GFR $\alpha$  member, with only 35 % identity with GFR $\alpha$ 1 and GFR $\alpha$ 2. However, all GFR $\alpha$  receptors share some basic structural features. All lack a transmembrane domain and are anchored to the extracellular membrane via a glycosylphosphatidylinositol (GPI)-linkage. GFR $\alpha$ 1-4 all possess an amino-terminal, hydrophobic, putative signal peptide and the characteristic carboxy-terminal feature of GPI-linked proteins: a C-

terminal hydrophobic domain separated by a hydrophilic linker region from a cleavage/binding consensus sequence for GPI linkage (Gerber *et al.*, 1992). In addition, the GFR $\alpha$  family contains a highly conserved cysteine backbone; all 30 cysteines are conserved between chicken GFR $\alpha$ 1 and GFR $\alpha$ 2 and 28 are conserved in GFR $\alpha$ 3 and GFR $\alpha$ 4. This suggests similarities in the three-dimensional structure of these receptors which may be of functional significance regarding ligand-receptor interactions.

Although it is clear that Ret activation and signalling by GDNF family ligands requires one of these GPI-linked coreceptors, some controversy exists over the specificity of interaction between the various ligands and receptors. Initial studies using equilibrium and competition binding experiments (Klein *et al.*, 1997) demonstrated a straightforward GFR $\alpha$ 1/GDNF and GFR $\alpha$ 2/NTN receptor-ligand pairing. It is now known that the presence of Ret modifies these interactions enabling GFR $\alpha$ 1 and GFR $\alpha$ 2 receptors to interact with two or three ligands, however the physiological relevance of some of these interactions remains unclear (reviewed in Rosenthal, 1999; Saarma and Sariola, 1999). Recent data comparing mice with null mutations in the genes encoding GDNF ligands or GFR $\alpha$  receptors suggests that the importance of GFR $\alpha$ 1 and GFR $\alpha$ 2 in mediating responses to GDNF and neurturin differs among certain populations of neurons (Chapter 3; Cacalano *et al.*, 1998; Rossi *et al.*, 1999). Nevertheless, it can be generalised that GFR $\alpha$ 1 acts as the primary coreceptor for GDNF, whereas GFR $\alpha$ 2 mediates neurturin signals to Ret. GFR $\alpha$ 4 has been shown to be a receptor for persephin (Enokido *et al.*, 1998) and most recently, the orphan GFR $\alpha$ 3 receptor has been found to be the preferred coreceptor for artemin (Baloh *et al.*, 1998).

Expression of GFR $\alpha$  family members has been considered in several studies, all investigating mRNA levels using Northern blotting, RT-PCR or *in situ* hybridisation techniques. GFR $\alpha$ 1 and GFR $\alpha$ 2 mRNAs, although having distinct spatial and temporal expression patterns, are both widely distributed in neuronal and non-neuronal tissues during development and into adulthood (Trupp *et al.*, 1997; Sanicola *et al.*, 1997; Nosrat *et al.*, 1997; Buj-Bello *et al.*, 1997; Jing *et al.*, 1997; Widenfalk *et al.*, 1997; Wang *et al.*, 1998a; Worby *et al.*, 1998; Thompson *et al.*, 1998). GFR $\alpha$ 3 is expressed in several peripheral tissues and organs, but, the greatest levels of expression are associated with nerves and ganglia of the peripheral nervous system (Trupp *et al.*, 1998; Widenfalk *et al.*, 1998; Baloh *et al.*, 1998a; Worby *et al.*, 1998; Jing *et al.*, 1997; Masure *et al.*, 1998). Within the central nervous system, however, GFR $\alpha$ 3 mRNA has a very limited expression, being detected only at very early embryonic stages (Masure *et al.*, 1998). GFR $\alpha$ 4 mRNA has been detected in developing chicken brain and peripheral tissues (Thompson *et al.*, 1998). These overlapping but distinct patterns of GFR $\alpha$  mRNA expression underscore the unique physiological roles of these receptors.

As protein expression patterns will not necessarily mirror those of their respective mRNAs, it is vital to study protein expression of the GFR $\alpha$  receptors to understand further the nature of the ligand-receptor interactions. Analysis of protein expression may also yield useful information regarding protein distribution within intracellular compartments or on different parts of the plasma membrane. With this in mind, this study aimed to produce polyclonal antibodies specific to two of the chicken GFR $\alpha$  receptors, recently cloned by colleagues, GFR $\alpha$ 2 (Buj-Bello *et al.*, 1997) and GFR $\alpha$ 4 (Thompson *et al.*, 1998). Given a successful immunisation program, the specific antibodies generated are powerful tools for studying various aspects of receptor expression, regulation and function, using a number of different assays.

Such an approach has been used previously to evaluate and characterise several neurotrophic factor proteins and their signalling receptors. For example, specific antibodies to TrkA, TrkB and TrkC have been used to identify the subsets of sensory neurons that express these receptors (Loy *et al.*, 1994; Lefcort *et al.*, 1996; Fariñas *et al.*, 1998; Huang *et al.*, 1999).

Using immunoblotting, the presence, quantity and size of antigen can be detected and it was hoped to utilise antibodies against the GFR $\alpha$  receptors to indicate the levels of receptor protein expression in various chicken peripheral tissues and brain. Furthermore, it would be informative to ascertain the pattern of receptor expression in populations of peripheral neurons known to respond to members of the GDNF ligand family. In the developing chicken, it has been demonstrated that in many peripheral neuronal populations, responsiveness to GDNF ligands differs between family members and responsiveness to a single factor can change throughout development (Chapter 2; Forgie *et al.*, 1999). Such changes in responsiveness may be, at least in part, attributed to changes in receptor expression. This was investigated by considering GFR $\alpha$ 1, GFR $\alpha$ 2, GFR $\alpha$ 4 and Ret mRNA levels. However, developmental changes in responsiveness to GDNF and neurturin were not consistently paralleled by changes in the relative levels of GFR $\alpha$  transcripts (Chapter 2; Forgie *et al.*, 1999). As it is possible that the levels of GFR $\alpha$  proteins are not reflected by the steady state levels of GFR $\alpha$  transcripts, this study aimed to address this question by considering receptor protein expression during development. It was hoped to use a semi-quantitative or quantitative immunoblotting technique to analyse GFR $\alpha$  expression in whole ganglia and purified neurons at various stages in chicken embryogenesis, corresponding to the periods at which the most dramatic changes in neuronal responsiveness are observed. Specific antibodies are also very useful for immuno-staining of either cultured cells or

histological specimens. Hence, it was hoped to undertake immunocytochemistry and immunohistochemistry experiments to demonstrate receptor expression within certain neuronal populations and to allow cellular localisation of these signals. A further application of antibodies is in neutralising experiments. Antibodies against neurotrophic proteins or their receptors are occasionally useful as function-blocking reagents. Such function-blocking antibodies against GFR $\alpha$  receptors would be useful tools for investigating ligand-receptor cross talk in various neuronal populations.

The success of these various applications of antibody technology depend on the quality of the initial product, i.e. the polyclonal antibodies generated as a result of an immunisation schedule. Although anti-GFR $\alpha$  antibodies were produced, the poor specificity of these antibodies and problems with their purification limited their usefulness.

## 4.2. Methods

### 4.2.1. Peptide selection and coupling

The use of synthetic peptides as immunogens is a well recognised technique that has both advantages and disadvantages over the alternative method of using bacterially expressed proteins. With careful selection of the sequence and coupling technique, most peptides elicit a good immunological response. Also, this approach is preferred if the protein is a member of a highly conserved protein family. Although anti-peptide antibodies generally bind well to denatured proteins, their major disadvantage is that they may not recognise the native antigen. However, because of the close sequence similarity of GFR $\alpha$  family members, synthetic peptides were employed as the antigen in this study. To increase the chances of success, two peptide sequences were selected from each of the predicted amino acid sequences of chicken GFR $\alpha$ 2 and GFR $\alpha$ 4 (Figure 4.1.). When designing peptides, there are no "hard and fast" rules guaranteeing a successful outcome. What works well for one antigen may not work at all for another. Nevertheless, there are some general guidelines to assist in choosing the appropriate peptide sequence. It should be noted that most peptide sequences will induce antibodies specific to the peptide itself; the key is to devise a peptide sequence such that the antibodies will recognise the native protein. Hence, it is important that the sequence chosen is exposed at the surface of the native protein and not buried within the tertiary structure. Internal hydrophilic regions or hydrophilic carboxyl or amino terminal regions are likely to be exposed and therefore, make good targets. It is also suggested that mobility of amino acid residues is important with flexible stretches more likely to be epitopes. Peptide size is also important with less than 6 residues being too small to elicit antibodies that will bind to the original protein and more than 20 being difficult to synthesize and couple. A compromise is to prepare

multiple small peptides (10-18 amino acids in length) from various regions of the sequence. As well as the above factors, two other criteria contributed to peptide selection in this study. First, within the GFR $\alpha$  family there are many cysteines, believed to be involved in the spatial organisation of the receptor molecule. Where the distance between adjacent cysteine residues is short, tight knotted structures are likely and these regions may therefore be hidden from the surface of the protein. For this reason, peptides were located in stretches of sequence where the interval between adjacent cysteines was as long as possible. Second, the aim of this study was to generate specific antibodies to each of the GFR $\alpha$  receptors. Therefore, the respective peptides had to be selected from regions of least homology between these proteins. Four peptides (5  $\mu$ mole scale), designated NT1 and NT2 from GFR $\alpha$ 2 and PF1 and PF2 from GFR $\alpha$ 4, were synthesized and purified by Alta Bioscience (University of Birmingham). The peptide sequences were as follows:

NT1 . . . CIHLGLAEGEEFYEA  
NT2 CKVEKSPALPDDINDSNT  
PF1 . . . CIHHTLMEGMNVLES  
PF2 CSISP TTQMYKQERNANR

N-terminal cysteines were necessary in all peptides to allow chemical coupling of the peptide to a carrier protein, as described below.

cGRF $\alpha$ 2	<u>MILANAF</u> <u>CIVL</u> <u>FVDE</u> TLR-SLAAPPS-PPGQDLQGWRVPVDCIRANKICAAEGSCS SRYR	58
cGRF $\alpha$ 4	<u>M</u> <u>R</u> <u>G</u> <u>I</u> <u>L</u> <u>Y</u> <u>F</u> <u>C</u> <u>T</u> <u>L</u> <u>I</u> <u>L</u> <u>L</u> <u>E</u> <u>G</u> ---M-AEAVSSR-----DCLQAGESCTNDPICSSKFR	44
cGRF $\alpha$ 2	TLRQCLAGRDRN----TML-ANK-ECQAALVLEL-QESPLYDCRCKRGMRKEIQCLQVYWS	111
cGRF $\alpha$ 4	TLRQCIAGNGANKL--GPDA-KNQC RSTVTALLS-SQLYGCKCKRGMKKEKHCLSVYWS	99
cGRF $\alpha$ 2	<u>IHLGLAEGEEFYEA</u> SPYEPITSRLSDFRLASIFSG--MDPATNSKSNHCLDAAKACNLN	169
cGRF $\alpha$ 4	<u>IHHTLMEGMNVLES</u> SPYEPFIRGFDYV-RLASIT A--GSENEVTQVNRCLDAAKACNVD	155
cGRF $\alpha$ 2	DNCKRLRSGYISTCSKEISATEHCSRKCHKALRQFFDNVPSEYTYRLLFCSC--KDQAC	227
cGRF $\alpha$ 4	EMCQRLRTEYVSCIRRLARADTCNRSKCHKALRKFFDRVPPPEYTHELLEFCPC--EDTAC	213
cGRF $\alpha$ 2	AEPRRQTIVPFCSEYEDDKEKPNCLDLRNVCRADHLCRSRLADFHANCAQSFQSLTSCPGD	287
cGRF $\alpha$ 4	AERRRQTIVPACSEY-SKEKPNCLAPLDSRENYVCRSRYAEFQFNCQPSLQTASGCRRD	272
cGRF $\alpha$ 2	NYQACLGSYTGLIGFDMTPNYVDASTTSITISPWCSCKGSGNLEEECEKFLRDFTENPCL	347
cGRF $\alpha$ 4	SYAACLLAYTGIIGSPITPNYIDNSTSSI--APWCTCNASGNRQEECESFLHLEFTDNVCL	330
cGRF $\alpha$ 2	RNAIQAFNGTVDVNLSPKNPSPPIITMLKVEKSPALPDDINDSNTMYDTSIITT-----	401
cGRF $\alpha$ 4	QNAIQAFNGTYLNAATA-HSISPTTQMYKQERNANRAAATLSENI FEHLQPTKVAGEER	389
cGRF $\alpha$ 2	CTSI----QEHGQKLNKSKEQSLCYSETQLTTDTMPDQKTFVDQKAA-GSRHRAAR-ILP	454
cGRF $\alpha$ 4	LLRGSTRLSSETSSPAAPCHQAASLLQLWLPPTLAVLSHFMM	431
cGRF $\alpha$ 2	<u>AVPIVLLKLLL</u>	466

Figure 4.1. Aligned amino acid sequences of chicken GFR $\alpha$ 2 and GFR $\alpha$ 4.

Conserved cysteines are shown in red, and amino acids in GFR $\alpha$ 2 that are identical to those in GFR $\alpha$ 4 are shown in blue. The N-terminal hydrophobic, putative signal peptides are underlined in red, the C-terminal hydrophobic domain is underlined in green. The sequences of the synthetic peptides chosen for antibody production are enclosed in green boxes. Adapted from Thompson *et al.*, 1998.

To raise antibodies, synthetic peptides must be coupled to a larger soluble protein. In this case, keyhole limpet hemacyanin (KLH) was used. This coupling allows the synthetic peptide to serve as the epitope for antibody binding on the B-cell surface while the carrier provides the class II MHC/T-cell receptor binding site.

The first stage in the coupling process was cross linking of KLH to MBS (3-maleimidobenzoic acid N-hydroxysuccinimidine ester). First, dialysis tubing (Sigma) was prepared by boiling in 0.1 M EDTA followed by several cycles of rinsing and boiling in de-ionised, distilled water. 2 ml of KLH (25 mg/ml, present as an ammonium sulphate liquid slurry) was loaded into the tubing and dialysed twice against 2 l of 1xPBS, pH6.0 at 4 °C. Dialysis brought about a 2-3 fold increase in volume with the final concentration of KLH being approximately 10 mg/ml. 10 mg of dialysed KLH was placed in a Bijou tube, one for each of the four peptides, and each solution was stirred very vigorously for 5 minutes using a small magnet. 50 µl of MBS was pipetted under the surface of the KLH and each solution stirred vigorously for a further 30 minutes. Next, this KLH-MBS complex was separated from any remaining free MBS and other contaminants such as DMSO. A Sephadex G-25 column (P10, Pharmacia) was pre-equilibrated with 4 volumes of 1xPBS, pH7.4, at room temperature. The KLH-MBS solution was then loaded onto the column and 1 ml fractions of eluate were collected. The KLH-MBS complex eluted as a cloudy greyish solution after the void volume (tube 2-3). The optical density (at 280 nm) of the samples was checked to ensure correct selection, after which the fractions of interest (generally, tubes 3-5) were pooled. The expected recovery of the complex is around 80 %. Later fractions (from tube 6 onwards) were not included as these may contain free MBS and other contaminants.

Before the peptides could be coupled to the KLH-MBS complex, they had to be solubilised. Peptide solubility can be a problem and is another reason why it is always advisable to select more than one peptide from each antigen. 5 mg of each peptide was weighed and dissolved in 200 µl of PBS, pH7.4. If fully soluble, as for the peptides NT1 and NT2, this was made up to a final volume of 500 µl with PBS. For the insoluble peptides PF1 and PF2, 2 µl portions of 10 M NaOH, up to a maximum of 5 portions, were added and the solubility was checked after each addition. This was sufficient for PF2 and the solution was then made to a final volume of 500 µl with PBS. The peptide, PF1, was still insoluble and was made up to 500 µl with DMSO instead of PBS to further aid solubilisation. The solubilised peptides were stored on wet ice. 500 µl of each peptide was then mixed with approximately 3 ml of KLH-MBS solution in a Bijou tube. The solution was vortexed vigorously for 1 hour at room temperature, after which 200 µl aliquots were prepared and stored at -20 °C.

#### **4.2.2. Immunisation regime and production of antisera**

The peptides, coupled to KLH, were sent to Eurogentec (Belgium) for immunisation into rabbits. Assuming that the availability of antigen is not limiting, rabbits represent the best choice of animal for polyclonal antibody production; up to 500 ml of antisera can be obtained from a single animal throughout the course of an immunisation schedule. It is also important that immunisations are carried out in animals that are as far in evolutionary distance as possible from the source of the antigen (in this case, chicken). Even in genetically identical animals, a single preparation of antigen will elicit the production of different antibodies. These differences are heightened when using outbred animals such as rabbits. Hence, two rabbits were used for each peptide immunisation scheme and the antisera from individual animals were screened separately. Prior to injection, the coupled

peptides (approximately 1 mg/ml) were mixed (1:1), by vigorous and prolonged vortexing, with Freund's adjuvant. 400 µl of this mixture, representing a delivery of around 200 µg of peptide, was administered per injection, per animal. Adjuvants are nonspecific stimulators of the immune response and their use is essential to induce a strong antibody response to soluble antigens. Freund's adjuvant has two main components. Firstly, it contains nonmetabolizable mineral oils which, when mixed with the immunogen in a water-in-oil emulsion, form a deposit around the antigen protecting it from rapid catabolism. This depot action allows the use of much smaller doses of antigen as well as ensuring that the antibody response is more persistent. The second component is heat-killed bacteria, commonly *Mycobacterium tuberculosis*. The active component of *M. tuberculosis* (muramyl dipeptide) acts by raising the level of lymphokines which in turn, stimulate the activity of antigen-processing cells causing a local inflammatory response at the site of injection. Freund's adjuvant can be either complete (CFA, both components present) or incomplete (IFA, without *M. tuberculosis*). The principal disadvantage to the use of Freund's adjuvant is that it can invoke aggressive and persistent granulomas. To minimise such effects, the primary injection was given in CFA, while all boosts were done in IFA. A standard immunisation schedule was used on two animals for each peptide as shown below, with injections given at multiple intradermic sites.

For the antigens, PF1 and PF2, in one animal per peptide only, an additional three boosts were given at 28 day intervals with bleedings 10 days after each boost. At the end of each immunisation schedule, a final bleeding was performed.

Day 0	first immunisation in CFA	pre-immune serum taken
Day 14	boost 1 in IFA	
Day 28	boost 2 in IFA	
Day 56	boost 3 in IFA	
Day 66		large bleed I
Day 84	boost 4 in IFA	
Day 94		large bleed II
Day 112	boost 5 in IFA	
Day 122		large bleed III

The first injection elicits a primary response which is often weak, especially against a soluble antigen which is readily broken down. When the same antigen is re-introduced into a primed animal two weeks later (boost 1), the response is very different. B-cells bearing antigen-specific cell surface antibodies rise exponentially and peak after 3-4 days. Antibody levels in the serum peak after 10-14 days and persist for 2-4 weeks after the injection. A second boost was given after a further 14 days and thereafter, the interval between injections was 28 days. This allows circulating levels of antibody to drop sufficiently to prevent a rapid clearance of newly injected antigen. The response to subsequent injections broadly mirrors that to the second injection although higher titres of antibody are achieved. More importantly however, during this time there is a maturation of the immune response and high affinity antibodies are generated. A pre-immune serum sample was taken to act as a control in later experiments. Serum was not sampled after the primary injection or after boosts 1 and 2, as although antibodies may be present at this stage, they are typically of lower affinity than in later samples. Serum was sampled 10 days after boost 3 and all subsequent injections as this corresponds to the predicted peak in antibody titres. Serum samples were received from Eurogentec (Belgium) and stored at -70 °C until use.

Sequential serum samples were monitored for the production of specific antibodies and the immunisation schedules were curtailed or lengthened as appropriate.

#### **4.2.3. Immunoblotting**

Immunoblotting combines the resolution of gel electrophoresis with the specificity of immunochemical detection and was used first, to check for the presence and specificity of antibodies from different samples of polyclonal sera, and later, to detect the presence and measure the quantity of antigen. Additionally, it was also used in an attempt to purify specific antibodies from polyclonal sera.

The first stage in immunoblotting is the preparation of the antigen sample. In preliminary experiments to determine whether specific antibodies were present in the polyclonal sera or not, samples of recombinant protein (i.e. antigen) were required. Samples of GFR $\alpha$ 2 recombinant protein and GFR $\alpha$ 4 recombinant protein were received from colleagues, Phillip Strachan and Natalia Ninkina respectively. GFR $\alpha$ 2 and GFR $\alpha$ 4 protein solutions were mixed with an equal volume of 2x SDS/sample buffer and placed in a boiling water bath for 5 minutes. Any precipitates were removed by centrifugation and the samples, now ready for electrophoresis, were stored at -20 °C until use. In later experiments, tissue samples were used in place of recombinant proteins in order to analyse the expression patterns of GFR $\alpha$ 2 and GFR $\alpha$ 4 proteins. Tissue samples and organs were removed from E10 White Leghorn chicken embryos and placed immediately on dry-ice. A x10 volume of 1x SDS/sample buffer was added to each tissue sample and mixed vigorously. The samples were then boiled for 5 minutes after which chromosomal DNA was sheared, to lower viscosity, by passing the sample repeatedly through a 20- and then 26-gauge needle. The samples were centrifuged and any pellets

discarded. These total cell lysates were ready for electrophoresis without further purification. Samples were diluted by 10 to 1000x. Up to 150 µg of total protein present in a complex mixture of tissue-extracted protein can be loaded per 5x0.1 mm slot. However, if a sample contains only 1 or 2 proteins, a maximum of only 10 µg should be loaded. Excessive loading leads to distortion as the proteins migrate through the gel.

Tris/glycine discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) was carried out, essentially according to the method of Laemmli (1970). A 15x15x0.1 cm polyacrylamide gel was prepared, comprising a 10 % SDS polyacrylamide resolving gel with a 6 % SDS polyacrylamide stacking gel above. Since lower percentage gels allow a better transfer of proteins from the gel, the lowest percentage gel that yields the desired resolution is used for PAGE.

The resolving gel was prepared as follows (a 10 % gel is suitable for resolving denatured proteins of approximately 16-70 kDa):

ddH <sub>2</sub> O	9.9 ml
30 % acrylamide mix	8.3 ml (acrylamide: bis-acrylamide= 30:1)
1.5 M Tris/HCl, pH8.8	6.3 ml
10 % SDS	0.25 ml
10 % APS	0.25 ml
TEMED	0.015 ml

The above reagents, added in order, were gently mixed and poured into the gel casting stand. 1-2 ml of water-saturated butanol was pipetted onto the surface of the gel to prevent oxidation of the polymerisation initiators by air. Once the resolving gel had polymerised (around 30 minutes), the butanol

was removed, the top of the gel was rinsed with water and the stacking gel was prepared as below:

ddH <sub>2</sub> O	6.54 ml
30 % acrylamide	2.0 ml
1.0 M Tris/HCl, pH6.8	1.26 ml
10 % SDS	0.1 ml
10 % APS	0.1 ml
TEMED	0.015 ml

The stacking gel was gently poured onto the resolving gel and a comb was placed at the top of the gel to form wells for sample application. The gel was left to set at room temperature for at least a further 30 minutes.

Once the gel had polymerised, it was placed in a gel chamber with the top and bottom of the gel immersed in running buffer (see appendix). The comb was removed and each well was thoroughly flushed out with running buffer. Also, any air bubbles were removed. 10 µl of each protein sample was loaded per well and in at least one lane per gel, a 10 µl sample of Rainbow™ coloured protein molecular weight markers was also loaded (Rainbow marker was prepared in 2x sample buffer as for other protein solutions). A constant current of 25 mA (approximately 250 V) was applied. The migration of the gel front was visualised by the bromophenol blue stain and the electrophoresis was terminated soon after this stain reached the bottom of the gel (approximately 5 hour run). After protein separation, the stacking gel was removed and discarded and the resolving gel was incubated with transfer buffer (see appendix) for 30 minutes with gentle agitation. When analysing chicken tissues and organs, sets of samples were run in duplicate with half the gel being used for transfer and the remaining half soaked in

Coomassie Brilliant Blue R Solution for 30 minutes at room temperature. The gel was then destained with several washes of 10 % acetic acid. This permitted visualisation of total protein and allowed adjustments to be made to ensure equal loading in all lanes.

The next stage in the immunoblotting procedure was the transfer of the separated polypeptides from the gel to a PVDF membrane (Hybond™ P, Amersham). This was achieved using electrophoretic elution. One sheet of PVDF membrane and four sheets of absorbant filter paper (Whatman 3MM) were cut to the size of the gel. The membrane was soaked in 100 % methanol for 2-3 minutes to reduce organic contamination and decrease hydrophobicity, and then rinsed twice in de-ionised, distilled water. At this stage, care must be taken not to allow the membrane to dry out. The membrane and filter papers were then equilibrated in transfer buffer. A gel-membrane transfer sandwich was then assembled as shown in Figure 4.2. and the sandwich completely immersed in transfer buffer between platinum electrodes with the membrane closest to the anode.

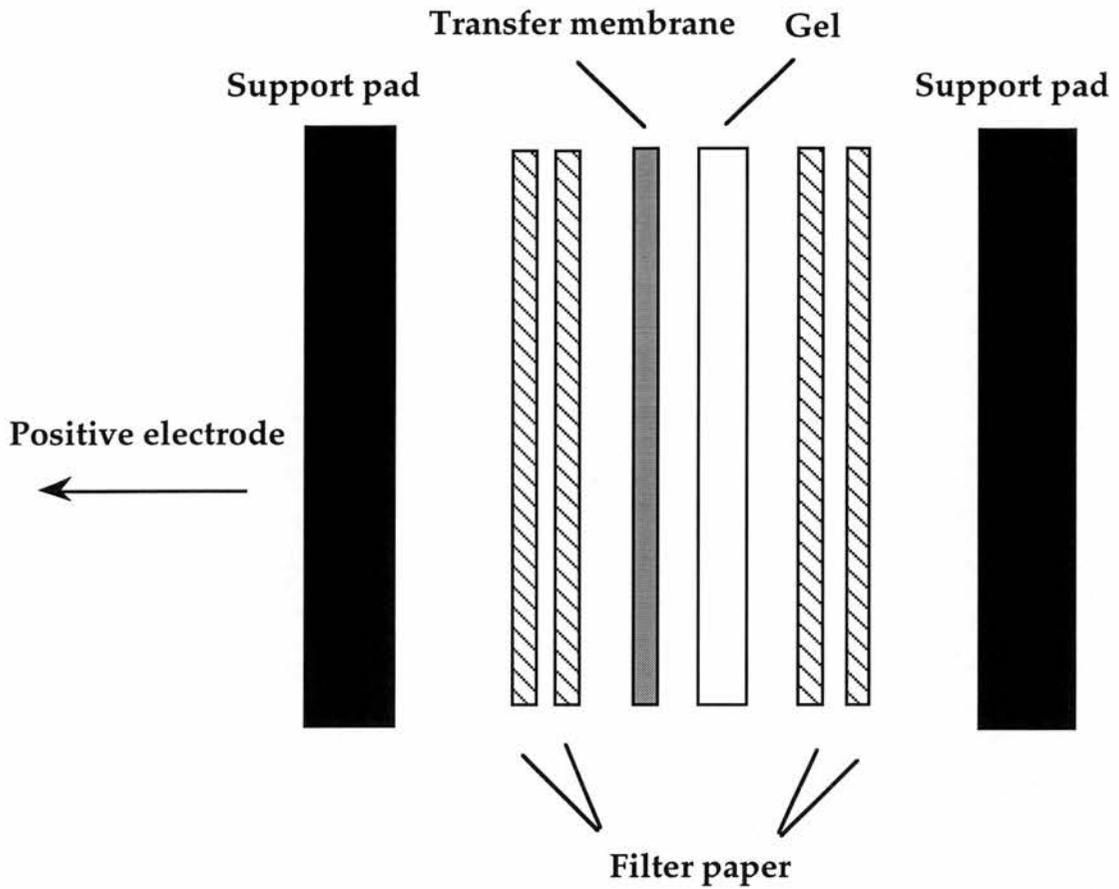


Figure 4.2. Wet/submerged electrophoretic transfer.

A transfer sandwich is assembled comprising support pads, filter papers and the gel and membrane in apposition. All components must be kept wet and the sandwich must be tightly assembled. Air bubbles are eradicated by rolling a pipette over the sandwich. For wet transfer, the gel-membrane sandwich is completely immersed in transfer buffer in a transfer tank with the membrane closest to the positive electrode.

Transfer took place at 25 V, overnight at 4 °C. A low temperature is required, because the temperature rises substantially during the run which could result in the formation of gas bubbles within the sandwich and uneven transfer. After transfer, the sandwich was disassembled and the PVDF membrane was clipped at one corner for orientation. The working surface (i.e. that next to the gel) was also marked. The use of pre-stained protein

markers served to confirm protein transfer as well as indicating the relative positions of variously sized proteins. The membrane was then washed with three changes of PBS for 15 minutes each, before being incubated in blocking buffer (see appendix) with agitation for either one hour at room temperature or overnight at 4 °C. This is an essential stage before antigen detection, as nonspecific binding sites on the blot must be blocked to eliminate any further reaction with the membrane, i.e. to prevent nonspecific adsorption of immunological reagents to the membrane.

After transfer of proteins, the next stage was to bind antibodies to the proteins immobilised on the membrane. An indirect detection technique was employed, i.e. the antibodies were located with a labelled secondary antibody. At this point, the membrane was often cut into strips to enable samples to be incubated with various antisera (e.g. anti-GFR $\alpha$ 2 or anti-GFR $\alpha$ 4). The blots were incubated in solutions of the relevant antiserum, diluted in blocking buffer, for 2 hours at room temperature. All antibody incubations (both primary and secondary) were carried out in shallow trays with gentle agitation in order to minimise antibody consumption. For polyclonal antisera, the generally recommended final antibody concentration is between 1 and 50  $\mu$ g/ml. Here, the concentrations of the antibody solutions were unknown, hence, several dilutions were tried to determine the correct range. Following primary antibody binding, the blot was washed with three changes of blocking buffer, for 10 minutes each, after which it was incubated with a labelled secondary antibody for 1 hour at room temperature. A donkey anti-rabbit IgG, conjugated with horseradish peroxidase (HRP) was used at a dilution of 1:1000 in blocking buffer. Thereafter, the blot was washed for 10 minutes in blocking buffer followed by two washes of 10 minutes each in PBS/0.1 % Tween 20.

The membrane was now ready for detection by enhanced chemiluminescence (ECL). ECL is a light-emitting method that detects immobilised specific antigens conjugated indirectly with HRP-labelled antibodies. The principles of this method are shown in Figure 4.3.. A commercially available kit was used (ECL Western blotting detection kit, Amersham) and all steps were carried out in a dark room under safe light conditions. First, the membrane was blotted with filter paper to remove excess liquid. It was then incubated in detection solution for 1 minute (working side of the membrane must be in contact with the detection solution) and again dried on filter paper. The membrane was carefully wrapped in cling film, avoiding air bubbles, and placed in a film cassette. X-ray film (Kodak X-OMAT) was exposed for varying lengths of time depending on the strength of the signal, usually 2-5 minutes was sufficient, and then developed.

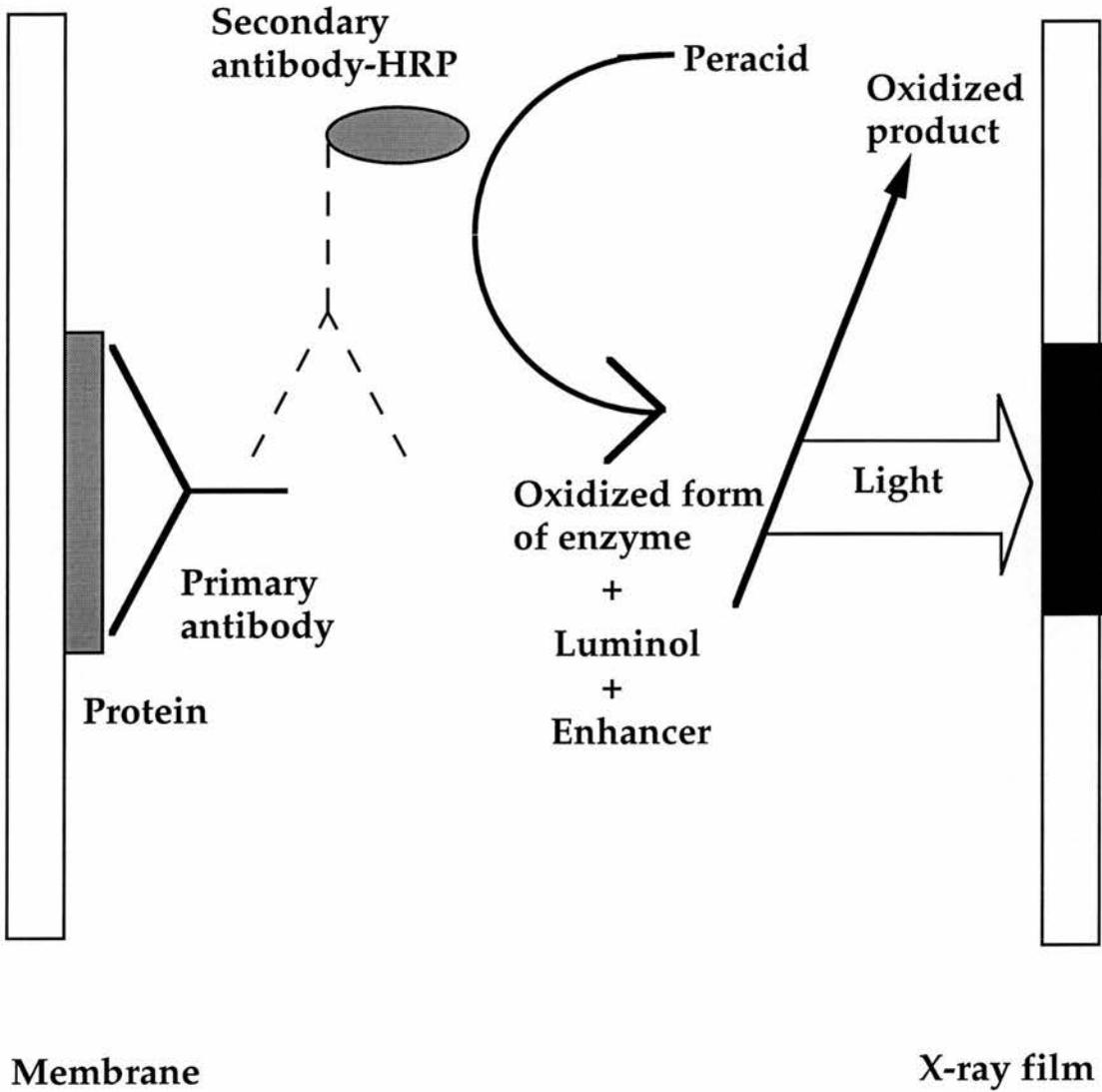


Figure 4.3. Principles of ECL Western blotting.

Horseradish peroxidase and hydrogen peroxide catalyse the oxidation of luminol in alkaline conditions. The resultant excited state luminol immediately decays to ground state luminol via a light emitting pathway. This emission of light is enhanced if the oxidation occurs in the presence of chemical enhancers such as phenols. The light produced from this enhanced chemiluminescent reaction peaks after 5-20 minutes and decays slowly thereafter. The maximum light emission is at a wavelength of 428 nm which can be detected by a short exposure to autoradiography film.

#### 4.2.4. Antibody purification

Many methods are available for the purification of antibodies, e.g. ammonium sulphate precipitation combined with purification using caprylic acid, a DEAE matrix or hydroxyapatite. These techniques yield relatively pure antibody preparations and are useful for purifying specific monoclonal antibodies from tissue culture supernatants (>95 % pure) or ascites (>90 % pure) where either no or very few contaminating antibodies are present. The total antibody concentration in serum is generally around 10 mg/ml of which only 1 mg/ml, maximum, will be the specific antibody of interest. Using these purification methods, the purity of specific antibody will be only 10 % at best, the remainder being contaminating serum antibodies. The only method available to purify antigen-specific antibodies from polyclonal sera is immunoaffinity purification on an antigen column. In this procedure, pure antigen is bound covalently to a solid support and the antibodies within the polyclonal pool that are specific to that antigen are allowed to bind. The unbound antibodies are then removed by washing, and the specific antibodies are eluted. A major disadvantage of this method is the requirement for large amounts of pure antigen and due to technical difficulties in the large-scale production of recombinant GFR $\alpha$ 2 and GFR $\alpha$ 4 proteins, it was not possible to proceed with this method here. Nevertheless, two variations based on the same principle of purification by specific antigen-antibody interaction were attempted.

First, antibodies that bound to the recombinant protein band were purified from polyclonal sera by a simple modification of the immunoblotting technique. A gel was prepared and all lanes (with the exception of one at either side for the marker) were loaded with undiluted sample of recombinant GFR $\alpha$ 2. The electrophoresis, transfer and blocking were carried out as previously. Thereafter, a thin strip of the blot containing the

recombinant protein band (approximately 50 kDa), as determined by the relative positions of the protein markers, was excised using a sharp scalpel. This thin strip of membrane was incubated with the appropriate serum (NT2/rabbit-SK235), prepared in a 1:1 ratio with 2x PBS/0.05 % Tween 20, overnight at 4 °C with gentle agitation. After three washes of 10 minutes each with PBS/Tween 20, the membrane strip was cut into small pieces and placed in microfuge tubes. The strips were incubated with 500 µl 100 mM glycine, pH2.6 for 10 minutes to elute the antibodies. The buffer was removed and immediately neutralised with 50 µl of 1M Tris, pH8.0. The eluted fractions were combined and dialysed for 48 hours against two changes of PBS, pH7.4 at 4 °C. A small volume was removed from the dialysis tubing and used to check for the presence of antibodies by immunoblotting. The remainder was dialysed against PBS/glycerol for a further 24 hours, which concentrated the antibody solution some 2-4 fold. The antibodies were stored at -20 °C until further use.

As an alternative to using recombinant protein as the antigen in affinity purification, the original peptide against which the antibodies were raised was used. Peptides NT2 and PF2, which appeared to have elicited a stronger response than NT1 and PF1, were synthesized linked to controlled pore glass (250 mg, Alta Bioscience). The following protocol was then carried out for each peptide. The glass beads were washed three times for 10 minutes each in PBS, pH7.4 before being loaded onto a 5 ml column (Pharmacia), also pre-equilibrated in PBS, pH 7.4. The beads were washed with 3 volumes of 0.1 M citric acid, pH3.0 and then equilibrated again in PBS. Meanwhile, the appropriate serum was prepared by centrifuging at 10, 000 rpm, at 4 °C, for 10 minutes or until the supernatant was clear. 4 ml of supernatant was loaded onto the column which was sealed at both ends and placed in a rotating mixer for 2 hours at room temperature. After adsorption of the

serum to the beads, the serum effluent was collected and kept aside to be passed through the column again. The column was washed thoroughly (10 volumes) with PBS until the optical density (at 280 nm) of the effluent was zero. The bound antibodies were eluted using first, one volume of 0.1 M citric acid, pH3.0, followed by one volume of 0.1 M glycine, pH2.6. Eluate samples in 0.5 ml aliquots were immediately neutralised with alkali (citric acid elution- 25  $\mu$ l 1 M Tris, pH9.0, glycine elution- 28  $\mu$ l 1 M Tris, pH9.5) and placed on ice. UV absorbance at 280 nm was ascertained. Samples with a high protein content were combined (mean OD for samples combined for NT2 purification was 0.07 equivalent to approximately 0.105 mg/ml, for PF2 0.52, 0.39 mg/ml) and dialysed against PBS and PBS/glycerol as previously. The column was washed thoroughly with PBS and stored containing PBS/Na azide (0.1 %) at 4 °C for further use. Specific antibody activity in the dialysed solution was checked by immunoblotting.

#### **4.2.5. Neutralising experiments**

Low density, dissociated cultures of ciliary neurons from E6 White Leghorn chicken embryos were established as in Chapter 2, section 2.2.1.. Survival was monitored, as previously, in cultures containing CNTF, GDNF, neurturin and antiserum against GFR $\alpha$ 2.

#### **4.2.6. Immunocytochemistry**

Low density, dissociated cultures of ciliary and trigeminal neurons from E8 White Leghorn chicken embryos were established as in Chapter 2, section 2.2.1.. After 48 hours in culture, the neurons were fixed in 4 % paraformaldehyde dissolved in PBS, pH7.3 for 30 minutes at room temperature and then processed for surface staining. After three washes of 10 minutes each with PBS, the cells were blocked in S blocking buffer (see appendix) for 30 minutes at room temperature. Cells were then incubated in

the relevant antisera, diluted in S blocking buffer (1:20 to 1:200), for 1 hour at room temperature. After three washes in PBS, the secondary antibody, diluted 1:100 in S blocking buffer, was applied for one hour at room temperature. The secondary antibody of choice was a fluorescein-conjugated goat anti-rabbit IgG, hence, all further stages were carried out in the dark. Following three final washes in PBS, the cells were mounted in Citifluor (Agar) and examined under a Zeiss Axioskop microscope with fluorescent light and suitable filters. Appropriate controls, i.e. omitting the primary or secondary antibodies, were performed.

### 4.3. Results

#### 4.3.1 Detection of antibodies to GFR $\alpha$ 2 in polyclonal sera

Recombinant GFR $\alpha$ 2 protein (undiluted and diluted 1:100 and 1:500) was used as a target for immunoblotting to check for the presence of antibodies to GFR $\alpha$ 2 in polyclonal sera taken from three rabbits (one animal died during the immunisation regime) immunised with peptides (NT1 and NT2) from the chicken GFR $\alpha$ 2 amino acid sequence. The protein was produced in insect cells transfected with recombinant virus, hence, as a negative control, untransfected insect cell lysates were used. Serum samples were diluted 1:100 or 1:500 before use. Serum samples from the final or penultimate bleedings were used to maximise the presence of high affinity antibodies. Figure 4.4. demonstrates a clearly defined band in each of the lanes containing recombinant GFR $\alpha$ 2 protein with no bands apparent in the neighbouring control lanes. Hence, all three polyclonal sera contained antibodies to GFR $\alpha$ 2. The serum from rabbit SK235, injected with peptide NT2, clearly contained either the most abundant or most specific antibodies against GFR $\alpha$ 2, demonstrating the importance of always selecting more than one peptide and using several animals. This particular serum was therefore used in all further experiments. The size of the band, relative to various molecular weight markers, was as predicted, around 50 kDa. This is approximately 10 kDa smaller than the size of native GFR $\alpha$ 2, as during construction of the expression vector, the GPI linkage and a hydrophobic N-terminal region were removed to aid solubilisation of the recombinant protein.

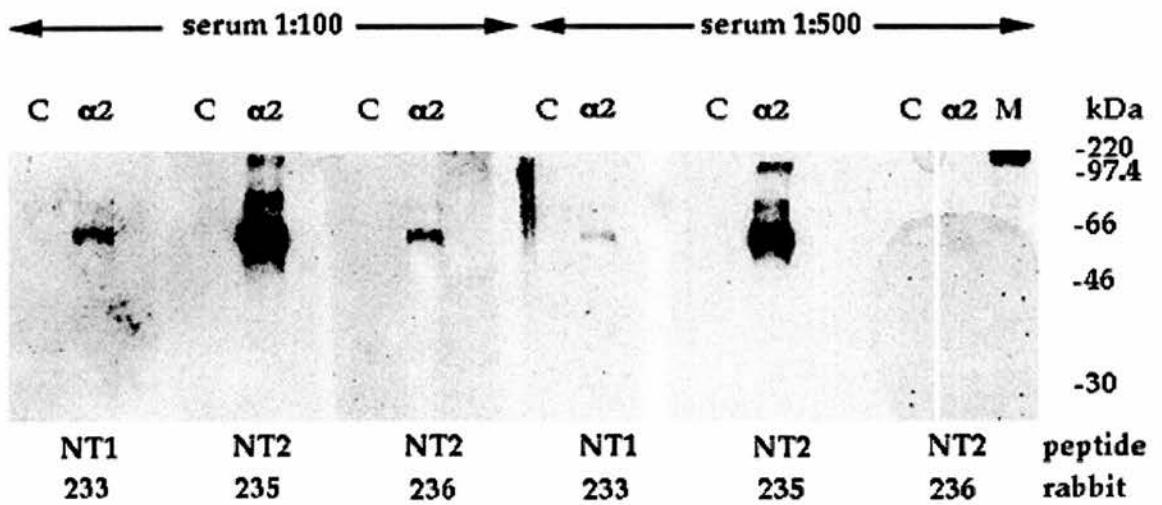


Figure 4.4. Immunoblot showing the presence of GFR $\alpha$ 2 binding antibodies in polyclonal sera.

A clear band is present in the lanes containing recombinant GFR $\alpha$ 2 (diluted 1:100) ( $\alpha$ 2). Lanes loaded with total cell lysates of insect cells only (C) are negative. The size of the recombinant GFR $\alpha$ 2 band is approximately 50 kDa relative to proteins of known size in the Rainbow<sup>TM</sup> marker (M). Each of the three antisera, at a dilution of 1:100, could detect the recombinant protein. A dilution of 1:500 was sufficient but for two of the sera, elicited only a very weak signal. The serum from rabbit SK235, injected with peptide NT2, clearly elicited the greatest response.

#### 4.3.2. Detection of antibodies to GFR $\alpha$ 4 in polyclonal sera

Recombinant GFR $\alpha$ 4 protein was used as a target in immunoblotting to check for the presence of antibodies to GFR $\alpha$ 4 in polyclonal sera taken from four rabbits immunised with peptides (PF1 and PF2) from the chicken GFR $\alpha$ 4 amino acid sequence. Recombinant protein, fused to GST, was produced in a bacterial overexpression system. Protein from several preparations was used (coded 19a and 19b) and GST only was used as a negative control. Serum samples were diluted 1:50 before use. Serum samples from the final or penultimate bleedings were used to maximise the presence of high affinity antibodies. Figure 4.5. demonstrates a clear band in each of the lanes containing recombinant GFR $\alpha$ 4 protein with no bands apparent in the neighbouring control lanes. Hence, all four polyclonal sera contained antibodies to GFR $\alpha$ 4. From this and other blots, serum from rabbit SK334, injected with peptide PF2, appeared to exhibit the strongest antibody activity, therefore, this particular serum was used in all further experiments. The size of the band, relative to various molecular weight markers, was as predicted, around 66 kDa. This is approximately 20 kDa greater than the size of native GFR $\alpha$ 4, as although the GPI linkage and a hydrophobic N-terminal region (approximately 10 kDa) were removed during construction of the expression vector to aid solubilisation of the recombinant protein, GST (approximately 27 kDa) was added.

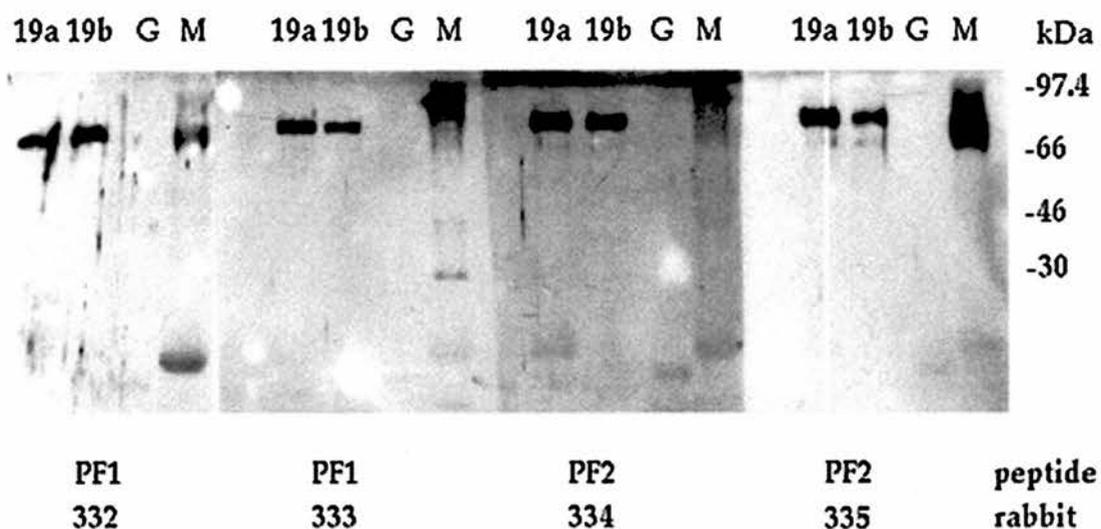


Figure 4.5. Immunoblot showing the presence of GFR $\alpha$ 4 binding antibodies in polyclonal sera.

A clear band is present in the lanes containing recombinant GFR $\alpha$ 4 (19a, 19b). Some nonspecific binding (27 kDa) is apparent in the lanes loaded with GST only (G). The size of the recombinant GFR $\alpha$ 4 band is approximately 66 kDa relative to proteins of known size in the Rainbow<sup>TM</sup> marker (M). Each of the four antisera, at a dilution of 1:50, could detect the recombinant protein.

#### 4.3.3. Expression of GFR $\alpha$ 2 in chicken tissues and organs

Several tissues and organs from E10 chicken embryos were analysed for expression of GFR $\alpha$ 2 protein using an immunoblotting technique and a polyclonal serum known to interact with recombinant GFR $\alpha$ 2 protein. Despite the appearance of a discrete band in the lane corresponding to recombinant GFR $\alpha$ 2, multiple bands were apparent in the lanes containing tissue samples (Figure 4.6.). As these bands were of various sizes and intensities (no one band predominated), it was not possible to glean any information regarding GFR $\alpha$ 2 expression from this blot. The multiple bands either reflected nonspecific binding of the GFR $\alpha$ 2 antibodies themselves, the presence of contaminating antibodies in the polyclonal mixed pool or both. Antibodies raised against the original peptide represent  $\leq 10$  % of the total antibodies present in rabbit serum hence, multiple nonspecific interactions may occur. This underscores the importance of affinity purification which regrettably was unsuccessful in this case.

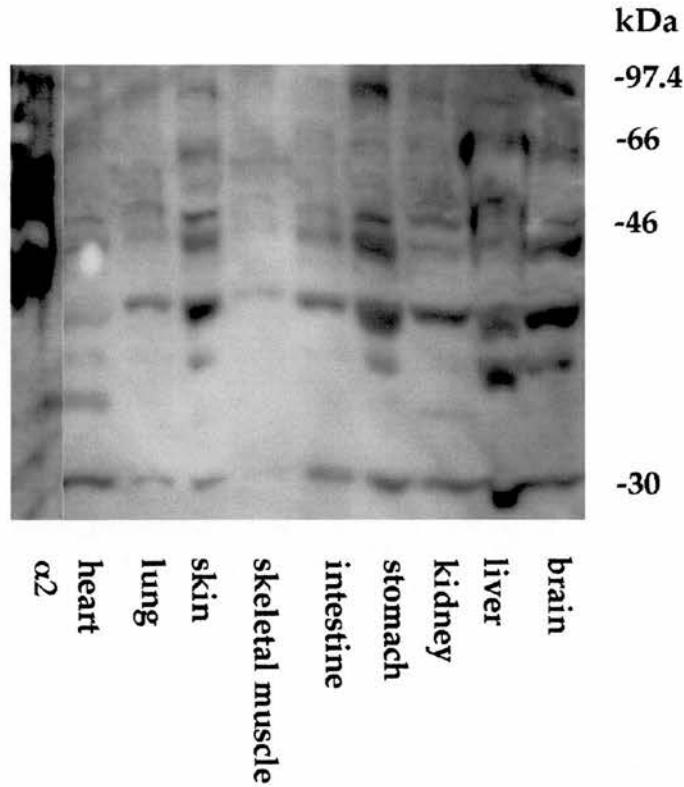


Figure 4.6. Immunoblot showing nonspecific binding of polyclonal antibodies to proteins extracted from chicken tissues.

A strong band is present in the lane containing recombinant GFR $\alpha$ 2 protein ( $\alpha$ 2). Multiple bands are present in the lanes containing E10 chicken tissues and organs, representing nonspecific binding of the GFR $\alpha$ 2 antibodies themselves and/or nonspecific binding of contaminating antibodies present in the mixed polyclonal pool. Total protein loaded to each lane was matched by considering Coomassie Blue stained gels. The serum (NT2, rabbit SK235) was used at a 1:100 dilution.

4.3.4. Partial purification of polyclonal antibodies against GFR $\alpha$ 2 and GFR $\alpha$ 4

Antibodies to GFR $\alpha$ 2 were purified from an immunoblot as previously described in section 4.2.4.. Antibodies in the dialysed eluate were confirmed using recombinant GFR $\alpha$ 2 protein as shown in Figure 4.7..

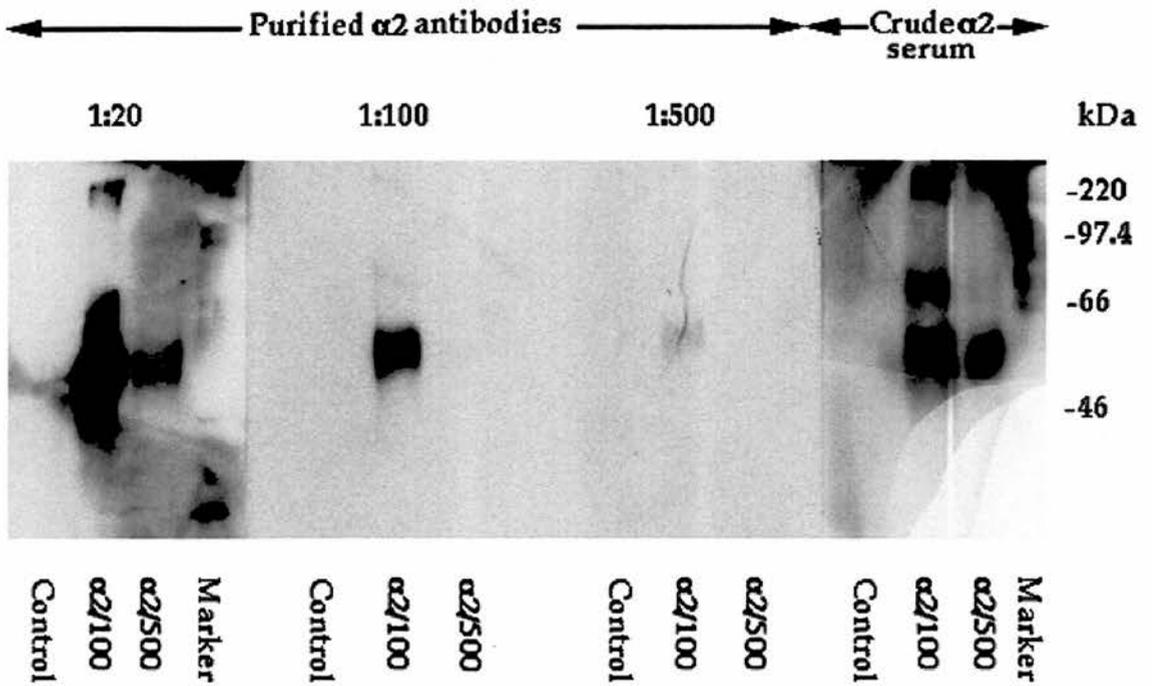


Figure 4.7. Presence of GFR $\alpha$ 2 antibodies after purification from an immunoblot.

After the purification process, antibodies were detected in the eluate and produced a strong signal when bound to recombinant GFR $\alpha$ 2 at 1:100 ( $\alpha$ 2/100) and 1:500 ( $\alpha$ 2/500). No bands were observed in control lanes (Control). With purified antibodies present at 1:20, 1:100 and 1:500 dilutions, a discrete band is observed. This band is of the same size, relative to several protein markers (Marker), but lesser intensity than a similar one observed in a parallel blot incubated with crude GFR $\alpha$ 2 antiserum (diluted 1:100).

These partially purified GFR $\alpha$ 2 antibodies were then tried again on an immunoblot with protein extracted from chicken tissues and organs. Although fewer bands were observed than in Figure 4.6. above, multiple nonspecific bands were still present precluding any interpretation of GFR $\alpha$ 2 expression (data not shown). In an attempt to decipher these multiple bands, two identical sets of tissue samples were run and processed for immunoblotting in parallel. The only difference was that for one of the blots, the purified antibody preparation was preincubated with an excess of original NT2 peptide. After ECL detection, the blots were compared; the expectation being that any bands absent in the peptide-preincubated blot as compared to the normal blot represented a specific interaction of GFR $\alpha$ 2 antibody and GFR $\alpha$ 2 protein. However, the same multiple bands were witnessed in both blots and no differences could be seen (data not shown). It was concluded, therefore, that all interactions were of a nonspecific nature.

In a final attempt to overcome the problem of lack of specificity, affinity purification using peptides linked to controlled pore glass (CPG) beads was undertaken and after dialysis, the presence of purified antibodies to GFR $\alpha$ 2 and GFR $\alpha$ 4 was checked using recombinant protein samples. The highly acidic elution conditions required can abolish antibody activity, however, antibodies to GFR $\alpha$ 2 and GFR $\alpha$ 4 were present after the purification process. Nevertheless, when each was used to probe blots of chicken tissue samples, multiple bands were still evident. Figure 4.8. demonstrates that although nonspecific binding has been reduced as a result of the purification process of GFR $\alpha$ 2 antiserum, nonspecific binding of GFR $\alpha$ 2 antibodies and possibly remaining contaminating antibodies is still a major problem limiting the scope of these antibodies in immunoblotting assays to the detection of recombinant protein only. A similar blot was obtained with purified GFR $\alpha$ 4

antibodies, again demonstrating multiple nonspecific interactions (data not shown).

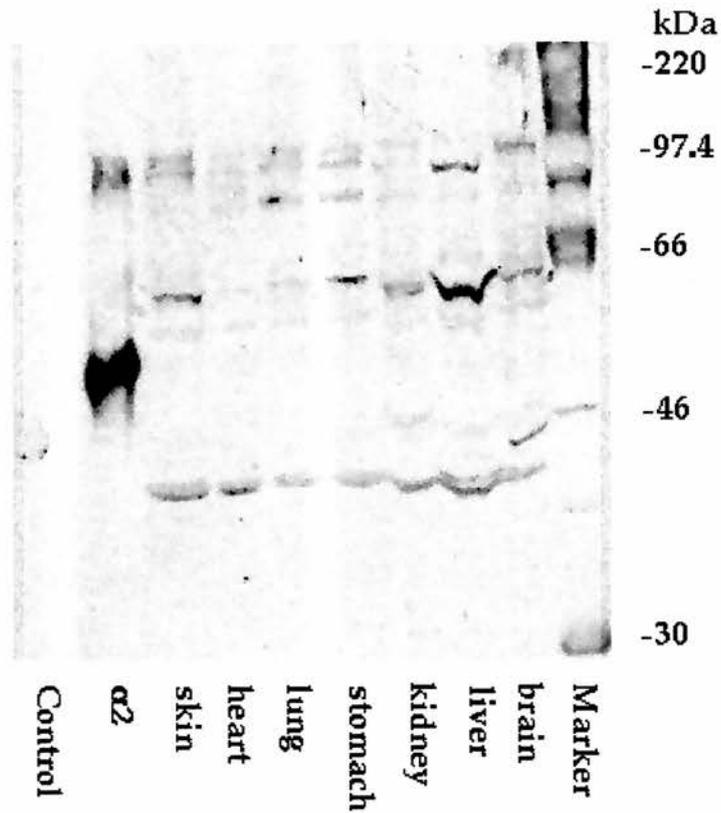


Figure 4.8. Immunoblot showing nonspecific binding of purified GFR $\alpha$ 2 antibodies to proteins extracted from chicken tissues.

After affinity purification of GFR $\alpha$ 2 antisera using a CPG-linked peptide, multiple bands are still evident in the lanes containing proteins extracted from E10 chicken tissues and organs. Because no single band predominates, it is not possible to draw any conclusions regarding GFR $\alpha$ 2 expression in these tissues. Lanes containing recombinant GFR $\alpha$ 2 ( $\alpha$ 2), insect cells (Control) and a protein marker (Marker) were run as previously.

#### 4.3.5. Effects of antibodies to GFR $\alpha$ 2 on the survival of ciliary neurons

Having established the presence of antibodies to GFR $\alpha$ 2 in the serum of rabbits injected with peptides, NT1 and NT2, from the chicken GFR $\alpha$ 2 amino acid sequence, possible neutralising effects of these antibodies were investigated. Low density, dissociated cultures of ciliary neurons from E6 chicken embryos were established. This particular population was selected as the neurons are very responsive to GDNF and neurturin and in accordance, express GFR $\alpha$ 1 and GFR $\alpha$ 2 mRNA (Chapter 2, Forgie *et al.*, 1999). Neuronal survival after 48 hours in control cultures (i.e. with no added neurotrophic factors) and in cultures supplemented with CNTF, GDNF and neurturin was determined in the presence and absence of a GFR $\alpha$ 2 antibody-containing serum, present at a dilution of either 1:100 or 1:1000. Figure 4.9. demonstrates that although the survival of ciliary neurons with neurturin was reduced in the presence of antibodies to GFR $\alpha$ 2, its preferred receptor, this was not a specific, neutralising effect as survival in control cultures and cultures supplemented with CNTF, which utilises entirely different receptor machinery, was also compromised to a similar degree. This suggests a more general, toxic effect of components within the serum.

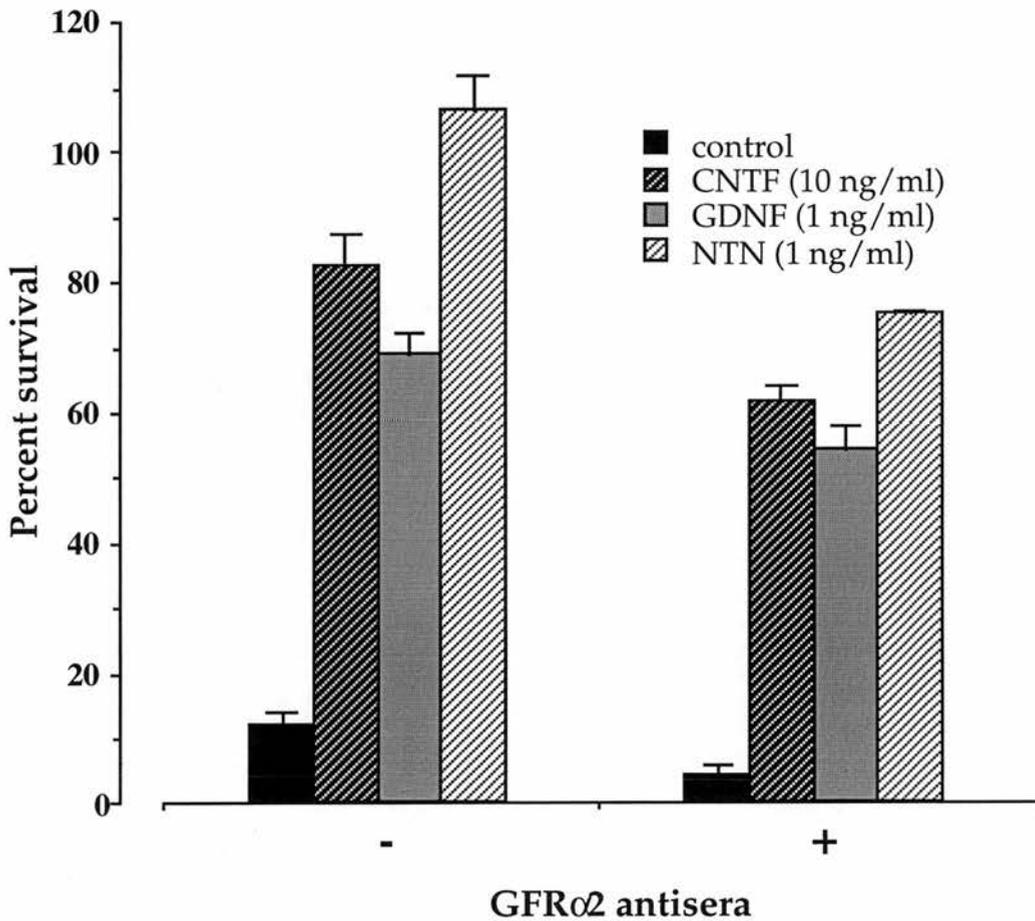


Figure 4.9. Bar chart showing the effects of GFRα2 antibodies on the survival of ciliary neurons grown in the presence of neurturin.

The number of neurons surviving after 48 hours is expressed as a percentage of the number of attached neurons counted 3 hours after plating. CNTF was present at a saturating concentration (10 ng/ml). GDNF and NTN were present close to their EC<sub>50</sub> values (1 ng/ml). Specific effects of GFRα2 antibodies (1:100) on the survival of NTN-supplemented neurons were not apparent. The mean ± standard errors are shown (n=3).

#### 4.3.6 Expression of GFR $\alpha$ 2 and GFR $\alpha$ 4 in cultured neurons

In a limited number of experiments, immunocytochemistry using GFR $\alpha$ 2 and GFR $\alpha$ 4 antisera was performed on cultured ciliary and trigeminal neurons to investigate expression of these receptors in neuronal populations with strikingly different responses to neurturin and persephin, the preferred ligands for GFR $\alpha$ 2 and GFR $\alpha$ 4 respectively. E8 ciliary neurons are highly responsive to neurturin whereas E8 chicken trigeminal neurons are unresponsive to this factor. Neither population responds to persephin (Chapter 2, Forgie *et al.*, 1999). Accordingly, marked differences in the levels of GFR $\alpha$ 2 and GFR $\alpha$ 4 mRNAs are detected in purified neurons from these ganglia, for example, in E8 ciliary neurons expression of GFR $\alpha$ 2 mRNA is relatively high compared to negligible levels of GFR $\alpha$ 4 mRNA (Forgie *et al.*, 1999, Doxakis, personal communication). A strong fluorescent signal was detected in ciliary and trigeminal neurons stained with antibodies to both GFR $\alpha$ 2 and GFR $\alpha$ 4. The cell bodies and neurites were labelled, as were any non-neuronal cells present in the cultures (data not shown). Very little background staining was apparent in controls where either the primary or secondary antibodies were omitted. Although this staining pattern may represent widespread expression of GFR $\alpha$ 2 and GFR $\alpha$ 4 receptors in ciliary and trigeminal neurons, it seems probable that this ubiquitous staining of neurons with very different ligand responsiveness may reflect the nonspecific nature of these antibodies as demonstrated in the other assays undertaken.

#### 4.4. Discussion

The results of immunoblots using recombinant GFR $\alpha$ 2 and GFR $\alpha$ 4 confirm that antibodies to these proteins are present in polyclonal sera derived from rabbits injected with peptides derived from chicken GFR $\alpha$ 2 and GFR $\alpha$ 4 amino acid sequences, respectively. These antibodies, present in crude sera, specifically cross-reacted with the respective recombinant protein, generating a discrete band on the blot. However, when used to probe GFR $\alpha$ 2 and GFR $\alpha$ 4 expression in chicken tissues and organs, multiple nonspecific interactions were apparent. Measures taken to purify the specific antibodies to GFR $\alpha$ 2 and GFR $\alpha$ 4 away from contaminating antibodies present in rabbit sera had limited success. Despite exposure to the highly acidic elution conditions which can cause inactivation, the purified antibodies retained their activity against recombinant protein, however, nonspecific interactions continued to pose a significant problem. A small number of immunocytochemistry experiments further highlighted the nonspecific nature of the antibodies generated in this study and the need for better purification methods.

There are several reasons why expression of GFR $\alpha$ 2 and GFR $\alpha$ 4 could not be detected. Although capable of detecting relatively large amounts of recombinant protein, the antibodies raised against GFR $\alpha$ 2 and GFR $\alpha$ 4 may not be specific enough to detect the relatively small amounts of protein present in tissues. It is possible that even if the antibodies were highly specific, there may have been insufficient antigen to be detected. Because the total amount of protein that can be resolved on a gel is limited to 150  $\mu$ g per lane, some minor proteins within a complex mix may be present below the detection limit of the assay, a minimum of 1 ng protein per sample. Therefore, within a complex protein mix, the antigen must be present at

1:150000 to be detected. To detect proteins present at lower levels, it is necessary to partially purify the protein of interest before running on an electrophoresis gel. This can be achieved using chromatographic and extraction protocols or immunoaffinity techniques. Another means of increasing the sensitivity of immunoblots is to increase the strength of the detection method, for example by amplifying the signal with a triple layer detection method or including streptavidin as an intermediate. However, caution must be exercised as this also increases nonspecific background. Previous studies investigating GFR $\alpha$ 2 and GFR $\alpha$ 4 expression in development have considered mRNA levels. Although the pattern of mRNA expression may not reflect protein levels, it is interesting to note that at the mRNA level, the expression of GFR $\alpha$ 2 and GFR $\alpha$ 4 in some chicken peripheral tissues is very low. Using Northern blotting and semi-quantitative RT-PCR, high levels of GFR $\alpha$ 2 mRNA are detected in the liver, however, only very low levels of expression are found in other peripheral tissues of E10 and E18 chicken embryos (Buj-Bello *et al.*, 1997; Thompson *et al.*, 1998). GFR $\alpha$ 4 mRNA is expressed at high levels in the E10 chicken kidney whereas expression is low elsewhere in the periphery and in brain (Thompson *et al.*, 1998). These expression patterns contrast to the more abundant tissue distribution of GFR $\alpha$ 1 mRNA (Buj-Bello *et al.*, 1997; Thompson *et al.*, 1998), and may contribute to sub-detectable levels of antigen in the cell lysates. One further explanation for insufficient antigen may be that the GFR $\alpha$ 2 and GFR $\alpha$ 4 proteins are found in an SDS-insoluble fraction of cell lysate and therefore, are not extracted in Laemmli buffer.

Alternatively, the amount of antigen present may not have been the limiting factor. The specificity of the antibodies raised against the antigen is also brought into question. Additionally, when using polyclonal antibodies present in whole sera, the entire repertoire of circulating antibodies found in

the immunised animal at the time of serum collection are also present. This serum may well contain high titred antibodies which may specifically recognise spurious antigens. If the molecular weight of these spurious antigens is similar to that of the antigen of interest, the additional bands generated will obscure those under investigation. Such specific contamination cannot be removed by methods designed to lower nonspecific background such as more extensive blocking of the membrane and vigorous washing. Adsorption techniques can be employed to block the specific contaminating antibodies. For example, one way to remove contaminating antibodies is pre-incubation of the serum with a preparation containing the contaminating proteins, but not the antigen of interest. Despite such specificity problems, polyclonal antibodies are the most commonly used antibody preparation for immunoblotting purposes. Although monoclonal antibodies demonstrate considerable specificity of interaction, many do not recognise epitopes destroyed by the denaturing conditions used in electrophoresis sample preparation. Polyclonal antibodies, on the other hand, can bind to a number of denaturation resistant epitopes on the antigen. Nevertheless, the denaturing conditions may still have affected the antibody-antigen interactions in this assay. To fully address the specificity problems encountered here, new peptides could be selected from the chicken GFR $\alpha$ 2 and GFR $\alpha$ 4 amino acid sequences and a new immunisation program undertaken. Further work is also required to generate the recombinant GFR $\alpha$  proteins in sufficient quantity to be used for affinity purification. This was beyond the time scale of this project.

In a limited number of experiments, the activity of the antibodies in non-denaturing conditions was investigated. Tissue culture experiments indicated that antibodies against GFR $\alpha$ 2 could not bind to this receptor in such a way as to block its function. This does not rule out the possibility that

these antibodies can bind to native GFR $\alpha$ 2 protein, just that they did not bind to and block the ligand binding site of this receptor. The small reduction in survival on addition of the antiserum in the neurturin-supplemented cultures did not represent a specific neutralising effect of the antibodies. Because there was a drop in survival in all conditions, it is more likely that the antiserum mediated a general toxic effect. It should be noted that antibodies specific only to GFR $\alpha$ 2 could, in theory, have blocked neurturin binding to GFR $\alpha$ 2 receptors in these experiments without reducing survival if the survival response was, in fact, mediated by GFR $\alpha$ 1. The physiological relevance of such ligand-receptor cross talk has been demonstrated in ciliary neurons. In the neurturin knockout animal, there is a 48 % deficit in ciliary ganglion neurons compared to the wild-type (Heuckeroth *et al.*, 1999). However, in the GFR $\alpha$ 2 knockout animal, the ciliary ganglion is intact (Rossi *et al.*, 1999) demonstrating that the essential role of neurturin in the development of this neuronal population can be mediated by a receptor other than GFR $\alpha$ 2, most likely GFR $\alpha$ 1. Given the general, inhibitory effect of the serum on neuronal survival and the lack of specificity observed in previous experiments it seems unlikely that this is the scenario here. Neutralising antibodies to GFR $\alpha$ 1 and GFR $\alpha$ 2, added in combination to the cultures would be required to fully address this issue. Moreover, if antibodies could be generated with a neutralising function against each of the GFR $\alpha$  family members, similar experiments could prove extremely useful in resolving some of the controversies still surrounding the degree of promiscuity in GDNF family ligand and GFR $\alpha$  receptor interactions.

GFR $\alpha$ 1-3 mRNAs are found in several peripheral ganglia (Nosrat *et al.*, 1997; Widenfalk *et al.*, 1997, 1998; Worby *et al.*, 1998; Baloh *et al.*, 1998a) and indeed, their expression patterns are known to overlap within particular neuronal populations, for example, GFR $\alpha$ 1, GFR $\alpha$ 2 and GFR $\alpha$ 3 mRNAs are expressed

in distinct, yet overlapping, subsets of neonatal mouse trigeminal ganglion neurons (Naveilhan *et al.*, 1998). The availability of antibodies specific to GFR $\alpha$  family proteins would provide a powerful tool to investigate their spatial and temporal expression patterns in peripheral ganglia and their cellular distribution in neuronal subpopulations, and to correlate receptor expression with ligand responsiveness. Moreover, regulatory influences on GFR $\alpha$  receptor expression could be considered.

To summarise, polyclonal antibodies to GFR $\alpha$ 2 and GFR $\alpha$ 4 were successfully generated, however, due to technical problems in the production of recombinant GFR $\alpha$  proteins, purification of the antibodies was incomplete and problems of specificity and contaminating antibodies in the sera considerably limited the scope of this work.

## Chapter 5

### *In vivo* survival requirement of a subset of nodose ganglion neurons for nerve growth factor

#### 5.1. Introduction

Sensory neurons of the nodose ganglion are generally thought to be the classic example of a population of peripheral nervous system neurons that do not require NGF for survival during development but are dependent on other neurotrophins. In the course of experiments surveying the effects of a wide range of factors on a variety of embryonic mouse neurons in culture, with particular interest in the GDNF family (Chapter 3), a subpopulation of nodose neurons surprisingly demonstrated a survival response to NGF.

*In vitro* and *in vivo* studies of cranial sensory neurons have been important in defining which neurotrophins different kinds of sensory neurons require for their survival. These neurons are segregated into groups that are derived from either the neural crest or neurogenic placodes and in most cases subserve distinctive sensory modalities. Indeed, the first evidence that the neurotrophin requirements of different kinds of sensory neurons are related to sensory modality came from studying different populations of cranial sensory neurons (Davies, 1987a); a conclusion that has been substantiated by the analysis of mice with null mutations in the neurotrophin and *Trk* genes (Snider, 1994).

One of the most extensively studied populations of cranial sensory neurons are those of the nodose ganglion. These placode-derived neurons provide sensory innervation to thoracic and abdominal viscera. Numerous *in vitro*

and *in vivo* studies have established that neurotrophins other than NGF, namely BDNF and to a lesser extent NT-3 and NT-4, are required to maintain the survival of nodose neurons during development. These neurotrophins promote the survival of embryonic nodose neurons in culture (Lindsay *et al.*, 1985; Davies *et al.*, 1986, 1993a; Vogel and Davies, 1991; Buj-Bello *et al.*, 1994). Similarly, administration of BDNF to quail embryos *in ovo* during the period of naturally occurring neuronal death reduces the number of nodose neurons that die (Hofer and Barde, 1988). The physiological role of these factors is clearly demonstrated by the increased neuronal death observed in mouse embryos that have null mutations in the *BDNF*, *NT-3* and *NT-4* genes (Conover *et al.*, 1995; Liu *et al.*, 1995; Erickson *et al.*, 1996; ElShamy and Ernfors, 1997). In contrast, evidence for a role of NGF in the development and maintenance of nodose neurons and indeed, other placode-derived sensory neurons, is less clear cut. A large number of studies have indicated that NGF has no role to play in promoting the survival of nodose neurons. Results from *in vitro* experiments demonstrate that embryonic (Thaler *et al.*, 1994) and neonatal (MacLean *et al.*, 1988) rat nodose ganglion neurons in dissociated neuron-enriched cultures are totally unresponsive to NGF. Similarly, NGF does not influence the survival of chicken nodose ganglion neurons, cultured over a wide range of developmental stages (Davies and Lindsay, 1985; Lindsay and Rohrer, 1985; Buj-Bello *et al.*, 1994). Likewise, NGF administration *in ovo* fails to rescue nodose neurons from cell death whilst preventing the death of populations of NGF-dependent neurons (Dimberg *et al.*, 1987). The administration of anti-NGF antibodies to quail embryos has been reported to reduce the size of NGF-dependent populations such as those of dorsal root and sympathetic ganglia, but not to affect the number of nodose neurons (Rohrer *et al.*, 1988). Placental transfer of maternal anti-NGF antibodies in rodents also brought about profound reductions in the numbers of sympathetic and certain

sensory neurons whilst nodose neurons were unaffected (Pearson *et al.*, 1983; Johnson *et al.*, 1980). Furthermore, the volume of the nodose ganglion in *NGF*<sup>-/-</sup> mouse embryos has been reported not to be significantly smaller than that of wild-type embryos (Crowley *et al.*, 1994).

Although the above studies concluded that NGF does not play a role in regulating the survival of nodose ganglion neurons, others have provided rather conflicting evidence. For example, several studies have reported the existence of a small subpopulation of chicken nodose ganglion neurons responsive to NGF early in their development (Hedlund and Ebendal, 1978, 1980; Hiscock and Straznicky, 1986), and NGF has been shown to promote a modest elevation in the number of process-bearing neurons in E6, but not older, cultures of nodose neurons (Lindsay *et al.*, 1985). In other studies, results between dissociated and explant nodose ganglion cultures have been anomalous, with neurons in dissociated culture being entirely refractory to NGF whereas NGF promotes enhanced neurite outgrowth from early nodose ganglia in explant culture (Davies and Lindsay, 1985; Lindsay and Rohrer, 1985). Although a systematic analysis of the response of mammalian nodose neurons to NGF has not been carried out, NGF has been shown to support newborn rat nodose neurons in dissociated, neuron-enriched, long-term cultures (Baccaglini and Cooper, 1982), and to increase neurite outgrowth and neuronal somata size in rabbit nodose neurons (Sato, 1985). E14 rat nodose neurons in dissociated culture also respond to NGF with enhanced survival and neurite outgrowth. This specific effect was blocked with an NGF antiserum (Katz *et al.*, 1990). The response of nodose neurons of the chicken (Lindsay *et al.*, 1985) and rat (Katz *et al.*, 1990) at early stages in development and absence of response (Lindsay *et al.*, 1985, 1985a; Thaler *et al.*, 1994) later on raises the possibility that some nodose ganglion neurons that require NGF for survival in culture during early stages of

gangliogenesis are either eliminated by cell death or switch dependence to other factors at later ages.

Initial  $^{125}\text{I}$ -NGF binding experiments failed to demonstrate NGF receptors on chicken nodose neurons (Lindsay and Rohrer, 1985; Lindsay *et al.*, 1985a). However, specific  $^{125}\text{I}$ -NGF binding has since been demonstrated (Raivich *et al.*, 1987), as has the expression of TrkA mRNA by developing and adult rat nodose neurons (Verge *et al.*, 1992; Zhuo and Helke, 1996). In addition, adult nodose neurons are capable of retrogradely transporting NGF from their peripheral targets (Helke *et al.*, 1998). However, because of the compelling body of evidence against a survival promoting role for NGF in the nodose ganglion, these receptors have been assumed to mediate other effects of NGF. For example, in cultures of neonatal rat nodose neurons, NGF increases expression of substance P and to a lesser extent CGRP (MacLean *et al.*, 1988, 1989), increases functional nicotinic acetylcholine receptors (Mandelzys *et al.*, 1990; Mandelzys and Cooper, 1992) and promotes the growth of dendrite-like processes (De Koninck *et al.*, 1993).

Given the inconsistencies apparent in the literature and the availability of mice with a null mutation in the *NGF* gene (Crowley *et al.*, 1994), it was decided to re-examine, in a rigorous manner, the physiological significance of NGF in the development of nodose neurons. Three experimental approaches were employed: first, *in vitro* experiments assessing the effects of NGF on wild-type nodose ganglion neurons at various stages in their development, secondly, a histological evaluation of the total neuronal number in the nodose ganglion of the *NGF*<sup>-/-</sup> compared to wild-type animal, and finally, in a collaborative study, a comparison of TrkA mRNA and GAPDH mRNA expression between genotypes. The results clearly

demonstrate that a subset of nodose ganglion neurons depend on NGF for survival during development.

## 5.2. Methods

### 5.2.1. Neuronal cultures

This study considered the effects of NGF on mouse nodose ganglion neurons in dissociated, low density cultures. Embryos (E11-E18) and neonates (P1) were obtained from overnight matings of CD-1 mice. At earlier developmental stages (E11 to E16), the nodose ganglion contains relatively few non-neuronal cells and, with a clean dissection, nodose neurons were obtained relatively free of contaminating non-neuronal cells. At E18 and P1, a separation technique was required to ensure neuronal cultures of >95 % purity. This allowed any direct neurotrophic effects of a particular factor to be more clearly elucidated.

The dissections, differential sedimentation and subsequent preparation of neuronal cultures were carried out as previously described in sections 2.2.1. and 3.2.1.. Neurons were plated onto 35 mm plastic tissue culture dishes (Nunc, Gibco) at a density of 500-2000 neurons per dish and grown in 2 ml of F-14 medium with glutamine and SATO supplement (see appendix), in a humidified 5.5 % CO<sub>2</sub> environment at 37 °C. Purified recombinant human BDNF, NGF, or both in combination, were added to the cultures at the time of plating. A concentration of 5 ng/ml was used for both these factors as this is saturating for the effects of these neurotrophins in promoting the survival of embryonic nodose and trigeminal neurons, respectively (Buchman and Davies, 1993; Davies *et al.*, 1993, 1993a).

Three to six hours after plating, the number of attached neurons in a 12 x 12 mm standard graticule in the centre of each dish was counted with the aid of an inverted phase-contrast microscope. 24 and 48 hours after plating, the number of surviving phase-bright, process-bearing neurons in the same area

was ascertained. The survival response of neurons to BDNF or NGF was expressed as percent survival, i.e. the number of surviving cells at 24/48 hours expressed as a percentage of the number present at 3 hours. Within each experiment, triplicate cultures were established for each condition and all experiments were repeated at least three times.

### **5.2.2. Quantification of neurons in the nodose ganglion of NGF null mutant mice**

A small number of C57BL/6 male and female mice, heterozygous for disruption of the *NGF* gene, were received as gift from Heidi Phillips, Genentech Inc. USA. These were crossed into the CD1 background which improved the general health status and breeding potential of the colony. In accordance with Home Office regulations governing the use of animals in scientific procedures, offspring were anaesthetised with halothane and tail-tipped for genotyping purposes at four weeks of age. At this time, the mice were also labelled with numbered ear tags (IMS). Wild-type animals were eliminated from the colony at a later date. Mice homozygous for a null mutation in the *NGF* gene were not viable post weaning and hence, did not reach genotyping age. This premature death is thought to be related to ineffective ingestion of food, secondary to sensory nervous system deficits (Crowley *et al.*, 1994). The knockout animals were smaller than their littermates at birth and subsequently developed at a slower rate, for example, with respect to hair growth and eye opening. Heterozygote mice on the other hand, demonstrated normal growth and breeding characteristics and could not be distinguished from normal littermates on the basis of appearance or behaviour. For experimental purposes, heterozygous crosses were set up in order to generate embryos of all three possible genotypes, i.e. wild-type, heterozygote and knockout. The predicted ratio of

1:2:1 was borne out, indicating that the gene disruption had not compromised survival *in utero*.

Pregnant females were killed by cervical dislocation after 16 or 18 days gestation and the embryos were removed and collected in individually labelled dishes of PBS. Using fine scissors, the embryos were sub-dissected by making a transverse cut just rostral to the upper limbs and another just above the eyes. The nose, whisker pad area and brain were also removed to allow maximal penetration of the fixative. The remaining head and neck region of each embryo was then fixed in neutral buffered formalin for up to one week. Approximately 20x the volume of the head was used. The legs and/or tail of each embryo were placed in a correspondingly labelled 1.5 ml microfuge tube and used for DNA extraction and genotyping (see section 5.2.3.). The genotypes of the embryos under investigation were not revealed until after the counting was complete in order to prevent observer bias.

After fixation, the specimens were washed twice in de-ionised, distilled water and left to decalcify in 10 % (w/v) EDTA for 7-10 days, followed by dehydration of the tissues through a graded series of alcohols. The samples were cleared in chloroform overnight, in order to bleach the tissues prior to staining, incubated in three changes of paraffin wax (BDH) at 56 °C and finally embedded in a paraffin wax block. The heads were then coronally sectioned at 8 µm using a microtome. Short "ribbons" of sections were mounted on polysine coated slides (BDH) and allowed to dry for 48 hours, at 37 °C, before being stained with cresyl fast violet. Briefly, the staining protocol involved clearing the sections in xylene, rehydration through a series of graded alcohol steps and staining with 0.01 % (E16) and 0.1 % (E18) cresyl fast violet. Due to their higher water content, younger cells took up

the stain more rapidly than older ones and so were stained for less time (approximately 15 minutes for E16 tissues and 20 minutes at E18) with a weaker stain. After staining, the sections were gradually dehydrated and individual cells were differentiated in 96 % alcohol with glacial acetic acid until the cell nucleoplasm was seen to be free of stain. The time required for differentiation varied between samples and batches of stain. After a final dehydration step in 100 % alcohol and clearance in xylene, the specimens were mounted under glass coverslips (BDH) with DPX (Sigma).

The total number of neurons and pyknotic nuclei in both nodose ganglia (i.e. from left and right sides) were quantified using a recognised technique (Piñón *et al.*, 1996) which involved three main steps: measurement of ganglia size, quantification of neuronal number within a defined area and finally, estimation of ganglion total cell number. To ascertain ganglia size, the nodose ganglion was identified under x100 magnification and *camera lucida* drawings were made of its profile in every fifth section along its rostrocaudal axis. Using a scanner and an Image Processing and Analysis Program (NIH Image), the cross-sectional area of each section was calculated in mm<sup>2</sup>. The next stage was to quantify the number of neuronal cells in a standard area of every fifth section drawn previously. Neurons were counted using a Zeiss Axioskop microscope with an eye piece-mounted 0.01 mm<sup>2</sup> grid, under x1000 magnification with oil immersion, and identified by virtue of their Nissl substance and large, round, pale-stained nuclei (Konigsmark, 1970). Non-neuronal cells were clearly distinguishable by their small size, more irregular shape and very darkly stained appearance (Figure 5.1. A). The counting graticule was moved randomly around each section with a minimum of 3-5 grids counted per section. In cases where the area of the section was less than that of the grid, all neurons in the section were counted. Hence, for every fifth section, a mean number of neurons per 0.01 mm<sup>2</sup> was

calculated, and from the area of each section, the total number of neurons per section was quantified. These figures were added together and multiplied by five to give an estimation of total ganglion neuron number (uncorrected). A correction for split nuclei was made by the method of Abercrombie (Abercrombie, 1946) as shown below:

$$n_c = n_u \times T / (P + T) \quad \text{where,}$$

$n_c$  = corrected number of neurons  
 $n_u$  = uncorrected number of neurons  
 $T$  = section thickness  
 $P$  = mean nuclear diameter.

Mean nuclear diameter was estimated by drawing approximately 100 nuclear profiles per ganglion using a drawing tube under x400 magnification. Nuclear diameter was measured from the profiles using NIH Image software. This was undertaken for both wild-type and knockout embryos to control for any neuronal size differences between the genotypes.

To evaluate the extent of cell death, pyknotic nuclei were also counted. These were recognised as one or more very darkly stained spherical structures, representing condensed chromatin, contained within a clearly visible membrane (Clarke and Hornung, 1989; Piñón *et al.*, 1996) (Figure 5.1. B). The great majority of these pyknotic nuclei were observed in large degenerating cells, suggesting that these were neurons (Oppenheim, 1991). Any pyknotic nuclei observed in very small cells, of similar size to satellite cells, were excluded. The total number of pyknotic nuclei in every fifth section was counted under x400 magnification and extrapolated to give an estimation of total pyknotic nuclei per ganglion as described above.

In rodents, the nodose ganglion is juxtaposed to another, much smaller, population of placode-derived neurons that constitutes the petrosal ganglion. Because it is not always easy to discern the precise junction between these ganglia, this minor population of placode-derived sensory neurons was included in this analysis.

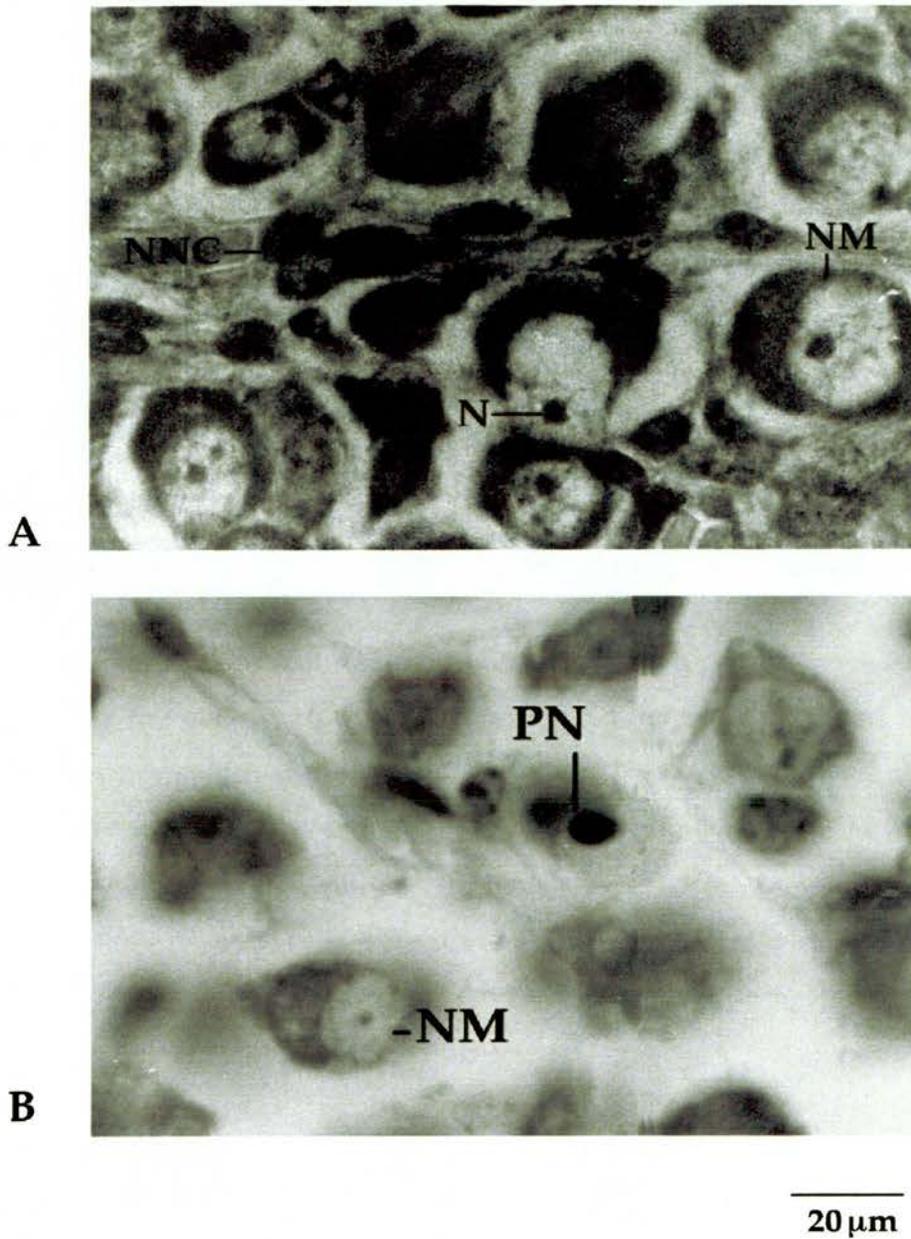


Figure 5.1. Photomicrographs of the histological appearance of the nodose ganglion stained with cresyl fast violet.

(A) The upper figure shows the appearance of E18 nodose ganglion neurons demonstrating the nuclear membrane (NM) and nucleolus (N). Small darkly stained non-neuronal cells are also visible (NNC).

(B) The lower figure demonstrates the typical appearance of a pyknotic nucleus (PN).

(x1000 magnification)

### 5.2.3. Genotyping of *NGF* null mutant mice by PCR

All stages in the preparation of DNA and subsequent PCR reactions were carried out in a plasmid DNA-free environment using standard procedures to avoid DNA contamination. Tissue from E16 and E18 mouse embryos was digested overnight in 500  $\mu$ l proteinase K solution at 55 °C, after which an equal volume of basic phenol was added and gently mixed. Following centrifugation, 100  $\mu$ l of the aqueous layer containing the DNA was removed, placed in a new tube and mixed with 120  $\mu$ l de-ionised water, 20  $\mu$ l 3M sodium acetate, pH5.2 and 550  $\mu$ l 100 % ethanol. The precipitated DNA was then pelleted by centrifugation and, after removal of the supernatant, the DNA pellet was washed with 70 % ethanol. After further centrifugation and removal of the ethanol, the DNA pellet was air dried and resuspended in a volume of water appropriate to the pellet size (usually around 20-100  $\mu$ l). This DNA solution was ready for amplification by PCR.

In the production of *NGF* knockout mice, a segment of the *NGF* gene was replaced by a neomycin resistance gene cassette by homologous recombination in embryonic stem cells (Crowley *et al.*, 1994). In this study, the polymerase chain reaction (PCR) was used to ascertain which combination of the two possible *NGF* alleles each embryo carried (i.e. wild-type or neo cassette). A three primer PCR reaction was used as this allowed identification of both wild-type and mutated alleles in a single PCR reaction. One primer was located in the *NGF* coding sequence and was common to both alleles (NGFC). A second primer was located in the region of the *NGF* coding sequence replaced in the mutant and therefore, detected the wild-type allele only (NGF1). To detect the mutant allele, a third primer that bound to the *neo* gene was used (NGF2). The use of these three primers together gave amplification of a single PCR product for wild-type animals, another unique single product for knockout animals and both PCR products

for heterozygotes. As these products were of different sizes (190 bp's for the WT band and 610 bp's for the KO band), they were readily resolved on an agarose gel.

A reaction master mix was prepared using the appropriate volumes of reagents for the number of tubes required. Two reactions were set up per animal in order to monitor the consistency of the results. Appropriate positive (known +/- DNA) and negative (no DNA) control PCR reactions were also performed. Each reaction was carried out in a 20  $\mu$ l reaction volume in a 500  $\mu$ l microfuge tube as below:

2 $\mu$ l	10x reaction buffer
4 $\mu$ l	25 mM magnesium chloride
1 $\mu$ l	5 mM dNTPs
0.16 $\mu$ l	NGF1
0.5 $\mu$ l	NGF2
0.24 $\mu$ l	NGFC
0.13 $\mu$ l	Taq thermostable DNA polymerase
1 $\mu$ l	template DNA
7.17 $\mu$ l	ddH <sub>2</sub> O
3.8 $\mu$ l	5x Q solution

The primer sequences were as follows:

NGF1	-5' ACAGATAGCAATGTCCCAG 3'
NGF2	-5' TCTGGATTCATCGACTGTG 3'
NGFC	-5' GGTGCTGAACAGCACACG 3'

Two drops of mineral oil were layered on top of the PCR reagents to prevent their evaporation during thermocycling. After an initial denaturation step of 95 °C for 15 minutes, the PCR program comprised 33 cycles of the following conditions: 95 °C for 1 minute (denaturation), 57 °C for 1 minute (primer annealing) and 72 °C for 1 minute 30 seconds (elongation). This was followed by a final elongation stage of 72 °C for 10 minutes and completed with a refrigeration step to 4 °C.

Gel loading buffer was added to the completed PCR reaction and the products of the PCR reaction were separated on 2 % agarose gels cast with TAE buffer and containing 1 µg/ml ethidium bromide. The wild-type lower band and mutant upper band were visualised under ultra-violet light. The size of the products in relation to a 1 kb reference ladder is demonstrated in Figure 5.2..

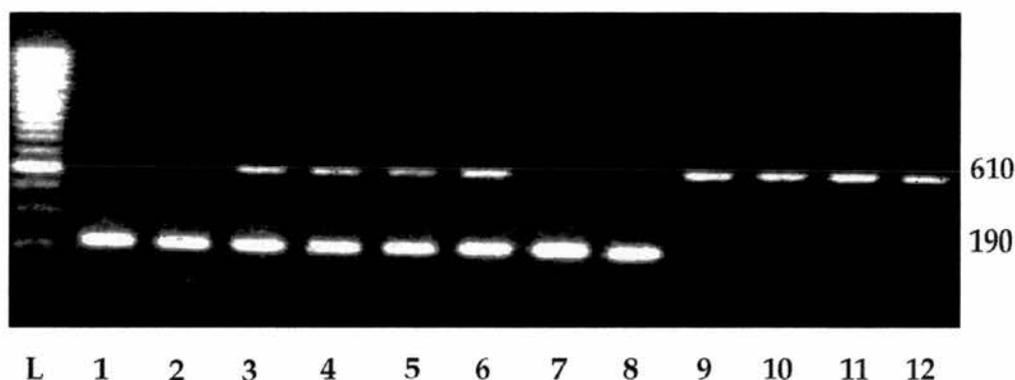


Figure 5.2. Gel showing the three possible genotypes of embryos from *NGF* +/- crosses.

Lanes 1, 2, 7 and 8 show the PCR products from wild-type DNA, lanes 3, 4, 5 and 6 show the PCR products from heterozygote DNA and lanes 9, 10, 11 and 12 show the PCR products from homozygote null mutant DNA. The size of the products is shown with respect to a 1 kb reference ladder (L).

#### 5.2.4. Measurement of TrkA and GAPDH mRNA levels

This part of the study was carried out by a colleague, Dr. Sean Wyatt, but is included here as the results substantiate other findings from the tissue culture and histology investigations.

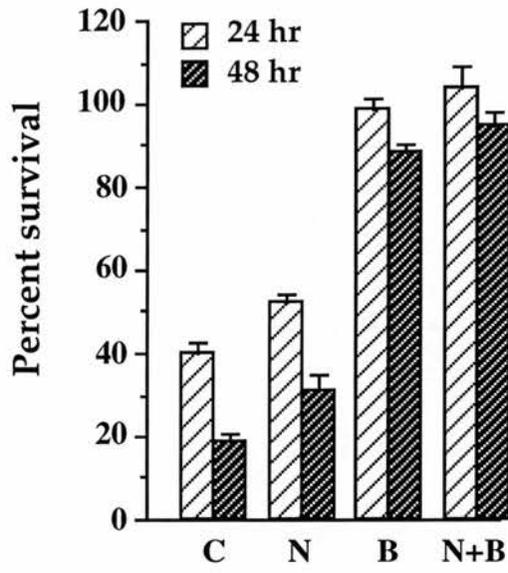
A quantitative RT-PCR technique (Wyatt and Davies, 1993) was used to measure the levels of mRNAs encoding TrkA mRNA and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA in total RNA extracted from pairs of nodose ganglia dissected from wild-type and *NGF* knockout mouse embryos. The dissection of nodose ganglia and genotyping of *NGF* mutant mice were as in sections 3.2.1. and 5.2.3. respectively. Details of the competitors, primers and reaction conditions can be found elsewhere (Wyatt and Davies, 1993, 1995; Wyatt *et al.*, 1997). Previous detailed comparisons of this method with quantitative Northern blotting have demonstrated its accuracy and reproducibility over a wide range of mRNA concentrations (Wyatt and Davies, 1993).

## 5.3. Results

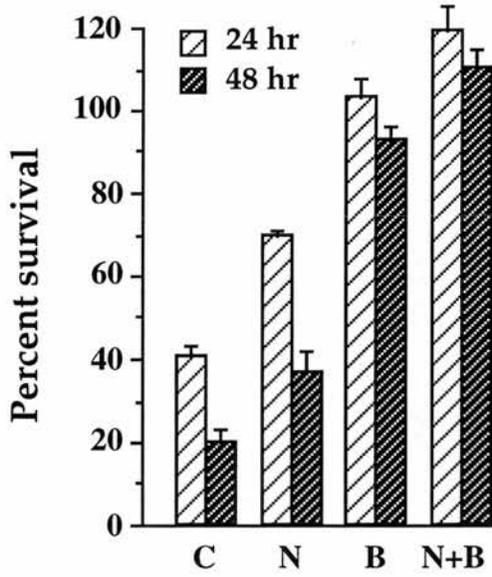
### 5.3.1. Survival response of developing nodose neurons to NGF

To investigate if NGF is capable of promoting the survival of nodose neurons *in vitro*, low density, dissociated cultures of these neurons were established at stages throughout their development. The number of surviving neurons was counted in control cultures (no neurotrophins present) and in cultures containing saturating concentrations of NGF, BDNF and NGF plus BDNF. After both 24 and 48 hours in culture, BDNF promoted the survival of the majority of neurons at all stages studied (Figure 5.3.). Neuronal survival in control cultures was very low except in E11 and E12 cultures where it was around 20 % after 48 hours incubation. This reflects the relatively long period of neurotrophin-independent survival of early nodose neurons (Vogel and Davies, 1991). At all stages examined, there were more neurons surviving in NGF supplemented cultures than in control cultures after both 24 and 48 hours incubation. This NGF survival response was, however, much less pronounced than the response of these neurons to BDNF. Nevertheless, as demonstrated in Figure 5.4., the NGF survival response appeared to peak at E12. After E12, the NGF survival response fell to almost zero by E16, however, a survival response to NGF re-emerged at P1. Additive effects of NGF and BDNF in combination were evident particularly at E12, the peak of nodose neuron NGF responsiveness. This indicates that at this age at least, the subpopulations of nodose neurons responding to NGF and BDNF are partially distinct.

# E11

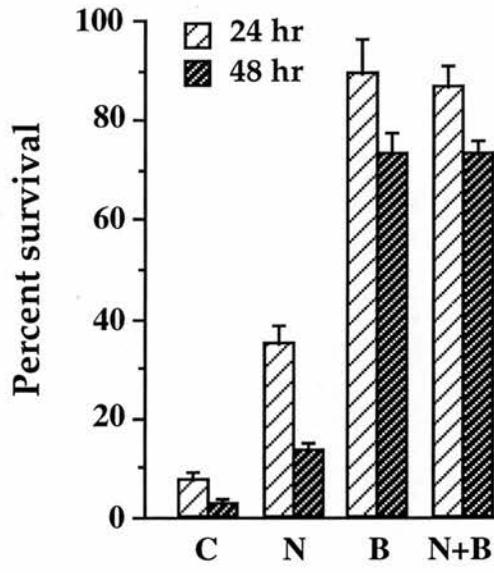


# E12

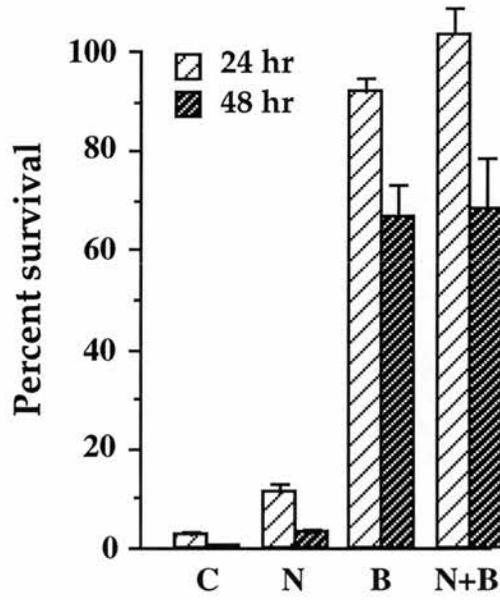


(Figure 5.3.)

# E13

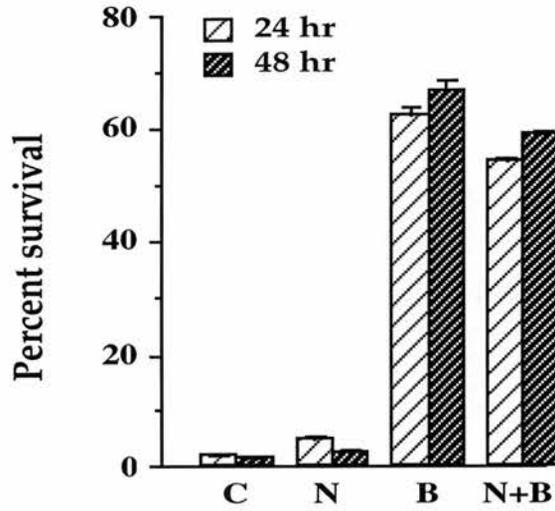


# E14



(Figure 5.3.)

## E18



## P1

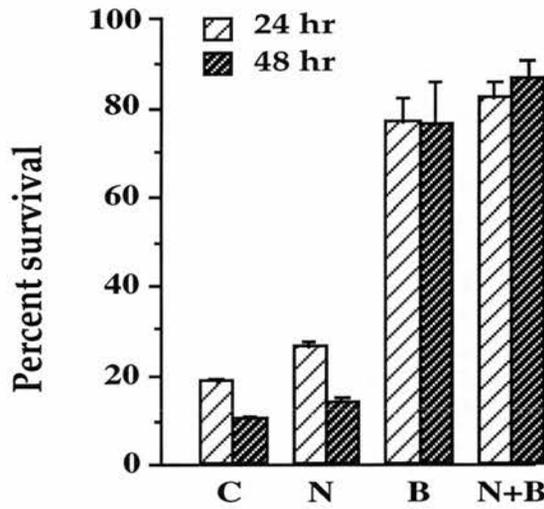


Figure 5.3. Bar charts of the survival responses of embryonic and newborn nodose ganglion neurons to NGF and BDNF.

These six panels show the percent survival of nodose neurons from E11 to P1 mice in control cultures (C) containing no neurotrophins and in cultures supplemented with 5 ng/ml NGF (N), 5ng/ml BDNF (B) or NGF plus BDNF (N+B). The number of neurons surviving after 24 and 48 hours incubation is expressed as a percentage of the number of attached neurons counted 3 hours after plating. The means and standard errors are shown ( $n \geq 3$  for each condition and at each age).

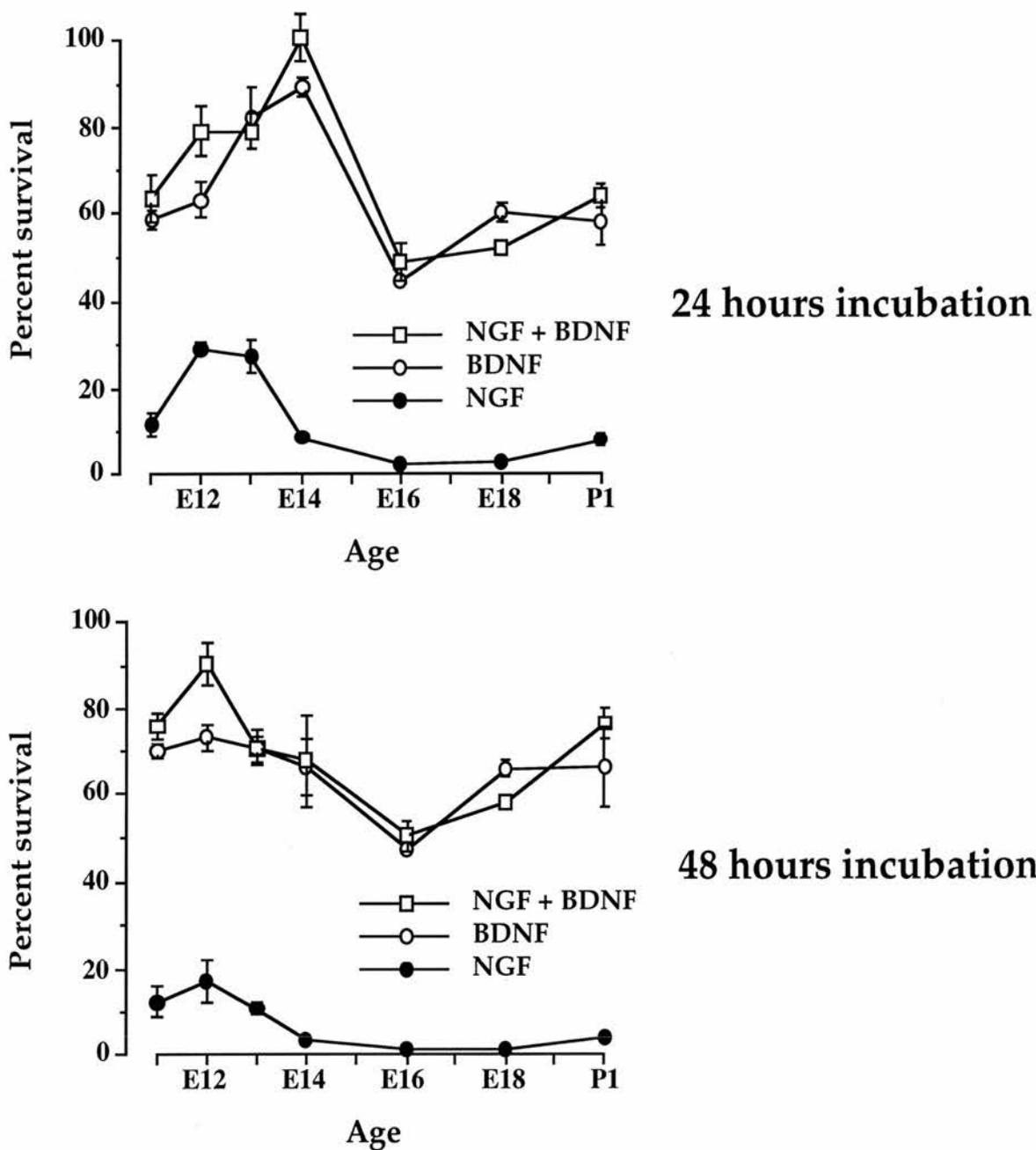


Figure 5.4. Graphs of the changes in NGF and BDNF responsiveness of nodose neurons throughout development.

These two panels summarise the changes with age in the response of nodose neurons to NGF and BDNF after 24 and 48 hours incubation. For clarity, the percent survival in control cultures is subtracted to show survival above control levels. The means and standard errors are shown (n≥3 for each condition at each age).

### 5.3.2. Loss of nodose ganglion neurons in *NGF*<sup>-/-</sup> embryos

The total number of neurons in the nodose ganglia of wild-type and *NGF* knockout embryos was estimated at E16 and E18 by counting neuronal profiles in 8  $\mu\text{m}$  serial sections and correcting for double counting of sectioned neuronal nuclei by the method of Abercrombie (Abercrombie, 1946). Figure 5.5. A demonstrates there were significantly fewer neurons in the nodose ganglia of *NGF*<sup>-/-</sup> embryos compared with wild-type embryos at both E16 (11 % decrease,  $p < 0.005$ , Student's t-test,  $n = 3$  WT animals and 4 KO animals) and E18 (15 % decrease,  $p < 0.0001$ , Student's t-test,  $n = 5$  WT and 5 KO animals). Total neuronal counts for wild-type ganglia were in line with previously published data (ElShamy and Ernfors, 1997; Liu *et al.*, 1995; Moore *et al.*, 1996). From the calculations of mean nuclear diameter for the Abercrombie Correction, it is interesting to note that average nuclear size was not significantly affected in the *NGF*<sup>-/-</sup> animals compared to the wild-type (at E18, WT- $11.16 \pm 0.16 \mu\text{m}$  and KO- $10.62 \pm 0.26 \mu\text{m}$ ).

To provide an indication of the number of dying cells at each of these ages, the total number of pyknotic nuclei was also counted. Figure 5.5. B shows that there was a 76 % increase in the number of pyknotic nuclei in the nodose ganglia of *NGF*<sup>-/-</sup> embryos compared with wild-type embryos at E16, suggesting that neuronal death is elevated in the absence of NGF at this stage of development. At E18, however, there was no significant difference in the number of pyknotic nuclei in the nodose ganglia of wild-type and *NGF*<sup>-/-</sup> embryos.

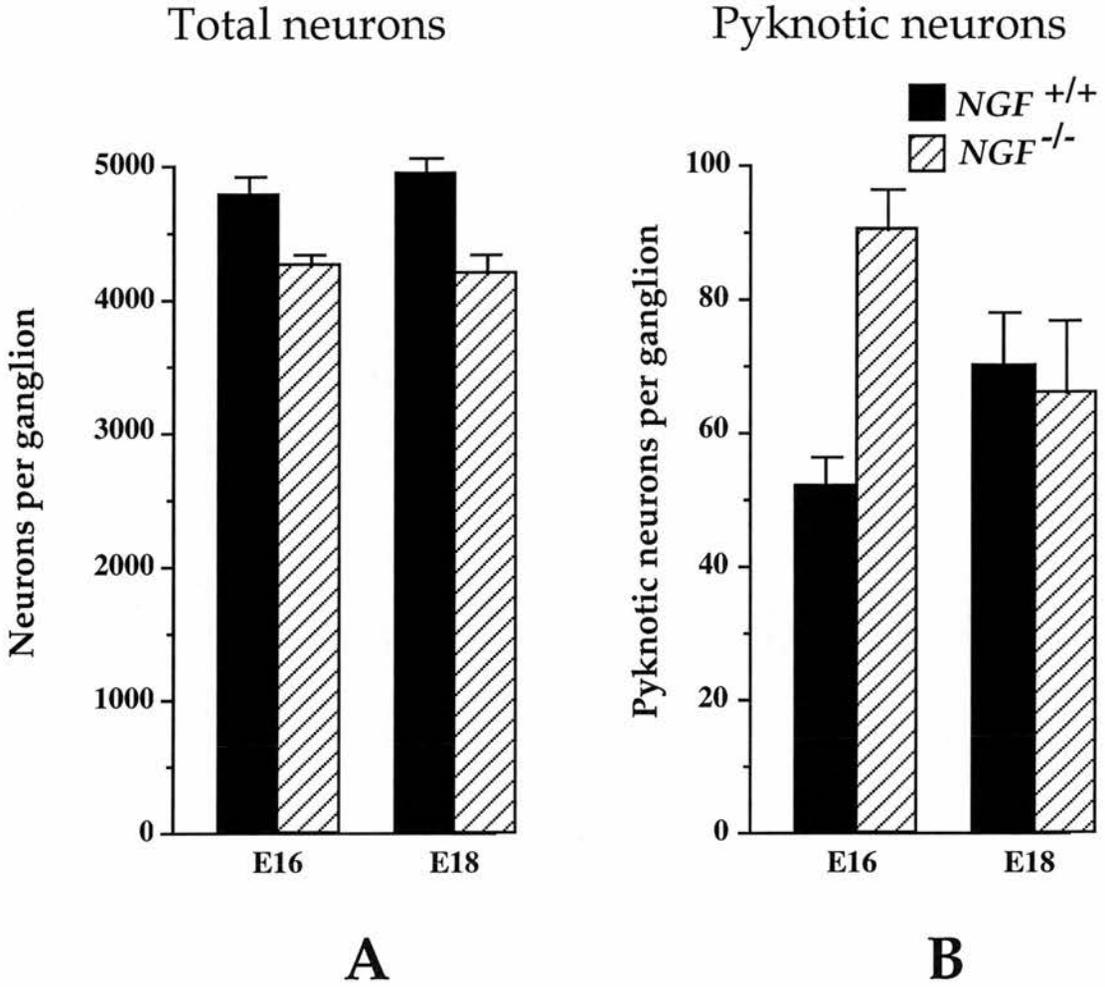


Figure 5.5. Bar charts of the total numbers of neurons (A) and pyknotic nuclei (B) in the nodose ganglia of E16 and E18 wild-type ( $NGF^{+/+}$ ) and knockout ( $NGF^{-/-}$ ) embryos.

The means and standard errors of the data obtained from 3 (E16) to 5 (E18) wild-type and 4 (E16) to 5 (E18)  $NGF^{-/-}$  embryos are shown.

An additional measure of the relative numbers of cells in the nodose ganglion of wild-type and *NGF*<sup>-/-</sup> embryos is provided from the levels of mRNA encoding glyceraldehyde phosphate dehydrogenase (GAPDH), a ubiquitous, constitutively expressed "housekeeping" protein, which was measured using a quantitative RT-PCR technique. Three different stages of development were analysed (E13, E15 and E18) and in each instance, the levels of GAPDH mRNA were lower in the nodose ganglia of *NGF*<sup>-/-</sup> embryos compared with the ganglia of wild-type embryos (Figure 5.6.). These differences were statistically significant at E15 and E18 ( $p < 0.05$ , Student's t-test). These results suggest that there are fewer cells in the nodose ganglia of *NGF*-deficient embryos in the latter half of development.

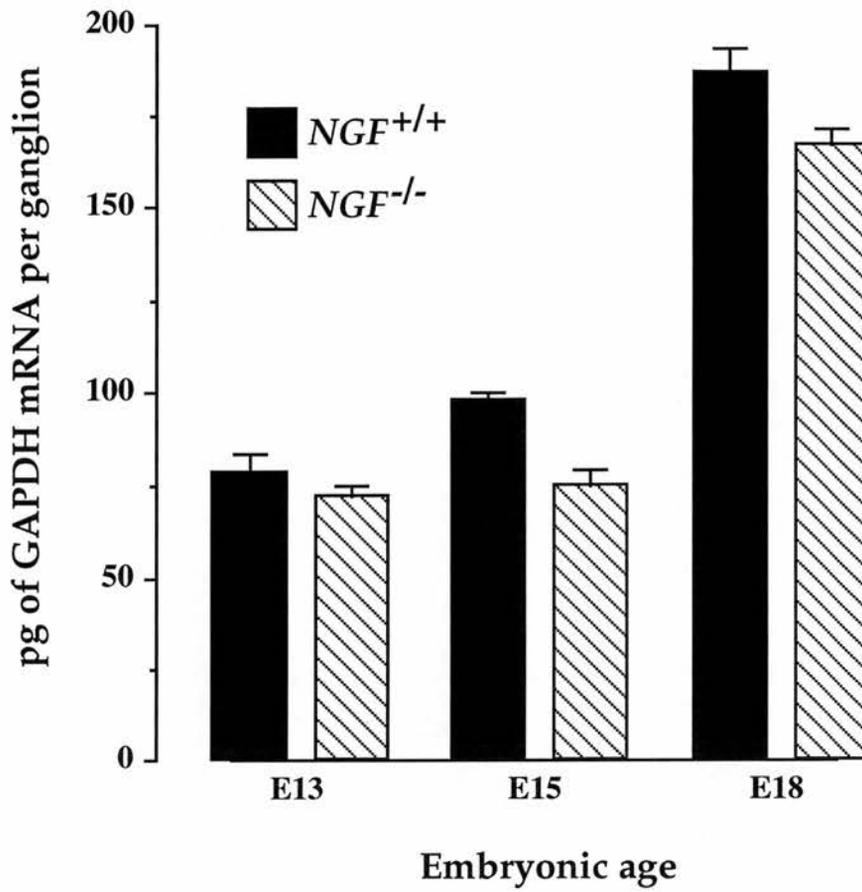


Figure 5.6. Bar chart of the level of GAPDH mRNA in the nodose ganglia of E13, E15 and E18 wild-type ( $NGF^{+/+}$ ) and knockout ( $NGF^{-/-}$ ) embryos.

The means and standard errors of data obtained from 4 to 7 embryos of each genotype at each age are shown.

### 5.3.3. TrkA mRNA expression in the nodose ganglia of wild-type and *NGF*<sup>-/-</sup> embryos

The NGF receptor tyrosine kinase, TrkA, is known to be essential for the survival response of neurons to NGF (Allsopp *et al.*, 1993; Xu *et al.*, 1994). Hence, if nodose neurons are responsive to NGF during development, TrkA must be expressed. TrkA expression was analysed using competitive RT-PCR which permits quantification of the levels of specific mRNA transcripts (Wyatt and Davies, 1993). TrkA mRNA was expressed as early as E11, shortly after the nodose ganglion becomes discernible. From E11 to E13, the mean level of expression increased and, after a small decrease at E14 and E15, rose to a peak at E18 (Figure 5.7.). These results suggest that NGF-responsive cells are present within the nodose ganglia during normal embryonic development.

In addition to ascertaining the normal developmental time-course of TrkA expression in the nodose ganglia, the effects of the *NGF* null mutation on TrkA mRNA expression in the nodose ganglia were also investigated. Figure 5.8. shows that the level of TrkA mRNA was lower in the nodose ganglia of *NGF*<sup>-/-</sup> embryos at all stages studied (E13, E15 and E18); the decrease being statistically significant at E15 and E18 ( $p < 0.01$ , Student's *t*-test). Moreover, the relative decrease in TrkA mRNA levels in *NGF*<sup>-/-</sup> embryos versus wild-type embryos at E15 and E18 (43 % and 67 % respectively) was much greater than the relative decrease in the total number of neurons in the nodose ganglia of *NGF*<sup>-/-</sup> embryos at these stages (11 % and 15 % respectively). This suggests that TrkA-expressing nodose neurons are preferentially lost in the absence of NGF.

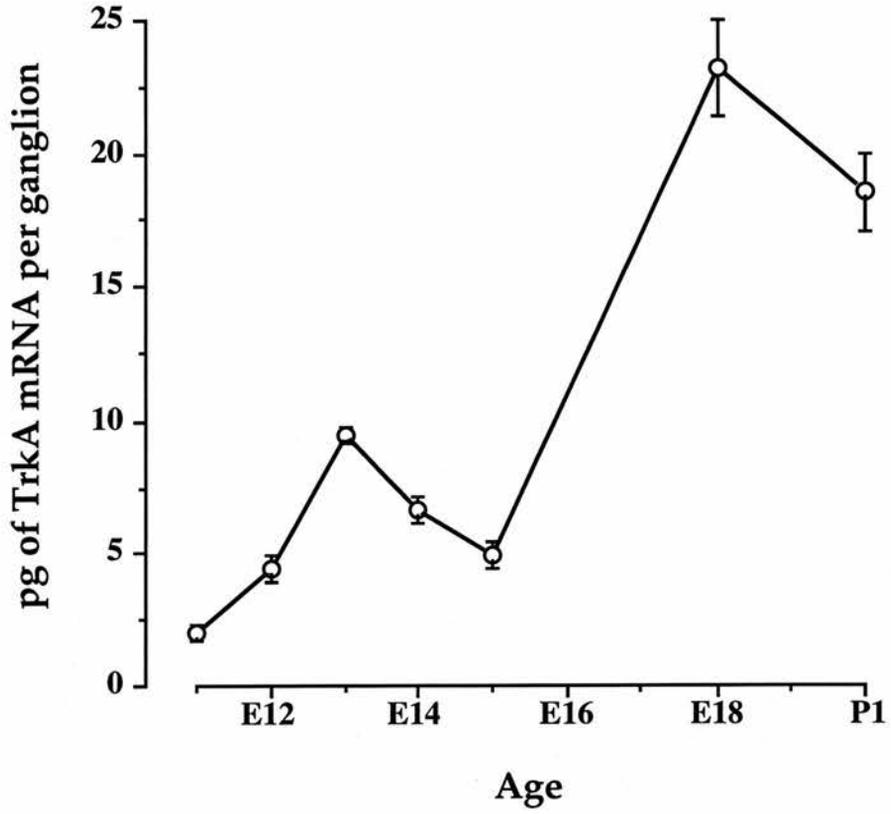


Figure 5.7. Graph of the level of TrkA mRNA in the nodose ganglia of wild-type embryos during development.

The means and standard errors of data obtained from 3 to 6 embryos at each age are shown.

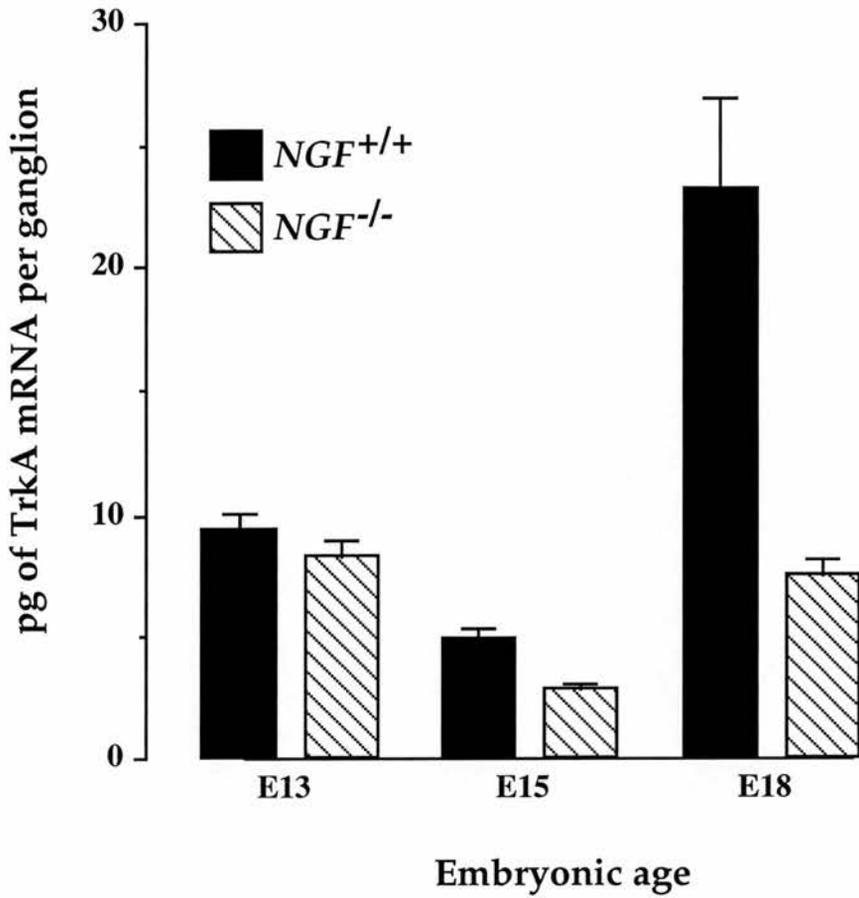


Figure 5.8. Bar chart of the level of TrkA mRNA in the nodose ganglia of E13, E15 and E18 wild-type ( $NGF^{+/+}$ ) and knockout ( $NGF^{-/-}$ ) embryos.

The means and standard errors of data obtained from 4 to 7 embryos of each genotype at each age are shown.

## 5.4. Discussion

Three lines of evidence demonstrate that the nodose ganglia of mouse embryos contain a subset of neurons that depend on NGF for survival. First, NGF promotes the survival of a small subset of nodose ganglion neurons in cultures established over a range of embryonic stages. At younger embryonic ages, this population is at least partially distinct from the major subset of BDNF responsive neurons. Second, detailed histological comparison of the nodose ganglia of wild-type and *NGF*<sup>-/-</sup> embryos has revealed that neuronal death is elevated in embryos lacking NGF, resulting in a significant reduction in the total number of neurons in the nodose ganglion by the end of embryonic development. This line of evidence is additionally supported by the finding that the level of GAPDH mRNA (which is proportional to cell number) is reduced to a similar extent in the nodose ganglia of *NGF*<sup>-/-</sup> embryos. Thirdly, quantitative RT-PCR has shown that TrkA mRNA is expressed in the nodose ganglia of wild-type embryos throughout embryonic development, indeed the peaks in TrkA expression closely mirror NGF responsiveness of nodose neurons in culture. Moreover, there is a marked decrease in TrkA mRNA expression in the nodose ganglia of *NGF*<sup>-/-</sup> embryos. This suggests that the neurons within the nodose ganglia that are capable of responding to NGF are preferentially lost in embryos lacking NGF. Rather than representing cell loss, it is possible that the reduction in TrkA mRNA levels merely indicates a down-regulation of TrkA in the absence of NGF. This is most unlikely, however, as NGF has been shown not to regulate TrkA expression in developing NGF-dependent trigeminal neurons (Wyatt and Davies, 1993; Davies *et al.*, 1995). The tissue culture data indicate that some NGF-responsive neurons may also be BDNF-responsive, i.e. the populations are only partially distinct. Hence, not all TrkA expressing cells are lost in the *NGF*<sup>-/-</sup> animal. Those cells that are lost

are clearly, critically dependent on NGF, demonstrating that other neurotrophic factors could not compensate for the loss of NGF action on these neurons. As the peak of responsiveness of nodose neurons in culture to NGF occurred at E12-E13, it would be interesting to consider any differences in total neuronal numbers and indeed, the degree of cell death at earlier ages than in the present study, for example E14. GAPDH levels at E13 indicate that there may be a reduction in cell number at this age, however, this difference was not significant.

These results contrast with many reports that have concluded that NGF does not influence the survival of nodose neurons either *in vitro* (Davies and Lindsay 1985; Lindsay and Rohrer 1985; MacLean *et al.*, 1988; Buj-Bello *et al.*, 1994; Thaler *et al.*, 1994) or *in vivo* (Pearson *et al.*, 1983; Dimberg *et al.*, 1987; Rohrer *et al.*, 1988; Johnson *et al.*, 1980; Crowley *et al.*, 1994). Some previous studies have suggested a role for NGF in promoting survival and neurite outgrowth from nodose ganglion neurons (Hedlund and Ebendal, 1978, 1980; Baccaglini and Cooper, 1982; Lindsay *et al.*, 1985; Katz, *et al.*, 1990). However, because these effects were modest in size, present only at limited stages early in development and inconsistent between reports, a role for NGF in the development of nodose neurons was largely ruled out. In an initial analysis of the nodose ganglion of *NGF*<sup>-/-</sup> mice, a reduction in ganglion volume was reported, albeit not statistically significant, and interestingly, a decrease in neuronal cell size was reported also (Crowley *et al.*, 1994). It is possible that had actual neuronal counts been undertaken, or if more ganglia had been analysed (n=3 in the analysis undertaken), a significant difference between the *NGF* null mutant and wild-type may have been revealed.

The small subset of NGF-dependent, mouse nodose ganglion neurons may represent a specific subpopulation of neurons as determined by size and/or phenotype. NGF-dependent sympathetic neurons express tyrosine hydroxylase (TH) (Black *et al.*, 1981; Hayashi *et al.*, 1985), a primary enzyme involved with catecholamine biosynthesis. There is a good deal of evidence to suggest that a subpopulation of mammalian (Katz *et al.*, 1983, 1987; Jonakait *et al.*, 1984; Katz, 1991; Katz and Erb, 1990), but not avian (Xue *et al.*, 1985), sensory neurons also have a catecholaminergic phenotype and this has been confirmed in the nodose ganglion (Katz *et al.*, 1983, 1986, 1987). It is tempting to speculate that there is a correlation between NGF dependency and a catecholaminergic phenotype in the nodose ganglion. This is currently being investigated by analysing the expression of TH, by RT-PCR, in NGF knockout mice. A reduction in TH expression in the NGF<sup>-/-</sup> animal may reflect a loss of TH-expressing cells in the absence of NGF, since TH expression in sensory neurons is not regulated by NGF (Katz *et al.*, 1986).

The findings from this study raise the question of the function of the subset of NGF-dependent neurons in the nodose ganglion. Previous studies of the response of different populations of sensory neurons to neurotrophic factors (Davies 1987a) and studies of Trk receptor expression (Mu *et al.*, 1993; McMahon *et al.*, 1994; Wright and Snider, 1995; Fundin *et al.*, 1997) and the kinds of neurons lost in mice with null mutations in the neurotrophin and neurotrophin receptor genes (Snider, 1994; Lewin and Barde, 1996) have indicated that the neurotrophin survival requirements of different kinds of sensory neurons are related to sensory modality. The available evidence, largely derived from studies of dorsal root ganglion neurons, suggests that NGF promotes the survival of nociceptive neurons. For example, rodents exposed to anti-NGF antibodies *in utero* are unresponsive to a variety of painful stimuli and display selective loss of small diameter DRG neurons,

substantially reduced numbers of unmyelinated and thinly myelinated axons in spinal nerve dorsal roots and almost no sensory axons projecting to laminae I and II in the dorsal horn (Johnson *et al.*, 1980, 1983; Goedert *et al.*, 1984; Ruit *et al.*, 1992). Likewise, the phenotype of *NGF*<sup>-/-</sup> and *TrkA*<sup>-/-</sup> mice is consistent with the loss of nociceptive neurons (Crowley *et al.*, 1994; Smeyne *et al.*, 1994). Depletion of NGF in neonates also reduces the number of nociceptive afferents in the skin and impairs responsiveness to noxious heat and mechanical stimuli (Ritter *et al.*, 1991; Lewin *et al.*, 1992; Lewin and Mendell, 1994). In addition, reducing NGF levels in adult mice leads to an impaired sensitivity of nociceptors to noxious thermal and chemical stimuli (McMahon *et al.*, 1995; Bennett *et al.*, 1998a). These effects are not due to a loss of NGF-dependent neurons, but rather reflect a modulation of phenotype in the absence of NGF. Nodose neurons provide a major source of afferents to the thoracic and abdominal viscera. Whether the subset of *TrkA*-expressing, NGF-dependent neurons in the nodose ganglion are nociceptors is not known. However, given these findings, it may be of potential clinical significance to ascertain which kinds of nodose sensory endings are lost or affected in the viscera of experimental animals depleted of endogenous NGF.

In conclusion, the data presented in this chapter demonstrates that a significant subset of mouse nodose neurons is dependent on endogenous NGF for survival during embryonic development. Although this finding is apparent from tissue culture and *TrkA* mRNA expression studies, the availability of mice lacking *NGF* (Crowley *et al.*, 1994) has provided the opportunity to re-examine and finally confirm the physiological significance of NGF in the development of nodose neurons.

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# Appendix I

## Abbreviations used in this thesis

AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
APS	Ammonium persulphate
ARTN	Artemin
ATP	Adenosine triphosphate
Bcl2	B-cell lymphoma/leukemia-2
BDNF	Brain-derived neurotrophic factor
BH	Bcl2 homology region
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene-related peptide
ChAT	Choline acetyltransferase
CHO	Chinese hamster ovary
CMF	Calcium-, magnesium-free
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CNTR $\alpha$	CNTF receptor alpha
CPG	Controlled pore glass
cRNA	Complementary ribonucleic acid
CT	Cardiotrophin
ddH <sub>2</sub> O	Double de-ionised, distilled water
DMSO	Dimethylsulphoxide
DMTG	Dorsomedial trigeminal ganglion
DNA	Deoxyribonucleic acid

dNTP	Deoxynucleotide triphosphate
DRG	Dorsal root ganglion
DVR	<i>Decapentaplegic-Vg1</i> -related
E	Embryonic day
EC <sub>50</sub>	Half maximally effective concentration
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
ENS	Enteric nervous system
ERK	Extracellular-signal regulated kinase
FGF	Fibroblast growth factor
FMTC	Familial medullary thyroid carcinoma
F12	Ham's nutrient mixture F12
F14	Ham's nutrient mixture F14
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial cell line-derived neurotrophic factor
gp130	Glycoprotein 130
GPA	Growth promoting activity
GPI	Glycosyl-phosphatidylinositol
GFR $\alpha$	GDNF family receptor alpha
GST	Glutathione-S-transferase
HBSS	Hank's balanced salt solution
HGF	Hepatocyte growth factor
HIHS	Heat-inactivated horse serum
HRP	Horse radish peroxidase
ICE	Interleukin-1 $\beta$ -converting enzyme
IFA	Incomplete Freund's adjuvant
IL	Interleukin
IL-6R $\alpha$	Interleukin-6 receptor alpha
JAK	Jak kinases

JNK	Jun kinase
kb	Kilobase
Kd	Dissociation constant
kDA	Kilo Dalton
KLH	Keyhole limpet hemacyanin
LIF	Leukemia inhibitory factor
LIFR $\beta$	Leukemia inhibitory factor receptor-beta
L-15	Liebowitz-15 medium
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MBS	3-Maleimidobenzoic acid N-hydroxysuccinimidine ester
MEN	Multiple endocrine neoplasia
MHC	Major histocompatibility complex
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
NGF	Nerve growth factor
NT	Neurotrophin
NTN	Neurturin
OD	Optical density
OSM	Oncostatin M
6-OHDA	6-Hydroxydopamine
P	Postnatal day
PAGE	Polyacrylamide gel electrophoresis
PARP	PolyADP-ribose polymerase
PCR	Polymerase chain reaction
PI3-kinase	Phosphatidylinositol 3-kinase
PIPLC	Phosphoinositide-specific phospholipase C
PLC	Phospholipase C
PBS	Phosphate buffered saline

PNS	Peripheral nervous system
PSP	Persephin
Ret	Rearranged during transfection
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SCG	Superior cervical ganglion
SDS	Sodium dodecyl sulphate
SH2	Src homology 2
SHP	SH2-containing tyrosine phosphatase
SNT	Suc1-associated neurotrophic factor target
STAT	Signal transducers and activator of transcription
TAE	Tris-acetate-EDTA
Taq	Thermus aquaticus
TEMED	NNN'N'-tetramethylethylenediamine
TGF- $\beta$	Transforming growth factor-beta
TH	Tyrosine hydroxylase
TMN	Trigeminal mesencephalic nucleus
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
Tris	Tris (hydroxymethyl) methylamine
Trk	Tropomyosin-related kinase
Tween 20	Polyoxyethylenesorbitan monolaurate
VLTG	Ventrolateral trigeminal ganglion

## Appendix II

### Solutions and media used in tissue culture studies

#### 0.1 % Trypsin

50 mg of trypsin was added to 5 ml of CMF-HBSS (Hank's balanced salt solution without calcium and magnesium, GibcoBRL) and sterilised with a 0.22  $\mu\text{m}$  filter (Nalgene). 100  $\mu\text{l}$  aliquots were stored at  $-20\text{ }^{\circ}\text{C}$ .

#### Poly-DL-ornithine

0.5 mg/ml poly-DL-ornithine (Sigma) was prepared in 0.15 M borate buffer (4.6g boric acid (BDH) in 500 ml de-ionised, distilled water, pH 8.4) and filter sterilised using a 0.22  $\mu\text{m}$  bell filter (Gelman Sc).

#### Laminin

Natural mouse laminin (GibcoBRL) was thawed at  $4\text{ }^{\circ}\text{C}$  and diluted in Ham's F-14 medium to give a final concentration of 20  $\mu\text{g}/\text{ml}$ .

#### L-15

A 1 l unit of L-15 powder (GibcoBRL) was dissolved in 1 l of de-ionised, distilled water containing 100 mg streptomycin (Sigma) and 60 mg penicillin (Sigma). The pH was adjusted to 7.3 and the solution sterilised with a 0.22  $\mu\text{m}$  bell filter (Gelman Sc).

#### Ham's F-12

A 1l unit of F-12 powder (GibcoBRL) was dissolved in 1 l of de-ionised, distilled water containing 100 mg streptomycin (Sigma) and 60 mg penicillin (Sigma). The pH was adjusted to 7.3 and the solution sterilised with a 0.22  $\mu\text{m}$  bell filter (Gelman Sc).

### **Ham's F-14**

10X stock: a 5 l unit of F-14 powder (Imperial Laboratories) was dissolved in 500 ml of de-ionised, distilled water containing 500 mg streptomycin (Sigma) and 300 mg penicillin (Sigma). The 10X stock was stored in 50 ml aliquots at -40 °C.

1X solution: a 50 ml aliquot of 10X F-14 stock was made up to 500 ml with de-ionised, distilled water containing 1 g  $\text{NaHCO}_3$  (BDH). The pH was adjusted to pH7 by adding a small pellet of dry ice, after which the solution was sterilised with a 0.22  $\mu\text{m}$  bell filter (Gelman Sc). To prepare serum-supplemented medium, 50 ml of HIHS (heat-inactivated horse serum, GibcoBRL) was added to 450 ml of 1x F-14.

### **SATO supplement**

A stock solution was prepared containing the following reagents: 100 ml pathocyte-4-BSA (ICN Biomedicals), 100 ml  $\text{ddH}_2\text{O}$ , 160 mg putrescine, 1 ml progesterone (0.625 mg/ml in ethanol, Sigma), 10 ml L-thyroxine (0.4 mg/ml in ethanol, Sigma), 1 ml sodium selenite (0.4 mg/ml in PBS, Sigma), 10 ml tri-iodothyronine (0.34 mg/ml in ethanol, Sigma). This stock solution was stored in 11.1 ml aliquots at -40 °C. To prepare the culture medium, 11.1 ml of SATO stock and 5 ml of 200 mM L-glutamine (GibcoBRL) were made up to 500 ml with 1x F-14 medium.

### **Neurotrophic factors**

All neurotrophic factors were kept in stock aliquots of 1 or 10  $\mu\text{g}/\text{ml}$  in F-12 plus 10 % HIHS, pH5.55, at -80 °C until use. Recombinant human CNTF was a gift from Regeneron Inc. USA. All other recombinant growth factors were received as gifts from John Winslow, Gene Burdon, Arnon Rosenthal and

Heidi Phillips, Genentech Inc. USA: recombinant rat NGF, BDNF, GDNF, recombinant human neurturin, artemin and murine persephin.

## **Solutions used in genotyping by PCR**

### **Proteinase K solution**

10 mg Proteinase K (GibcoBRL) was reconstituted in lysis buffer comprising 0.605 g Tris/HCl, 8.04 g NaCl, 0.37 g EDTA and 1 g SDS, prepared to 100 ml with ddH<sub>2</sub>O.

### **Basic phenol**

Aliquots of basic phenol (Sigma) were prepared and stored at 4 °C. This separated into phases; the phenol was located in the lower organic phase.

### **3 M NaAc, pH5.2**

22.8 g sodium acetate (BDH) was dissolved in 100 ml of ddH<sub>2</sub>O and the pH adjusted to 5.2 with glacial acetic acid.

### **Primers**

WT, AU, NEO (for *GFRα1*<sup>-/-</sup> mice), NGF1, NGF2 and NGFC (for *NGF*<sup>-/-</sup> mice) were synthesised to order by GibcoBRL. Primers were stored at a concentration of 200 μM in water. Before use, primers were diluted a further 10x with water to give a final concentration of 20 μM.

### **Taq thermostable DNA polymerase**

(Hotstar Taq, 5 units/μl, used as supplied, Qiagen)

### **TAE buffer**

A 50x stock was prepared containing 605 g Tris base, 142.75 ml glacial acetic acid and 250 ml 0.5 M EDTA, pH8.0, made up to 2.5 l with ddH<sub>2</sub>O. 20 ml of this stock was diluted to 1 litre before use.

### **Agarose gels**

To prepare an agarose gel, 2 g of agarose (Helena Biosciences) was dissolved in 100 ml TAE buffer by boiling in a microwave. 10 µl of ethidium bromide (10 mg/ml, Amresco) was then added to give a final concentration of 1 µg/ml.

### **6x Gel loading dye**

4 µl of loading buffer (MBI) was added per 20 µl PCR reaction.

**1kb DNA ladder** (used as supplied, MBI)

**25 mM MgCl<sub>2</sub>** (used as supplied, Qiagen)

**10x Reaction buffer** (used as supplied, Qiagen)

**5x Q solution** (used as supplied, Qiagen)

**De-ionised water** (Gibco, TC-tested)

## **Solutions used in immunoblotting and immunocytochemistry**

**MBS** (Sigma)

5 mg MBS was dissolved in 165 µl of DMSO (Sigma) to give a concentration of 30 mg/ml. The solution was stored at -20 °C wrapped in foil.

**0.1 M EDTA** (BDH)

18.7 g EDTA was dissolved in 500 ml ddH<sub>2</sub>O to give a concentration of 0.1 M. The pH of the solution was adjusted to pH8.0.

#### **1x PBS, pH6.0, pH7.4** (Oxoid)

Phosphate buffered saline was prepared by dissolving 5 tablets in 500 ml of ddH<sub>2</sub>O. If required, the pH of the solution was adjusted to 6.0 with HCl, prior to autoclaving.

#### **4x Tris.Cl/SDS**

6.05 g Tris was dissolved in 40 ml ddH<sub>2</sub>O and the pH adjusted to 6.8 with HCl. The solution was made to a total volume of 100 ml with ddH<sub>2</sub>O and passed through a 0.45 µm filter after which 0.4 g SDS was added.

#### **2x SDS/sample (Laemmli) buffer**

2x sample buffer was prepared with 25 ml 4xTris.Cl/SDS, pH6.8, 20 ml glycerol, 4 g SDS, 2 ml 2-mercaptoethanol and 1 mg bromophenol blue made up with ddH<sub>2</sub>O to a total volume of 100 ml.

#### **APS** (Sigma)

A 10 % APS solution was prepared by dissolving 100 mg APS in 1 ml ddH<sub>2</sub>O.

#### **1.5 M Tris/HCl, pH8.8** (Analar, BDH)

18.2 g Tris was dissolved in 100 ml ddH<sub>2</sub>O and the pH adjusted accordingly using HCl.

#### **SDS** (Sigma)

A 10 % SDS solution was prepared by dissolving 50 g SDS (99 %) in 500 ml ddH<sub>2</sub>O.

#### **1.0 M Tris/HCl, pH6.8, pH8.0, pH9.0, pH9.5** (Analar, BDH)

12.1 g Tris was dissolved in 100 ml ddH<sub>2</sub>O and the pH adjusted to the required value by addition of HCl.

### **Running buffer**

For convenience, running buffer was prepared as a 10x stock containing 30.25 g Tris, 144 g glycine and 10 g SDS, prepared to 1 l with ddH<sub>2</sub>O. This was diluted 10x before use.

### **Rainbow™ marker** (Amersham)

This provided a mixture of individually coloured and purified proteins ranging in size from 14.3 kDa to 220 kDa.

### **10 % Acetic acid** (BDH)

100 ml glacial acetic acid was made up to 1 l with ddH<sub>2</sub>O.

### **Transfer buffer**

For convenience, transfer buffer was prepared as a 10x stock containing 30 g Tris and 112.5 g glycine, prepared to 1 l with ddH<sub>2</sub>O. This was diluted 10x before use and 20 % methanol added. The solution was cooled to 4 °C before transfer.

### **Blocking buffer**

20 g dried, non-fat milk (Boots) and 2.5 ml 10 % Tween 20 were made up to 500 ml with PBS, pH7.4.

### **PBS/Tween 20 (0.05 %, 0.1 %)**

A 10 % solution of Tween 20 (BDH) was prepared by mixing 5 ml Tween 20 with 45 ml ddH<sub>2</sub>O for several hours. 2.5 or 5 ml of 10 % Tween 20 was then made up to 500 ml with PBS to give a 0.05 or 0.1 % solution respectively.

**100 mM Glycine, pH2.6** (BDH)

0.75 g glycine was dissolved in 100 ml ddH<sub>2</sub>O and the pH adjusted accordingly.

**0.1 M Citric acid, pH3.0** (BDH)

2.1 g citric acid was dissolved in 100 ml ddH<sub>2</sub>O and the pH adjusted accordingly.

**PBS/glycerol**

A 1:1 solution was prepared by mixing equal volumes of 2x PBS and glycerol (Prolabo).

**PBS/Na azide (0.1 %)**

100 mg Na azide (Sigma) was dissolved in 100 ml PBS, pH7.4 to give a 0.1 % solution.

**4 % Paraformaldehyde** (Fisons)

20 g paraformaldehyde was dissolved in 500 ml PBS by heating to 66 °C. NaOH pellets were added to aid solubilisation of the paraformaldehyde. The pH was adjusted to 7.4 and the solution filtered through a 0.22 µm bell filter.

**S-blocking buffer**

1 g BSA and 2 ml goat serum (i.e. of the same origin as the secondary antibody) were made up to 100 ml with PBS.

**Coomassie Brilliant Blue R solution** (Sigma)

**KLH** (Calbiochem)

**TEMED** (Sigma)

**Acrylamide mix (acryl:bis=30:1)** (Anachem)

<b><u>Butanol</u></b>	(BDH)
<b><u>Methanol</u></b>	(BDH)
<b><u>HRP-conjugated donkey anti-rabbit IgG</u></b>	(Amersham)
<b><u>FITC-conjugated goat anti-rabbit IgG</u></b>	(Jackson Laboratories)

### Solutions used in histology

#### **PBS**

Phosphate buffered saline was prepared by dissolving 5 tablets (Oxoid) in 500 ml of de-ionised, distilled water. The solution (pH7.4) was then autoclaved.

#### **10 % (w/v) EDTA**

50 g EDTA (BDH) was prepared in 500 ml of de-ionised, distilled water and left to dissolve overnight. The pH was later adjusted to 7.3.

#### **Cresyl fast violet**

50 mg (0.01 %) or 500 mg (0.1 %) cresyl fast violet (Sigma) was dissolved in 500 ml of de-ionised, distilled water by heating to 54 °C for 2 hours. The solution was passed through filter paper and 3-4 drops of glacial acetic acid (Analar, BDH) were added to each 100 ml just before use.

**Neutral buffered formalin** (Sigma)

**Chloroform** (Analar, BDH)

**Xylene** (BDH)