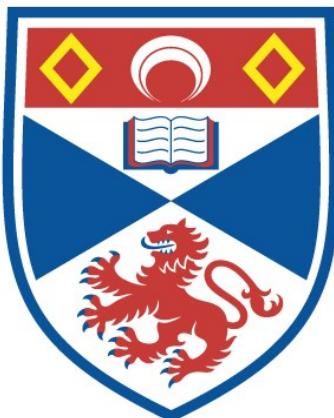


REGULATION OF EXPRESSION AND ROLE OF THE  
GDNF FAMILY RECEPTORS IN NEURONAL  
DEVELOPMENT

Epaminondas Doxakis

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



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**Regulation of expression and role of the GDNF family  
receptors in neuronal development**

A thesis submitted to the University of St-Andrews for the degree of Doctor of  
Philosophy (Ph.D.)

**Epaminondas Doxakis**

August, 1999

**Supervisor: Alun M Davies**

School of Biomedical Sciences

Bute Medical Buildings

St-Andrews



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Th D 437

***It is owing to wonder that people began  
to philosophize, and wonder remains the  
beginning of knowledge***

Aristotle

*I dedicate this thesis to my parents  
without whom I would not have got this  
far*

## **Declarations**

I, Epaminondas Doxakis, hereby certify that this thesis, which is approximately 54,000 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.

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## **Acknowledgements**

I would like to thank my supervisor, Professor Alun M Davies, for giving me the opportunity to carry out research in his laboratory. His effective guidance made the completion of this project possible.

I am grateful to Dr Sean Wyatt for his invaluable help and advice throughout the course of my work and for correcting this thesis.

I would like to thank Jimi Adu and Alison Forgie for their help with the tissue culture techniques.

I would also like to thank Vladimir Buchman and Natalia Ninkina for their advice and expertise in molecular biology.

I am grateful to all my friends and the rest of my colleagues for their support, encouragement and patience.

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## **Abbreviations**

**6-OHDA** 6-hydroxydopamine

**ART** artemin

**BDNF** brain-derived neurotrophic factor

**BH** bcl-2 homology

**BLAST** basic local alignment search tool

**BMP** bone morphogenic protein

**bp** base pairs

**BSA** bovine serum albumin

**C. elegans** *Caenorhabditis elegans*

**cDNA** complementary DNA

**ChAT** choline acetyl transferase

**CMF-PBS** calcium and magnesium free phosphate-buffered saline

**CNS** central nervous system

**CNTF** ciliary neurotrophic factor

**CT-1** cardiotrophin-1

**DA** dopaminergic

**DMTG** dorso-medial trigeminal ganglion

**DPP** decapentaplegic

**DR** death receptor

**DRG** dorsal root ganglia

**DVR** decapentaplegic-Vg1-related

**E** embryonic day

**E. coli** Escherichia coli

**EC50** effective concentration at 50%

**ENS** enteric nervous system

**EST** expressed sequence tag

**F12** Ham's nutrient mixture F-12

**F14** Ham's nutrient mixture F-14

**FGF** fibroblast growth factor

**FMTCA** familial medullary thyroid carcinoma

**GDNF** glial cell line-derived neurotrophic factor

**GFR $\alpha$**  GDNF family receptor alpha

**GM-CSF** granulocyte macrophage - colony stimulating factor

**Gp130** glycoprotein 130

**GPA** growth-promoting activity

**GPI** glycosyl-phosphatidylinositol

**h** hour

**HBSS** Hank's balanced salt solution

**HIFCS** heat-inactivated foetal calf serum

**HIHS** heat-inactivated horse serum

**HSCR** Hirschsprung disease

**HTGS** high throughput genomic sequences

**hOSM** human oncostatin M

**i.e.** id est, that is

**IL** interleukin

**IL-6R $\alpha$**  interleukin-6 receptor alpha

**JAK** JAK kinases

**JNK** jun kinase

**Kb** kilobase

**Kd** dissociation constant

**kDa** kilodaltons

**L** litre

**L15** Leibovitz's L15 nutrient mixture

**LB** Luria broth

**LIF** leukaemia inhibitory factor

**LIFR $\beta$**  LIF receptor beta

**M** molar

**MAP** mitogen-activated protein

**MEN2** multiple endocrine neoplasia type 2

**mg** milligram

**MH** mad homology

**MPTP** 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

**mRNA** messenger RNA

**NGF** nerve growth factor

**NR** neurokine receptor

**NT** neurotrophin

**NTR** neurotrophin receptor

**OSM** oncostatin M

**OSMR $\beta$**  oncostatin M receptor  $\beta$

**PBS** phosphate buffered saline

**PCR** polymerase chain reaction

**PI** isoelectric point

**PI3-kinase** phosphatidylinositol 3-kinase

**PIPLC** phosphatidylinositol-specific phospholipase C

**PLC- $\gamma$ 1** phospholipase C- $\gamma$ 1

**PNS** peripheral nervous system

**P-ORN** poly-DL-ornithine

**PSP** persephin

**PTC** papillary thyroid carcinoma

**r** rhombomere

**Ret** rearranged during transfection

**RNase** ribonuclease

**RT** reverse transcription

**RT** room temperature

**RTK** receptor tyrosine kinase

**s(CNTFR $\alpha$ )** soluble CNTFR $\alpha$

**s(IL-6R $\alpha$ )** soluble IL-6R $\alpha$

**SATO** Sato's nutrient mixture

**SC** sympathetic chain

**SCG** superior cervical ganglion

**SH2** Src-homology region 2

**SHP-1** SH2-containg tyrosine phospatase-1

**sLIFR $\beta$**  soluble LIFR $\beta$

**SNT** suc1-associated neurotrophic factor target

**SOS** son-of-sevenless

**STAT** signal transducers and activator of transcription

**Taq** *Thermus aquaticus*

**TGF- $\beta$**  transforming growth factor- $\beta$

**TK** tyrosine kinase

**TMN** trigeminal mesencephalic nucleus

**TNF** tumour necrosis factor

**Trk** tropomyosin-related kinase

**tRNA** transfer RNA

**UV** ultraviolet

**VLTG** ventrolateral trigeminal ganglion

**VRC** vanadyl-ribonucleoside complex

**W** watts

**$\mu$ g** microgram

**$\mu$ l** microlitre

## **Abstract**

The aim of this project was to determine the temporal and spatial pattern of expression of GDNF family receptors in the developing embryo, with particular emphasis on expression in the peripheral nervous system, and to investigate how expression of receptor mRNAs is regulated in developing neurons. It was hoped that the data obtained would prove useful in further characterizing the role that the GDNF family of neurotrophic factors play in embryonic development. Semi-quantitative PCR revealed that GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNAs are widely distributed with both complementary and overlapping, though distinct, patterns of expression in the chicken embryo during development.

Different populations of PNS neurons display different levels of responsiveness to GDNF and NTN and their sensitivity to these factors change throughout development. Examination of receptor expression by quantitative RT-PCR revealed that neurons that are more sensitive to GDNF express higher levels of GFR $\alpha$ -1 mRNA than GFR $\alpha$ -2 mRNA, and neurons that are more sensitive to NTN express higher levels of GFR $\alpha$ -2 mRNA compared to GFR $\alpha$ -1 mRNA. However, developmental changes in responsiveness of a population of neurons to these factors are not consistently paralleled by changes in the relative levels of GFR $\alpha$  transcripts. Furthermore, all neuronal populations express relatively high levels of ret mRNA. These results indicate the responsiveness of PNS neurons to GDNF and NTN is in part governed by the relative levels of expression of their GPI-linked receptors.

To determine how the expression of the GDNF family receptors is regulated, embryonic neurons were cultured under different experimental conditions. I found that GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNAs are not significantly regulated by GDNF and/or NTN. However, depolarizing levels of KCl cause marked changes in the expression of GFR $\alpha$  mRNAs. The effects of KCl are inhibited by L-type Ca $^{2+}$  channel antagonists, suggesting that they were mediated by elevation of intracellular free Ca $^{2+}$ . KCl treatment increases the response of neurons to GDNF and decreases their response to NTN. There is no marked effect of depolarization on ret mRNA expression.

# Chapter 1

## Introduction

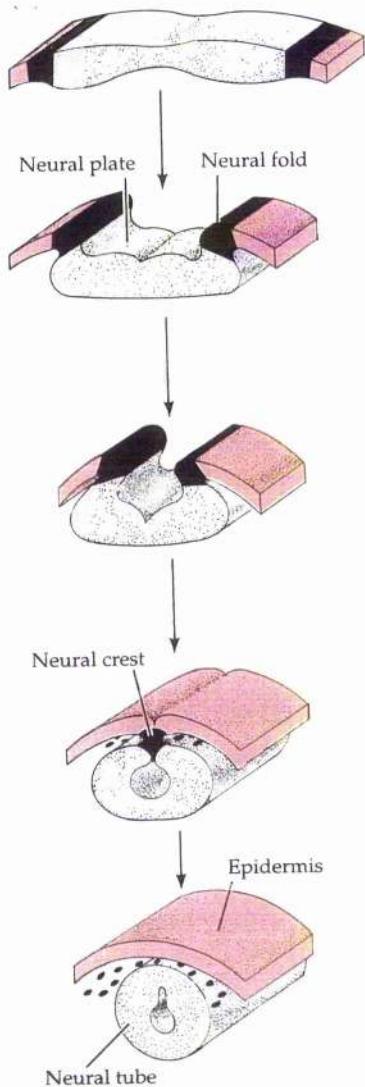
### 1.1. Development of the Vertebrate Nervous System

The diversity of cells in the nervous system and their intricate and precise interconnections present a developmental problem that has no parallel in other tissues. Tens of thousands of cell types must be generated at the right time and place, must extend their axons and dendrites, and must choose among millions of partners to make appropriate synaptic connections. Unraveling the mechanisms by which those events occur in an orderly and timely fashion is the ultimate puzzle in developmental biology.

In vertebrates, the development of the nervous system is initiated at the gastrula stage, when the embryo consists of three layers, namely, ectoderm, mesoderm, and endoderm. Triggered by chemical signals from the mesoderm, a region of the ectoderm, the neuroectoderm, begins to thicken and indent, forming a neural groove on the surface of the embryo along what will become its rostral-caudal (head-to-tail) axis. The lips of the neural groove start to swell, then meet and fuse to form a neural tube (Figure 1.1). The neural tube will give rise to the brain and the spinal cord, i.e., the central nervous system (CNS).

Along the line where the neural tube pinches off from the future epidermis, a number of cells break loose from the neuroectoderm and migrate through the mesoderm. These neural crest cells form almost all of the peripheral nervous system (PNS), including autonomic sympathetic and parasympathetic neurons,

most sensory neurons, and support cells of the PNS, such as the Schwann cells and satellite cells.



**Figure 1.1.** Formation of the neural tube and neural crest. The ectodermal cells are represented either as precursors of the neural crest (black) or as precursors of the epidermis (colour). The ectoderm folds in at the most dorsal point, forming a outer epidermis and an inner neural tube. The neural crest cells pinch off from the tube and migrate to their final destinations.

## **1.2. Ganglia of the peripheral nervous system**

The peripheral nervous system is composed of sensory and autonomic ganglia and peripheral nerves. Sensory ganglia are anatomically segregated into cranial (including the trigeminal, nodose, vestibulo-acoustic and geniculate ganglia) and dorsal root ganglia. The autonomic nervous system has three principal divisions: the sympathetic (including the prevertebral sympathetic ganglia and the paravertebral sympathetic chain), the parasympathetic (including the cranial parasympathetic ganglia and terminal parasympathetic ganglia) and the enteric nervous system (ENS).

Neurons of the peripheral nervous system offer many advantages for studying the different phases of neural development. They and their progenitor cell populations are well defined, have less extensive synaptic contacts than their CNS counterparts and are accessible to both *in vitro* and *in ovo* experimental manipulation from the earliest stages of their development.

## **1.3. Embryonic origin of the peripheral nervous system ganglia**

Vertebrate cranial sensory ganglia are derived from two distinct embryonic sources, the neural crest and the neurogenic placodes (Noden, 1978; D'Amico-Martel and Noden, 1983; Le Douarin, 1986). The neurogenic placodes are epithelial thickenings appearing in the cephalic ectoderm of the early vertebrate embryo. In the chicken embryo, the neurons of the ventrolateral part of the trigeminal ganglion, and the vestibulo-acoustic, geniculate, petrosal and nodose ganglia are derived from neurogenic placodes. The neuronal cells of the

dorsomedial trigeminal ganglion, the trigeminal mesencephalic nucleus (TMN), the jugular ganglion and the dorsal root ganglia are derived from different regions of the neural crest (D'Amico-Martel and Noden, 1983; Vogel, 1992). The Schwann cells and satellite cells associated with all sensory ganglia arise exclusively from the neural crest (D'Amico-Martel and Noden, 1983; Vogel, 1992).

Neural crest cells give rise to the neurons and glial cells of the sympathetic and parasympathetic ganglia (D'Amico-Martel and Noden, 1983; Bronner-Fraser, 1988; Le Douarin, 1980). Both neuronal and glial cells of the ENS are also derived from the neural crest. Enteric progenitors originate from the vagal, sacral, and truncal region of the neural crest (Le Douarin et al., 1973, 1974; Bronner-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 prior to migration (Bronner-Fraser, 1988). Their differentiation is influenced by the microenvironment of both the migratory pathway and the destination site.

#### **1.4. Apoptosis**

The construction of a functional nervous system is the culmination of both neurogenerative and neurodegenerative processes. In the vertebrate nervous system, approximately half of the neurons produced by neurogenesis die during embryonic and early postnatal development (Oppenheim, 1991). This developmentally programmed neuronal death generally occurs by apoptosis (Oppenheim, 1991; Sanders and Wride, 1995), an intrinsic mechanism of cellular suicide that is employed by most, if not all, cells that die during the course of development (Kerr et al., 1972). Common morphological features of apoptotic cells include cytoplasmic shrinkage, and chromatin condensation.

There are two major causes of neuronal cell death during development. One results from an inadequate supply of neurotrophic factors and the other results from factors that bind to death receptors. Of these, death resulting from inadequate trophic support has been the most intensively studied. Recently death-inducing receptors have been found in neurons. For example, several reports have shown that the p75 neurotrophin receptor, a member of the TNF/Fas family, transduces ligand-dependent apoptotic signals in chicken retina and TMN neurons (Frade et al., 1996; Davey and Davies, 1998). The intracellular events leading to apoptosis following activation of the p75 receptor have not yet been elucidated. Death-signaling receptors such as CD95 (a Fas receptor), p55 tumour necrosis factor (TNF) receptor, death receptor-3 (DR3), DR4 and DR5 have been studied in more detail in non-neuronal systems. In these cases, the apoptotic events that follow receptor activation have been fairly well documented (Yuan, 1997).

Depending on the specific apoptotic trigger and cell type, any one of many different intracellular signaling pathways can be activated during the induction phase of the apoptotic process. However, these pathways converge on one or two

control points in the execution or commitment phase of the apoptotic programme (Green and Kroemer, 1998). Investigation of the molecular mechanisms leading from the initial trigger to the culmination of the cellular apoptotic response have revealed that the components of the cell death machinery in species as distantly related as humans, *Drosophila melanogaster* and *Caenorhabditis elegans* are remarkably well conserved. Genetic analysis of *C. elegans* has identified three genes that are pivotal to programmed death of somatic cells in the nematode. These are ced-3, ced-4 and ced-9 (Ellis and Horvitz, 1986; Hengartner and Horvitz, 1994). ced-3 encodes a cysteine protease of the caspase family (Yan et al., 1993; Xue et al., 1996) whose activation is promoted by the product of ced-4. Together these proteins are responsible for the 131 somatic cell deaths that occur during normal nematode development. ced-9 works upstream of ced-3 and ced-4 and antagonizes their function in surviving cells (Hengartner et al., 1992). It is thought that ced-9 physically interacts with ced-4 thus preventing activation of ced-3 (Chinnaiyan et al., 1997; Spector et al., 1997; Irmler et al., 1997; Wu et al., 1997a, b). This death pathway has acquired higher levels of complexity in mammals. The caspase family of cysteine proteases, currently 13 in number, are the mammalian counterpart of ced-3 (Bergeron and Yuan, 1998), the Bcl-2 family of genes, currently 15 in number, are the ced-9 homologues (Bergeron and Yuan, 1998) and Apaf-1 is the homologue of ced-4 (Zou et al., 1997). Caspases are critical mediators of downstream apoptotic events. They are involved in a proteolytic cascade and induce disassembly as a result of cleavage of numerous cellular substrates (Nicholson and Thornberry, 1997; Cohen 1997).

Caspases are constitutively present in the cells and must therefore be inhibited for the cell to survive. It appears that the Bcl-2 family of proteins act upstream of caspases and regulate their activity. The Bcl-2 family contains both pro-apoptotic (Bcl-x<sub>S</sub>, Bax, Bak, Bad, Bik, Bid, Bim, Hrk, Bik) and antiapoptotic (Bcl-2, Bcl-

xL, Bfl-1 Bcl-w, A-1, Mcl-1) members (for review see Kelekar and Thompson, 1998). A common feature of the family members is their ability to homodimerize and heterodimerize through their conserved regions, BH1, BH2, BH3, and BH4. Many family members lack one or more of these domains. It has recently emerged that many of the pro-apoptotic members contain only the BH3 domain (Kelekar and Thompson, 1998). The level of expression of pro-apoptotic and anti-apoptotic proteins and their interactions seem to regulate cell death (Rao and White, 1997). Furthermore, post-translational phosphorylation of some Bcl-2 family members is also involved in controlling apoptosis to prevent caspase activation (Gajewski and Thompson, 1996). It has been shown that Apaf-1 binds pro-caspase-9 in the presence of cytochrome c and ATP. This event leads to the activation of caspase-9, which in turn cleaves and activates caspase-3. Caspases once active can cleave each other, resulting in a proteolytic cascade that leads to cell death (Zou et al., 1997; Li et al., 1997). It is thought that antiapoptotic Bcl-2 family members may interact with Apaf-1 (and possible, as yet undiscovered, related proteins) to prevent caspase activation (Chinnaiyan et al., 1997). Members of the Bcl-2 family are also thought to influence cell survival by regulating the release of cytochrome c from mitochondria which in turn can activate pro-caspase-9 directly in the cytosol.

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

### **1.5.1. Neurotrophic theory**

Neurons are generated in excess in the developing nervous system. Superfluous and inappropriately connected neurons are eliminated in a phase of cell death which begins shortly after neurons innervate their target fields (Oppenheim, 1981). It is now widely accepted that the target field plays an important role in regulating the number of neurons that survive by its production of a limited quantity of neurotrophic factors which the innervating neurons require for their survival. Neurons that are able to procure an adequate supply of this factor survive, whereas neurons that cannot, die. Evidence for this 'neurotrophic hypothesis' has come principally from work on nerve growth factor (NGF), the first neurotrophic factor to be identified and characterized (Levi-Montalcini and Angeletti 1968; Thoenen and Barde 1980; Davies, 1988a,b; Barde 1989).

The most important direct evidence is the finding that certain populations of developing neurons are dependent on NGF for survival *in vitro* and *in vivo*. Administration of anti-NGF antibodies during the phase of target field innervation eliminate these neurons, whereas exogenous NGF rescues neurons that would otherwise die (Johnson et al., 1980; Hamburger and Yip, 1984). Considerable indirect support for the theory comes from studies of the synthesis, expression and transport of NGF. Synthesis of NGF in the target tissues of NGF dependent neurons coincides with the beginning of target field innervation (Davies et al., 1987; Korschning and Thoenen, 1988) and the level of NGF expression is found to be proportional to the final innervation density (Harper and Davies, 1990). NGF is conveyed from target tissues to the cell bodies of the innervating neurons by rapid, retrograde axonal transport (Hendry et al., 1974; Korschning and Thoenen, 1983).

The purification of BDNF (Barde et al., 1982) extended the generality of the neurotrophic theory to a second neurotrophic factor. BDNF promotes the survival of subsets of embryonic neurons *in vitro* and prevents loss of these neurons *in vivo* when administered to embryos during the phase of naturally occurring neuronal death (Hofer and Barde, 1988). Likewise, these same neurons are lost in mice that have targeted null mutations in the BDNF gene (Ernfors et al, 1994; Jones et al., 1994) or the BDNF receptor tyrosine kinase (trkB) gene (Klein et al., 1993).

The discovery that NGF and BDNF are structurally related proteins led to the identification of additional members of this family of proteins: neurotrophin-3 (NT-3), NT-4/5, NT-6 and NT-7. In addition to these neurotrophins, several other families of neurotrophic factors have been identified. These include neuropoietic cytokines, TGF- $\beta$  and GDNF. Each of these families of neurotrophic factors will be discussed in detail in the following sections.

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

The term neurotrophin refers to a family of proteins that have common structural and functional features. To date, six neurotrophins have been characterized. These are: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5 or NT-4), neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7). These factors have been shown to influence the proliferation, survival, differentiation, growth, synaptic function and regeneration of a wide variety of neurons in both the peripheral and central nervous system. It has been suggested that NGF evolved as the result of a duplication event of a gene that might have been BDNF- or NT-3- like. It is also interesting to note that NGF acts as a neuron survival factor preferentially on those parts of the PNS that are generated late in ontogeny, and possibly phylogeny (e.g. sympathetic chain) (Barde, 1994). Neurotrophins interact with two structurally distinct classes of receptors on the cell surface. These are tyrosine kinase receptors of the Trk family and a transmembrane receptor called p75. Trk receptors mediate most actions of neurotrophins with a well-defined specificity. In general, NGF binds and activates TrkA, BDNF and NT-4 bind and activate TrkB and NT-3 binds and activates TrkC. However, there is some overlap in receptor specificity between members of the neurotrophin family. All neurotrophins are capable of binding to p75, albeit with lower affinity.

## Nerve growth factor

NGF was first discovered by Levi-Montalcini in 1951 (Levi-Montalcini, 1987). High concentrations of NGF were observed in the mouse submandibular salivary glands which facilitated its purification and cloning of the NGF gene in several species (Scott et al., 1983; Ullrich et al., 1983; Meier et al., 1986; Ebendal et al., 1986). NGF as purified from the submandibular salivary gland, consists of three subunits:  $\alpha$ ,  $\beta$  and  $\gamma$  and has a molecular weight of 130 kDa (Varon et al., 1968). The biological activity is conferred by the NGF  $\beta$  subunit, hence the active form of NGF is often referred as  $\beta$ -NGF. Mature  $\beta$ -NGF is synthesized from prepro- $\beta$ -NGF (26 kDa) and exists in its active form at physiological concentrations as a homodimer (Berger and Shooter, 1977; Scott et al., 1983; Edwards et al., 1988). Analysis of the three-dimensional structure of mature NGF has revealed that it contains a motif of three intrachain disulphide bridges, known as the "cysteine knot" and three antiparallel  $\beta$  strands (McDonald et al., 1991).

Extensive tissue culture studies have established that NGF promotes the survival of sympathetic neurons and subpopulations of neural crest-derived sensory neurons within neurons of the peripheral nervous system, but has no demonstrable effect on placode-derived cranial sensory neurons, enteric neurons, parasympathetic neurons or spinal motor neurons (Chun and Patterson, 1977; Hamburger, 1981; Davies and Lindsay 1985; Gorin and Johnson, 1980; Davies, 1994a). In these and other studies NGF was found to be a target derived growth factor.

*In situ* hybridization studies have shown that NGF is distributed at high levels within regions of the CNS that are innervated by the cholinergic neurons of the basal forebrain (hippocampus, olfactory bulb and neocortex) and regions containing the cell bodies of these neurons (septum, nucleus of the diagonal band

of Broca, nucleus basalis of Meynert). The highest level of NGF mRNA occurs within the hippocampus and cortex (Korschning et al., 1985). Comparatively low, but significant NGF levels have also been found in various other brain regions (Whittemore et al., 1986).

Confirmation of the specificity of NGF for certain peripheral nervous system neurons has been obtained from analysis of mice with targeted disruption of the NGF gene (Crowley et al., 1994). Mice that are homozygous for a null mutation in the NGF gene are born with virtually no sympathetic neurons and a loss of >70% of the normal complement of dorsal root and trigeminal ganglia neurons (Crowley et al., 1994). Interestingly, there is no marked loss of basal forebrain cholinergic neurons or other CNS neuronal populations (Crowley et al., 1994). NGF deficient mice die soon after birth (Crowley et al., 1994).

### **Brain-derived neurotrophic factor**

Brain-derived neurotrophic factor (BDNF), initially isolated from pig brain, was the second member of the neurotrophin family to be characterized. BDNF is a basic protein ( $pI \sim 10.1$ ) with a molecular weight of 12.3 kDa (Barde et al., 1982). The mature BDNF protein is 119 amino acids in length, one amino acid more than NGF, and like NGF exists as a stable homodimer linked by 3 disulphide bridges (Radziejewski et al., 1992). BDNF displays approximately 50% sequence identity to NGF, including conservation of all six of the structurally important cysteine residues (Leibrock et al., 1989).

BDNF expression is largely restricted to the CNS. BDNF mRNA and protein levels have been detected in the hippocampus, amygdala, thalamus, projection areas of the olfactory system, inner and outer pyramidal layers of the

neocortex, claustrum, septum, cerebellum, and the superior colliculus (Leibrock et al., 1989; Hofer et al., 1990; Wetmore et al., 1990; Phillips et al., 1990; Dugich-Djordjevic et al., 1995a; Gall et al., 1992; Huntley et al., 1992), suggesting that BDNF has a more widespread distribution than NGF. Outside the CNS, BDNF mRNA has been observed in the target tissues of populations of BDNF responsive neurons and, perhaps surprisingly, in a subset of the neurons of dorsal root, trigeminal, geniculate, and sympathetic ganglia, suggesting a possible autocrine or paracrine mechanism fashion (Robinson et al., 1996; Schecterson and Bothwell, 1992).

BDNF has been shown to support the survival of both neural crest-(trigeminal mesencephalic nucleus, jugular and dorsal root ganglion) and placode-(ventrolateral trigeminal ganglion, geniculate, petrosal, vestibular, and nodose ganglion) derived sensory neurons. In addition BDNF supports the survival of dopaminergic neurons within the substantia nigra, basal forebrain cholinergic neurons, hippocampal neurons, cerebellar granule cells, and retinal ganglion cells (Davies et al., 1986; Barde et al., 1987; Hofer and Barde, 1988; Alderson et al., 1990; Hyman et al., 1991; Knüssel et al., 1991; Oppenheim et al., 1992; for review see Korsching, 1993).

Homozygous mutant mice lacking the BDNF gene display substantially reduced numbers of cranial and spinal sensory neurons. In particular, significant numbers of sensory neurons are lost in the trigeminal, geniculate and vestibular ganglia and in the nodose-petrosal complex (Ernfors et al., 1994a; Jones et al., 1994). In contrast to previous studies showing that motor neurons are supported by BDNF *in vivo* (Yan et al., 1992; Oppenheim et al., 1992; Sendtner et al., 1992a, Vejsada et al., 1998) and *in vitro* (Henderson et al., 1993), motor neuron development is largely unperturbed by targeted disruption of the BDNF gene (Ernfors et al., 1994a; Jones et al., 1994). Although the CNS of BDNF deficient

mice shows no gross structural abnormalities, the expression of neuropeptide Y and calcium binding proteins is altered in many neurons, suggesting that these neurons may not function normally (Ernfors et al., 1994a; Jones et al., 1994).

### **Neurotrophin-3**

Neurotrophin-3 (NT-3), the third member of the neurotrophin family to be identified, was cloned on the basis of sequence homology between BDNF and NGF (Hohn et al., 1990; Maisonpierre et al., 1990; Jones and Reichardt, 1990; Rosenthal et al., 1990). The predicted size of the mature NT-3 polypeptide is 119 amino acids. NT-3 is a basic protein ( $pI \sim 9.3$ ) with a molecular mass of 13.6 kDa (Hohn et al., 1990; Maisonpierre et al., 1990). Mature rat NT-3 peptide displays 57% and 58% amino acid identity with rat NGF and BDNF respectively, and contains all six cysteine residues involved in forming the cysteine knot motif. Like NGF and BDNF, NT-3 exists as a homo-dimer at physiologically relevant concentrations (Radziejewski et al., 1992).

Despite the high structural homology with NGF and BDNF, NT-3 has distinct biological activity and a different spatiotemporal pattern of expression. In contrast to NGF and BDNF, the level of NT-3 mRNA is high during fetal developmental and reduced in the adult. NT-3 mRNA has a widespread tissue distribution. In the brain, levels of NT-3 mRNA are greatest in the cerebellum, medulla oblongata and hippocampus. In the periphery, NT-3 mRNA is mostly found in the heart, kidney, liver, skin, muscle, lung, spleen, and intestine, suggesting that it may act as a target-derived trophic factor for sympathetic and sensory neurons (Maisonpierre et al., 1990; Hohn et al., 1990; Rosenthal et al., 1990).

The neurotrophic activities of NT-3 have been analyzed using cell culture and *in vivo* approaches. Neurons responsive to NT-3 include subpopulations of the dorsal root ganglia (Maisonpierre et al., 1990), nodose ganglia (Rosenthal et al., 1990), trigeminal ganglia (Buchman and Davies, 1993), cochlear ganglia (Farinas et al., 1994), as well as proprioceptive neurons of the trigeminal mesencephalic nucleus (Hohn et al., 1990) and developing muscle sensory neurons (Hory-Lee et al., 1993). Moreover, NT-3 has been shown to induce survival, differentiation and/or proliferation of neuronal precursor populations (Kalcheim et al., 1992; Pinco et al., 1993; DiCicco-Bloom et al., 1993; Verdi and Anderson, 1994; Chalazonitis et al., 1994; Henion et al., 1995; Karavanov et al., 1995; Memberg and Hall, 1995). NT-3 also enhances the proliferation and survival of oligodendrocyte precursor cells *in vivo* and *in vitro* as well as promoting their clonal expansion when applied with platelet-derived growth factor (Barres et al., 1993; Barres et al., 1994; McTigue et al., 1998).

Neurotrophin-3 deficient mice display substantial neuronal cell loss in all peripheral sensory ganglia including the trigeminal ganglion, nodose ganglion, trigeminal mesencephalic nucleus and superior cervical ganglion (Ernfors et al., 1994b; Farinas et al., 1994; Tessarolo et al., 1994). Significantly, spinal proprioceptive afferents and their peripheral sense organs (muscle spindles and Golgi tendon organs) are completely absent in homozygous mutant mice. No gross abnormalities are seen in Pacinian corpuscles, cutaneous afferents containing substance P and calcitonin gene-related peptide, and deep nerve fibers in the joint capsule and tendon (Ernfors et al., 1994b). In contrast with the severe effects that NT-3 gene has on PNS neurons, neurons of the CNS do not appear to be seriously perturbed by the mutation (Ernfors et al., 1994b). Although these findings may not agree with many previous *in vitro* studies on the effects of NT-3, they are consistent with *in vivo* work showing that NT-3 does not effectively

promote the survival of motor neurons after transection (Sendtnert et al., 1992a; Koliatsos et al., 1993).

### **Neurotrophin-4/5**

Neurotrophin-4/5 (NT-4/5) is the fourth member of the neurotrophin family to be discovered. It was originally cloned from *Xenopus* and designated neurotrophin-4 (Hallböök et al., 1991). Subsequently, human and rat clones were identified, initially named NT-5, that share 65% amino acid identity with *Xenopus* NT-4 (Berkemeier et al., 1991). It is currently widely believed that *Xenopus* NT-4 and mammalian NT-5 are the same protein and are referred as NT-4/5 (Ip et al., 1992a). Mature NT-4/5 is a basic protein containing 123 amino acids, including all six conserved cysteine residues common to the entire family. The overall amino acid homology of the mammalian NT-4/5 with other neurotrophins is 50% with NGF, 54% with BDNF and 52% with NT-3 (Ip et al., 1992a).

NT-4/5 is widely distributed in both embryonic and adult tissues. These include the testis, muscles, lungs, prostate, thymus, heart, placenta, and brain (Ip et al., 1992a; Berkemeier et al., 1991; Timmusk et al., 1993). NT-4/5 shows strong developmentally regulated expression in the above tissues suggesting a target-derived role for neurons innervating them. In the CNS, NT-4/5 mRNA is detected in hypothalamus, pons, medulla, cerebellum, cerebral cortex, olfactory bulb and hippocampus (Timmusk et al., 1993). Overall, the amount of NT-4/5 mRNA expressed in the whole adult rat brain is considerably less (~10 times) than that of NGF, BDNF and NT-3 mRNAs (Ip et al., 1992a).

Several studies have demonstrated that different populations of neurons from the peripheral and central nervous system respond to NT-4/5 during

development and in adulthood. In general, these overlap with BDNF responsive neurons. In the PNS, NT-4/5 promotes the survival of early trigeminal, jugular, and nodose ganglia (Ibáñez et al., 1993; Davies et al., 1993a), and neurons of the dorsal root and paravertebral sympathetic ganglia (Hallböök et al., 1991; Berkemeier et al., 1991). Additionally, neurons of the vestibular and spiral ganglia in the inner ear can be rescued from the detrimental effects of different neurotoxins, by administration of NT-4/5 both *in vitro* and *in vivo* (Zheng et al., 1995a, b). In the CNS, NT-4/5 has been shown to prevent the death of developing and adult spinal motor neurons (Henderson et al., 1993; Friedman et al., 1995; Funakoshi et al., 1995) and facial motor neurons (Koliatsos et al., 1994).

NT-4/5 deficient mice exhibit loss of sensory neurons in the nodose-petrosal and geniculate ganglia (Conover et al., 1995; Liu et al., 1995). In contrast, motor neurons of the facial nucleus and sympathetic neurons are unaffected (Conover et al., 1995; Liu et al., 1995), suggesting that other factors could sustain these neurons in the absence of NT-4/5, and/or NT-4/5's primary role is not survival for these neuronal populations.

### **Neurotrophin-6**

Neurotrophin-6 (NT-6) was cloned from a genomic library of the teleost platyfish *Xiphophorus maculatus* by low stringency hybridization to overlapping sequences of NGF (Götz et al., 1994). Like the rest of the neurotrophins, NT-6 is synthesized as a precursor polypeptide that upon proteolytic cleavage gives rise to a 143-residues mature basic protein that contains all six cysteines conserved in the family. However, NT-6 contains an additional feature not shared by NGF, BDNF, NT-3 and NT-4/5: an insertion of 22 amino acids between the second and third

cysteine residues of the mature molecule which contains the heparin domain (Götz et al., 1994).

NT-6 mRNA is expressed in both developing and adult brain. It is also found in adult gill, liver and eye, with weak expression in skin, spleen, heart and skeletal muscle (Götz et al., 1994). Like NGF, NT-6 supports the survival of embryonic chick DRG and sympathetic neurons. However, lower levels of survival are seen with saturating concentrations of NT-6 compared to those observed with NGF. Cultured nodose and ciliary neurons do not survive with NT-6, suggesting that the spectrum of NT-6 activity is similar to that of NGF (Götz et al., 1994).

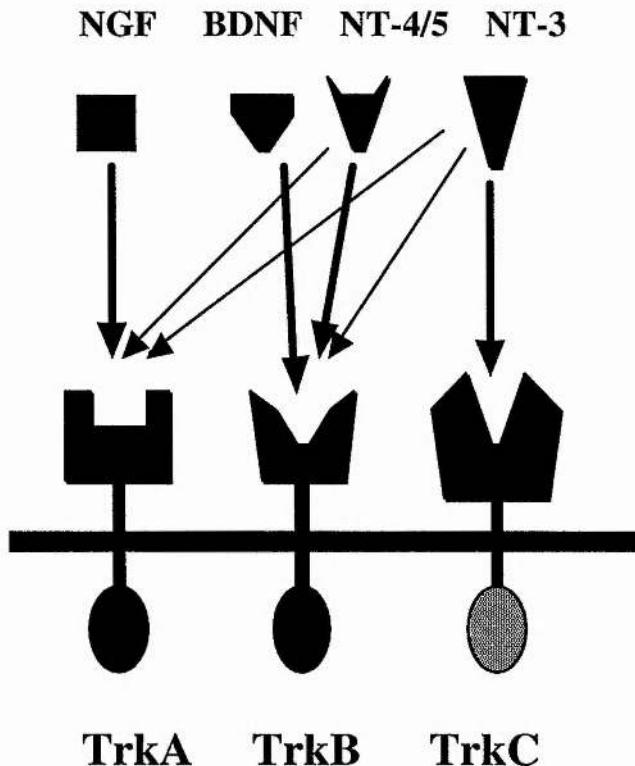
### **Neurotrophin-7**

Neurotrophin-7 (NT-7) is the most recent member of the neurotrophin family to be isolated. It was originally cloned from a genomic library of the teleost common carp *Cyprinus carpio* using the polymerase chain reaction (PCR) (Lai et al., 1998). Subsequently, zebrafish NT-7 was cloned (Nilsson et al., 1998). NT-7 shares about 66% amino acid identity to *Xiphophorus* NGF and NT-6. NT-7 possesses structural characteristics common to all known neurotrophins, such as the presence of six conserved cysteine residues and the flanking conserved sequences. In addition there is an insertion of 15 amino acids at the position corresponding to that observed for NT-6 (Lai et al., 1998; Nilsson et al., 1998).

Northern blotting analysis revealed that NT-7 mRNA is predominantly expressed in adult skin, heart and intestine, with weak expression in brain and testis (Lai et al., 1998). The neurotrophic activity of NT-7 was demonstrated by

its ability to promote neurite outgrowth and neuronal survival of chick dorsal root ganglia (Lai et al., 1998).

## Neurotrophin receptors



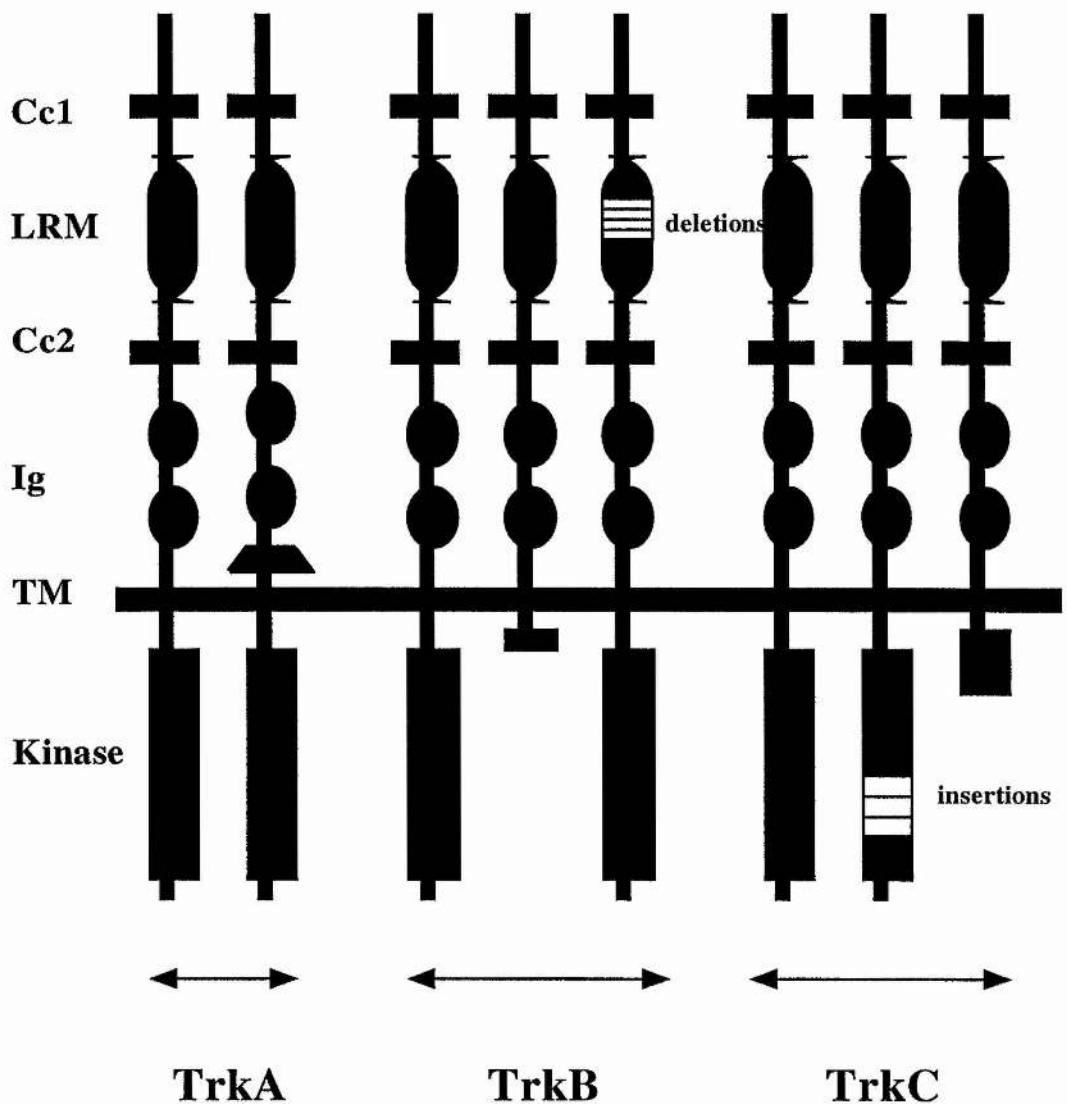
**Figure 1.2.** Neurotrophin receptors: binding patterns.

See text for details.

Two classes of neurotrophin receptors have been identified, members of the Trk family of receptor tyrosine kinases and the common neurotrophin receptor p75. Most of the biological activities of the neurotrophins are mediated by the Trk family (Chao, 1992). NGF interacts with the TrkA receptor (Hempstead et al., 1991; Kaplan et al., 1991; Klein et al., 1991a); BDNF and NT-4/5 bind with high affinity to TrkB (Berkemeir et al., 1991; Glass et al., 1991; Klein et al., 1991b, 1992; Soppet et al., 1991; Squinto et al., 1991; Ip et al., 1992a) and NT-3 associates strongly with TrkC (Lamballe et al., 1991). Both NT-4/5 and NT-3

appear to be somewhat promiscuous. NT-4/5 can bind and activate TrkA (Berkemeir et al., 1991; Ip et al., 1992a) while NT-3 can also signal through TrkA and TrkB (Lamballe et al., 1991; Soppet et al., 1991; Squinto et al., 1991) (Figure 1.2). However, the activation of TrkA by NT-3/NT-4/5 and TrkB by NT-3 is less efficient than activation of Trks by their preferred ligand. For example, NT-4/5 signaling via TrkA can support late mouse trigeminal (E12 onwards) and sympathetic (E15) neurons only when applied at high- 10 times above physiological- concentrations (Huber et al., personal communication). Whether NT-6 binds to any of these receptors is not known at the present time. NT-7 can bind TrkA but with considerable lower affinity than that of NGF (Lai et al., 1998).

Pairwise comparison of TrkA, TrkB and TrkC reveals 66-68% amino-acid sequence identity (Lamballe et al., 1991). The region that appears to be most conserved among Trks is the cytoplasmic domain. In common with other tyrosine kinase receptors, the catalytic domain is interrupted by a short insert sequence. However, certain amino acid substitutions in highly conserved kinase motifs and a short carboxy-terminal tail distinguish Trks from other subfamilies of tyrosine kinase cell surface receptors (Barbacid, 1994). The extracellular domain of the Trk receptors exhibit two distinct subsets of cell adhesion-related motifs (Schneider and Schweiger, 1991): two immunoglobulin-like (Ig) domains and an array of three tandem leucine-rich motifs (LRM) flanked by two distinct cysteine clusters (see Figure 1.3).



**Figure 1.3.** Neurotrophin receptors: isoforms.

Abbreviations: Cc, cysteine cluster; LRM, leucine-rich motifs; Ig, immunoglobulin-like domains; TM, transmembrane domain; JM, TK, tyrosine kinase domain. See text for details.

Several Trk receptor isoforms have been identified. The two TrkA kinase isoforms differ by the presence of six amino acid residues located in the extracellular domain near the transmembrane region (Barker et al., 1993;

Horigome et al., 1993). Both isoforms appear to have similar biological properties, but they have different pattern of expression. Whereas the TrkA isoform carrying the six amino acid insert is primarily expressed in neuronal cells, the other isoform has been found in other cell types (Barker et al., 1993; Horigome et al., 1992). With regard to TrkB, two variants with truncated intracellular domains (Klein et al., 1990a; Middlemas et al., 1991) and several isoforms with deletions in the leucine-rich motifs (Ninkina et al., 1997) have been identified. The truncated isoforms are the most abundant form in a variety of non-neuronal tissues, but they are also expressed in neurons (Valenzuela et al., 1993; Armanini et al., 1995; Ninkina et al., 1996). Truncated TrkB isoforms within the nervous system increase their abundance relative to the full length receptor as embryonic development progresses (Allendoerfer et al., 1994). Several functions of the truncated forms have been proposed including inhibitors of signaling of the full length TrkB receptor by the formation of functionally inactive receptor heterodimers (Eide et al., 1996; Ninkina et al., 1996), positive regulators of BDNF action by presenting BDNF to full length receptors (Klein et al., 1990a; Middlemas et al., 1991; Beck et al., 1993) and signal transducers of a presently unknown signaling pathway (Baxter et al., 1997).

Three TrkC isoforms containing inserts of varying length within the kinase domain, and four transcripts with no tyrosine kinase domain have been described (Tsoufas et al., 1993; Valenzuela et al., 1993). All forms are widely expressed in the nervous system, with the truncated forms predominating in non-neuronal tissues. Insert-containing forms of TrkC retain the ability to autophosphorylate in response to NT-3, but cannot mediate proliferation in fibroblasts or neuronal differentiation in PC12 cells (Tsoufas et al., 1993; Valenzuela et al., 1993). This suggests that the inserts alter the range of intracellular proteins that activated

TrkC can interact with and thus change the ultimate signaling capabilities of this receptor.

Trk kinase receptors become activated by a two-step process that involves their ligand-mediated dimerization followed by autophosphorylation on at least five tyrosine residues within three distinct domains of the intracellular portion of the receptor (Kaplan and Stephens, 1994). These phosphorylation events are required for the activation of the catalytic region of the Trk receptor as well as the recruitment of signaling protein substrates. At least four proteins, namely the SH2-containing adaptor protein (Shc) (Pelicci et al., 1992; Obermeier et al., 1993; Stephens et al., 1994) and its neural-specific isoform N-Shc (Nakamura et al., 1996), the phospholipase C- $\gamma$ 1 (PLC- $\gamma$ 1) (Ohmichi et al., 1991; Belia et al., 1991), SH2-containing tyrosine phosphatase-1 (SHP-1) (Vambutas et al., 1995) and the Suc1-associated neurotrophic factor target (SNT) (Rabin et al., 1993) directly associate with activated Trks. These proteins couple Trks to several intracellular signaling pathways. In brief, Shc activates the phosphatidylinositol 3-kinase (PI3-kinase) and Ras-MAP kinase pathways which are implicated in neuronal differentiation and survival. Activation of PLC- $\gamma$ 1 results in elevated activity of protein kinase C and induced calcium uptake (although there are some conflicting reports) (Guiton et al., 1994). Elevated calcium levels have been associated with survival, injury and plasticity (reviewed by Jiang and Guroff, 1997). The SNT and SHP-1 pathways are still unknown, but SNT might be involved in cell cycle control. An important point that has arisen from the neurotrophin signaling studies is that neuronal survival and differentiation are the result of co-operative processes, i.e. the activation and interaction of many different signaling pathways (for reviews see, Barbacid, 1994; Greene and Kaplan, 1995; Kaplan and Miller, 1997; Gunn-Moore and Tavaré, 1998).

Northern analysis and *in situ* hybridization studies have shown widespread expression of each of the Trk receptors throughout the CNS and periphery. In the CNS almost all neurons appear to express TrkB or TrkC (or both) (Klein et al., 1989, 1990a, b; Merlio et al., 1992; Tessarollo et al., 1993; Lamballe et al., 1994), whereas TrkA expression is highly restricted to cholinergic neurons of the basal forebrain or striatum (Vasquez and Ebendal, 1991; Holtzman et al., 1992; Merlio et al., 1992; Steininger et al., 1993). In the PNS, Trk receptors have been detected on all classes of neurons with the notable exception of parasympathetic neurons of the ciliary ganglion. TrkA transcripts have been found in DRG, sympathetic and other neural crest-derived ganglia (Martin-Zanca et al., 1990; Tessarollo et al., 1993; Schröpel et al., 1995), whereas TrkB and TrkC are expressed to some degree in all ganglia (Klein et al., 1989; Ninkina et al., 1996; Carroll et al., 1992; Tessarollo et al., 1993; Lamballe et al., 1994). Outside the nervous system, trkA mRNA expression has been observed in certain cells of the immune system (Ehrhard et al., 1993a, b). TrkB mRNA has been detected in lung, muscle and ovaries, whereas TrkC mRNA is expressed in several facial structures and tissues of the body cavity (Tessarollo et al., 1993; Lamballe et al., 1994).

In order to analyze the physiological relevance of the neurotrophin receptors during development, mutant mice lacking either functional TrkA, TrkB or TrkC genes have been generated by homologous recombination (Klein et al., 1993, 1994; Smeyne et al., 1994). In general, the phenotype of mice carrying a null mutation in each neurotrophin receptor gene is similar to that observed for its ligand (Crowley et al., 1994; Jones et al., 1994; Ernfors et al., 1994a, b). Mice lacking functional TrkA display severe sensory defects characterized by a complete loss of nociceptive activity. Neuroanatomical examination reveals extensive neuronal cell loss in trigeminal, dorsal root and sympathetic ganglia. Interestingly, cholinergic basal forebrain neurons shown to express TrkA are not

affected by the mutation (Smeyne et al., 1994). TrkB deficient mice display extensive neuronal cell loss in nodose-petrosal, dorsal root, and trigeminal ganglia. Finally, mice lacking the TrkC kinase receptors are severely defective in proprioception. This sensory defect is due to the complete absence of Ia muscle afferent projections to spinal motor neurons and reduced number of large myelinated axons in the dorsal root and posterior columns of the spinal cord. In addition, they show a 20% loss of DRG neurons (Klein et al., 1994).

The low-affinity neurotrophin receptor p75 binds all the members of the neurotrophin family (Rodrigues-Tebar et al., 1990, 1992). p75 is a 75 kDa protein with a richly glycosylated extracellular domain containing four cysteine-rich homologous segments and a short cytoplasmic domain that is not related to receptors with kinase activities (Johnson et al., 1986; Chao et al., 1986; Radeke et al., 1987). More than 10 other molecules with similar structure have been identified and are collectively referred to as tumor necrosis factor (TNF) receptor family members. In addition, p75 shares homology with some of these receptors within a short sequence of the cytoplasmic domain, referred to as the death domain (Feinstein et al., 1995), a region that has been associated with apoptosis (Brakebusch et al., 1992; for review see Baker and Reddy, 1996).

p75 is widely expressed in both developing and adult animals by both neurons and non-neuronal cells (Yan and Johnson, 1989; Ernfors et al., 1988; Buck et al., 1987). Contrary to the expectation that p75 plays an important role in the development of many tissues, mice homozygous for the mutation display no obvious defects in any major organ including the peripheral ganglia, suggesting no more than a modulatory function for p75 (Lee et al., 1992, 1994a). In line with this, higher concentrations of NGF are required to ensure the survival of p75 deficient neurons, compared to neurons obtained from wild-type animals (Davies et al., 1993b; Lee et al., 1994b). Further, it has been shown that p75 plays a role

in ligand discrimination by Trk receptors (Clary and Reichardt, 1994; Lee et al., 1994b Benedetti et al., 1993; Ryden et al., 1995). Recent findings indicate that in addition to modulating Trk function, p75 signals on its own. Specifically, in neural cell lines and glial cells neurotrophin binding to p75 stimulates the generation of ceramide (Dobrowsky et al., 1994, 1995; Casaccia-Bonelli et al., 1996), activation of nuclear factor (NF)-kB and translocation of this protein to the nucleus (Carter et al., 1996), and enhancement of jun kinase (JNK) activity (Casaccia-Bonelli et al., 1996). Both ceramide accumulation and JNK activation are correlated with apoptotic stimuli in a number of systems, and several observations suggest that p75 may play a role in regulating apoptosis (Barrett and Bartlett, 1994; Frade et al., 1996; Casaccia-Bonelli et al., 1996). Further, it has been shown that competitive signaling between Trks and p75 can determine cell survival (Yoon et al., 1998; Maliartchouk and Saragovi, 1997; Davey and Davies, 1998), a finding supported by evidence for the existence of receptor allosterism (Wolf et al., 1995; Ross et al., 1996, 1998). The apoptotic role of p75 has been confirmed in two transgenic systems. First, mice lacking a functional BDNF gene display an increased number of sympathetic neurons compared to normal littermates, suggesting that BDNF, which does not activate Trk receptors in these neurons, acts through p75 to cause apoptosis (Bamji et al., 1998). Second, mice over-expressing the intracellular domain of p75 show profound reductions in the number of sympathetic, peripheral sensory, and neocortical neurons, as well as increased neuronal death following axotomy (Majdani et al., 1997).

### **1.5.3. The family of neuropoietic cytokine and their receptors**

Neuropoietic cytokines, or neurokines, constitute a family of cytokines which exhibit pleiotropy and redundancy in biological activities. The family includes: ciliary neurotrophic factor (CNTF), growth-promoting activity (GPA), leukemia neurotrophic factor (LIF), interleukin-6 (IL-6), oncostatin M (OSM), cardiotrophin 1 (CT-1), and interleukin-11 (IL-11). Cytokines have been found to regulate a number of properties in cells of the developing and mature nervous system including, proliferation, survival, differentiation, regeneration and neurotransmitter phenotype. Analysis of the genes encoding these proteins reveals a shared exon organization suggesting evolutionary descent from a common ancestral gene (Bruce et al., 1992). Furthermore, although the cytokines share very little sequence identity (<30%), they adopt a very similar tertiary structure (Bazan, 1991). Family members signal through very similar multi-subunit receptors that contain a common transducing component. This later fact could partially explain the functional redundancy of the cytokines.

#### **Ciliary neurotrophic factor**

Ciliary neurotrophic factor (CNTF) was first identified in 1976 as a trophic activity found in heart conditioned medium that was able to support the survival of chick ciliary ganglion neurons *in vitro* (Helfand et al., 1976). Subsequent studies have led to the purification (Barbin et al., 1984; Manthorpe et al, 1986) and cloning of rat (Stöckli et al., 1989), rabbit (Lin et al., 1989, 1990) and human (Masikowski et al., 1991) CNTF. Biochemical analysis of CNTF has revealed that

it is a 200 amino acid long protein with a molecular weight of 22.8 kDa and pI of 5.78 (Stöckli et al., 1989; Lin et al., 1989). One unusual feature of CNTF is that it lacks a consensus sequence for glycosylation and a secretory signal peptide, suggesting that it is a cytosolic protein (Stöckli et al., 1989; Lin et al., 1989).

CNTF is widely expressed in the adult rat nervous system, but not elsewhere. High levels of CNTF mRNA can be found in sciatic and optic nerves, olfactory bulb and spinal cord. Lower levels of CNTF mRNA are expressed in the brain stem, cerebellum, septum, hippocampus, striatum and midbrain (Stöckli et al., 1991; Dobrea et al., 1992; Ip et al., 1993a). In these tissues, CNTF is mainly produced by glial cells.

It is now well established that in addition to ciliary neurons, a wide variety of peripheral and central neurons respond to CNTF. *In vitro*, CNTF enhances the survival of sensory neurons (Barbin et al., 1984), motor neurons (Sendtner et al., 1990; Arakawa et al., 1990; Oppenheim et al., 1991), cerebellar neurons (Lärkfors et al., 1994) and hippocampal neurons (Ip et al., 1991). Further, CNTF inhibits proliferation and enhances cholinergic properties of neuronal precursors from the sympathetic ganglion (Ernsberger et al., 1989a) and stimulates cholinergic differentiation of mature sympathetic neurons (Saadat et al., 1989). CNTF has a number of neurotrophic action *in vivo*. For example, CNTF can protect embryonic spinal motoneurons from naturally occurring cell death (Oppenheim et al., 1991). In addition, CNTF can rescue neonatal rat facial motor neurons (Sendtner et al., 1990), adult rat anterior thalamic neurons (Clatterbuck et al., 1993), medial septal neurons (Hagg et al., 1992) and neurons of the substantia nigra (Hagg and Varon, 1993) from post-axotomy degeneration. CNTF also protects adult rat retinal photoreceptors from constant light damage (LaVail et al., 1992).

In addition to these effects on neuronal cells, CNTF can act on the glial cells of the nervous system. For example, CNTF promotes the *in vitro*

differentiation of glial progenitors into astrocytes (Hughes et al., 1988; Lillien et al., 1988), and the maturation and survival of cultured oligodendrocytes (Louis et al., 1993). Outside of the nervous system CNTF has been shown to maintain the pluripotentiality of cultured embryonic stem cells (Conover et al., 1993). Additionally, CNTF has myotrophic actions on skeletal muscle *in vivo* (Helgren et al., 1994).

Although CNTF has many trophic effects on both embryonic and neurons, the actual physiological role of CNTF *in vivo* remains unclear. This is for three reasons. First, CNTF mRNA is only expressed postnatally (Stöckli et al., 1989). Second, CNTF is primarily synthesized by glial cells and not in neuronal target tissues such as skeletal muscle or skin (Stöckli et al., 1989, 1991; Lillien and Raff, 1990). Third, CNTF lacks a hydrophobic secretory sequence and is not released from transfected eukaryotic cells, implying that it is a cytosolic molecule (Friedman et al., 1992; Rende et al., 1992; Sendtner et al., 1992b; Reiness and Nishi, unpublished data).

It has been suggested that CNTF might act as a lesion factor under pathological conditions. In the PNS, CNTF protein has been detected in the extracellular fluid following nerve injury (Sendtner et al., 1992b). This is coupled to a sharp decrease in the levels of cytoplasmic CNTF in Schwann cells (Friedman et al., 1992) perhaps due to its release (by an unknown mechanism) in response to injury. In the CNS, mechanical lesions result in dramatic increase in CNTF mRNA and protein expression in tissue bordering the wound site (Ip et al., 1993b; Asada et al., 1995). Such an increase in CNTF production could be localized to reactive astrocytes in the resulting glial scar (Ip et al., 1993b; Rudge et al., 1994). The expression pattern of CNTF has led to the prediction that it does not play a critical role during normal development, but rather that it has a role in response to injury or in long-term maintenance. Consistent with this possibility, mice in which the

CNTF gene has been disrupted develop normally with only mild motor neuron loss and atrophy later in adulthood (Masu et al., 1993).

### **Growth-promoting activity**

Growth-promoting activity (GPA) was initially purified from chicken sciatic nerve (Eckenstein et al., 1990) and later cloned from embryonic chicken eye (Leung et al., 1992). GPA shares about 48% amino acid identity with mammalian CNTF, suggesting that it is the avian homologue. However, marked differences in secretion and expression suggest otherwise. GPA is a 195 amino acid residues protein with a predicted molecular weight of 21.3 kDa and pI of 5.1. (Eckenstein et al., 1990). Although the crystal structure of GPA has yet to be resolved, computer analysis predicts that GPA contains alpha helices in the same amino acid residues as CNTF (Sendtner et al., 1994). GPA, like CNTF, lacks an N-terminal secretory signal sequence, yet unlike CNTF it is detected in a biological active form in both choroid smooth-muscle-conditioned medium (Nishi, unpublished data) and cell lines transfected with a GPA cDNA (Leung et al., 1992). The ability to be secreted appears to be due to the presence of an internal signal sequence between amino acids 40 and 60 (Reiness et al., 1995).

GPA has been shown to promote the survival of a wide variety of neuronal cell types within the PNS, including ciliary ganglion neurons, lumbar sympathetic neurons and, in contrast to CNTF, E8 DRG neurons (Eckenstein et al., 1990; Finn et al., 1998). Moreover, GPA like CNTF, induces choline acetyltransferase expression in neonatal rat sympathetic neurons (Fann and Patterson, 1994) and vasoactive intestinal peptide expression in embryonic chick sympathetic neurons (Heller et al., 1994). Analysis of GPA expression has demonstrated that, unlike

CNTF, it is found in the developing chicken embryo. In particular, it is expressed in cells innervated by CG neurons (Finn and Nishi, 1996a) and in the embryonic sciatic nerve (Leung et al., 1992).

### **Leukemia inhibitory factor**

Leukemia inhibitory factor (LIF) was originally purified, characterized, and cloned by virtue of its ability to induce differentiation and suppress proliferation of the murine myeloid leukaemic cell line M1 (Tomida et al., 1984; Gearing et al., 1987; Hilton et al., 1988a, b; Metcalf et al., 1988). Subsequently, it has been shown that LIF is identical to hepatocyte-stimulating factor III, a glycoprotein capable of stimulating the synthesis of several acute phase plasma proteins (Baumann and Wong, 1989), the melanoma-derived lipoprotein lipase inhibitor (Mori et al., 1989) and cholinergic neuronal differentiation factor from heart cells (Yamanori et al., 1989).

Mature LIF protein comprises of 179 amino acid residues, and has a molecular weight of ~20 kDa and pI of ~8.8 (Gearing et al., 1987). Analysis of the three-dimensional structure of LIF has revealed a folding pattern similar to the other neurokines- four alpha helical cores connected by more linear linker regions (Bazan, 1991; Robinson et al., 1994).

LIF mRNA can be detected, albeit at low levels, in most tissues during development (Bhatt et al., 1991). In the adult, LIF transcripts are found in the brain, heart, thymus, liver and intestine (Bhatt et al., 1991). *In situ* hybridization and immunohistochemical analysis of the adult rat brain have shown that LIF mRNA is predominantly expressed by hippocampal pyramidal cells, hilar interneurons and granule cells of the dentate gyrus. Neurons of the cerebral

cortex, cerebellum, cholinergic basal forebrain nuclei and the striatum also express LIF mRNA (Lemke et al., 1996). Interestingly, LIF transcripts do not appear to be expressed by astrocytes *in vivo* (Lemke et al., 1996), although primary astrocyte cultures have been shown to express LIF mRNA (Murphy et al., 1995).

LIF exhibits pleiotrophic activities on a wide range of cell types. In many instances the effects of LIF are similar, if not identical, to those of CNTF. LIF regulates the transmitter phenotype and expression of many neuropeptides in cultured sympathetic and sensory neurons (Yamamori et al., 1989; Nawa et al., 1991; Bazan, 1991; Fan and Katz, 1993). LIF also stimulates the differentiation of neural crest cells into sensory-like neurons and enhances the survival of several populations of embryonic and postnatal sensory neurons in culture (for review see Horton et al., 1996). In addition, LIF promotes the survival of spinal motor neurons both *in vitro* (Martinou et al., 1992; Henderson et al., 1993) and after deafferentation or axotomy *in vivo* (Wei et al., 1994; Vejsada et al., 1995). Furthermore, LIF increases the survival of astrocytes and retinal pigment epithelial cells (Gadient et al., 1998; Gupta et al., 1997) in addition to promoting the generation, maturation, and survival of oligodendrocytes *in vitro* (Mayer et al., 1994; Vos et al., 1996). LIF also plays a role in the response to injury of both neural and peripheral tissues. In the CNS, mechanical lesions result in a dramatic increase (30-fold) in LIF mRNA close to, but not immediately adjacent to, the lesion site. Such an increase in LIF production is localized in astrocytes and a small number of microglial cells (Banner et al., 1997). In the periphery, LIF has been shown to stimulate skeletal muscle regeneration after injury by increasing the size, but not the number, of the muscle fibers. Furthermore in this lesion model, injured muscles appear to express LIF mRNA (Barnard et al., 1994).

Mice lacking the LIF gene have been generated to investigate its role *in vivo*. In contrast to its many effects in culture, LIF deficient mice appear to have

no major developmental abnormalities. However, homozygous mutant adult females have a defect in uterine function which prevents implantation of the developing embryo. This is consistent with the observation that LIF mRNA is expressed in the uterus prior to and during implantation in the uterus (Steward et al., 1992; Bhatt et al., 1991; Song et al., 1998). In addition, absence of LIF appears to result in reduced expression of neuropeptides in peripheral neurons both *in vitro* and *in vivo* (Rao et al., 1993). Finally, careful analysis of the brain in LIF knockout mice has revealed a significant deficit in myelin basic protein expression in female, but not male, young and adult mice, suggesting an alteration in the normal oligodendrocyte population (Bugga et al., 1998).

### **Interleukin-6**

Interleukin-6 (IL-6), originally named human B cell stimulatory factor-2 (BSF-2), was characterized and isolated as a T cell-derived factor that caused the terminal maturation of activated B cells to immunoglobulin-producing cells (Hirano et al., 1985, 1987). Subsequent studies have led to the cloning of IL-6 from several species (Hirano et al., 1986; Chiu et al., 1988; Northemann et al., 1989). Mature IL-6 protein is 184 amino acids long with a predicted molecular weight of about 20 kDa (Hirano et al., 1986; Chiu et al., 1988). Human and mouse IL-6 have very little sequence identity, with only 41% homology at the amino acid level (Chiu et al., 1988).

*In situ* hybridization analysis of the adult rat brain have shown that IL-6 mRNA is expressed in the pyramidal and granular neurons of the hippocampus, habenulae, dorsomedial and ventromedial hypothalamus, large pyramidal neurons of the piriform cortex, internal capsule, optic tract, scattered neurons of the cortex

and granular cells of the cerebellum (Schobitz et al., 1992, 1993; Gadiant and Otten, 1994).

IL-6 is perhaps the most extreme example of a pleiotropic cytokine with diverse functions throughout the body. Peripherally, IL-6 is known to stimulate the acute phase hepatocyte response (Gauldie et al., 1987) and the differentiation of B lymphocytes (Kopf et al., 1994; Lee, 1992). In addition, IL-6 plays a role in bone remodeling by osteoclasts (Poli et al., 1994) and stimulates protein breakdown in muscle (Goodman, 1994). In the central nervous system, IL-6 supports the survival of cultured embryonic and postnatal midbrain catecholaminergic neurons (Kushima et al., 1992). IL-6 also supports the survival of spinal cord (Kushima and Hatanaka et al., 1992) and basal forebrain cholinergic neurons (Hama et al., 1989). In the PNS, IL-6 promotes the survival of nodose and trigeminal ganglion neurons (Horton et al., 1996). IL-6 also modulates cholinergic differentiation and expression of neuropeptides such as substance P in cultured sympathetic neurons (Fann and Patterson, 1994), and induces differentiation of PC12 cells into a neuron-like phenotype (Satoh et al., 1988). Furthermore, IL-6 stimulates astrocyte proliferation (Campbell et al., 1993; Chiang et al., 1994; Fattori et al., 1995), and plays a role in the protection against neuronal damage (Akaneya et al., 1995; Hirohata et al., 1996; Ikeda et al., 1996) and modulation of pain (DeLeo et al., 1996; Geiss et al., 1997; Oka et al., 1995; Arruda et al., 1998).

IL-6 deficient mice are viable, fertile and do not present any evident phenotypic abnormality in the nervous system or periphery either during development or in adulthood. However, these mice display impaired immune and acute phase responses to infection (Kopf et al., 1994). IL-6 deficient mice also display defective hepatocyte regeneration (Cressman et al., 1996) and specific bone phenotype (Poli et al., 1994).

## **Oncostatin M**

Oncostatin M (OSM) was initially described as a growth regulatory molecule which could inhibit the growth of certain tumor cell lines and stimulate the growth of several normal fibroblast lines (Zarling et al., 1986). Subsequently, OSM was cloned from several species (Malik et al., 1989, 1995; Hara et al., 1997). Mature OSM protein is 196 amino acids long and has a molecular weight of ~28 kDa (Malik et al., 1989). The predicted crystal structure of oncostatin M reveals a four-helical bundle motif similar to other neurokines (Rose and Bruce, 1991; Bazan, 1991). The expression of OSM is largely restricted to haematopoietic cells, including activated peripheral T-lymphocytes and lipopolysaccharide induced human monocytes (Bruce et al., 1992).

The effects of OSM *in vitro*, which have been largely determined using various cell lines, overlap with those of other neurokines (Bruce et al., 1992). In the developing nervous system, OSM promotes the survival of nodose, trigeminal and DRG ganglion neurons in culture (Horton et al., 1996; Ware et al., 1995). OSM also plays a role in regulating oligodendrocyte cell survival and differentiation (Vos et al., 1996).

## **Cardiotrophin-1**

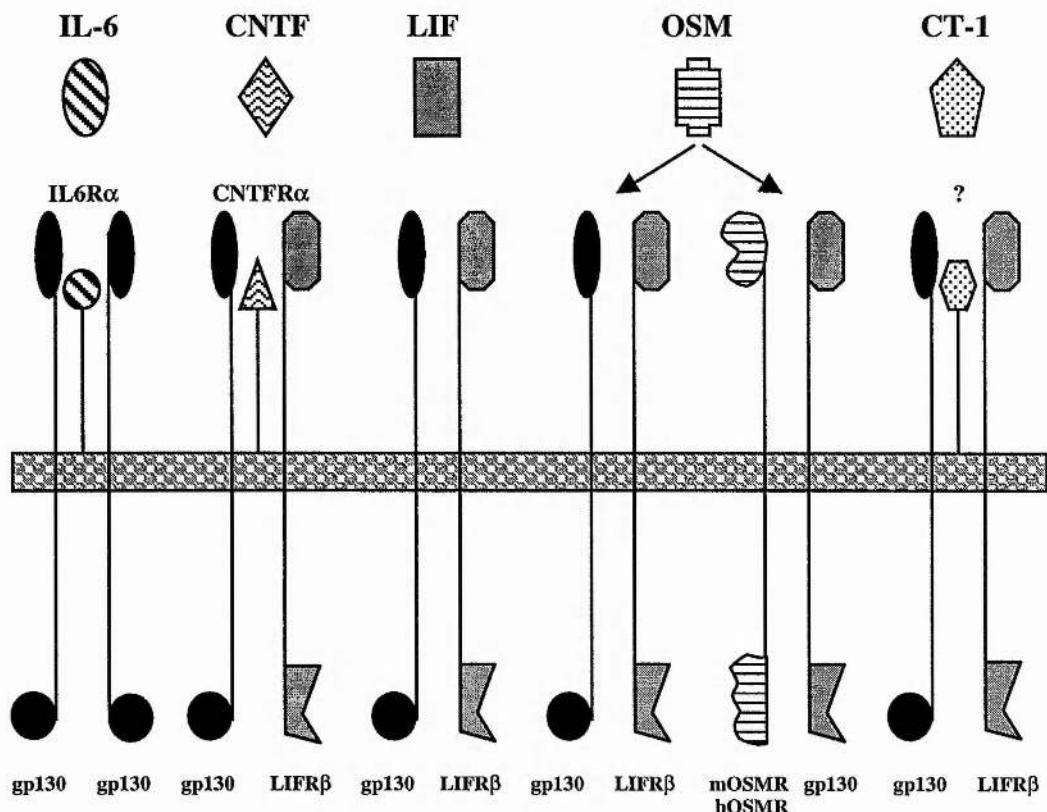
Cardiotrophin-1 (CT-1) is the latest member of the neurokine family to be isolated. It was initially identified based on its ability to induce cardiac myocyte hypertrophy *in vitro* (Pennica et al., 1995a). Mature CT-1 protein is 203 amino acids long with a molecular weight of 21.5 kDa. Like CNTF, CT-1 lacks a conventional amino-terminal secretion signal sequence; CT-1 is, however, found

in the medium of transfected mammalian cells. CT-1 mRNA is widely expressed in adult mouse tissues, including heart, kidney, skeletal muscle, liver, lung, testis, spinal cord, and brain (Pennica et al., 1995a).

The biological actions of CT-1 largely resemble those of other neurokines. For example, CT-1 inhibits the growth of the mouse M1 myeloid leukemia cell line, but unlike IL-6, CT-1 does not stimulate B cell expansion. CT-1 also inhibits the differentiation of mouse embryonic stem cells in a similar way to CNTF, LIF and OSM (Pennica et al., 1995b). In the nervous system, CT-1 promotes the survival of cultured rat hindbrain dopaminergic neurons, chicken ciliary neurons, mouse nodose neurons, late embryonic mouse trigeminal neurons and E14.5 rat motoneurons (Pennica et al., 1995b; Horton et al., 1998; Arce et al., 1998). In addition, CT-1 induces a phenotypic switch in rat sympathetic neurons *in vitro* (Pennica et al., 1995b) and protects neonatal sciatic motoneurons against the effects of axotomy *in vivo* (Pennica et al., 1996). Although no gene disruption experiments have yet been carried out, chronic administration of CT-1 to mice results in increased growth of heart, liver, kidney and spleen, in addition to atrophy of the thymus, and increased platelet counts. These effects suggest that CT-1 has a broad spectrum of biological activities *in vivo* (Jin et al., 1996).

## Neurokine receptors

The biological action of the neutropoietic cytokines is mediated by multimeric cell surface receptors that share a common subunit, the glycoprotein gp130 (see Figure 1.4).



**Figure 1.4.** Neurokine receptors.

See text for details.

For receptor activation, IL-6, CNTF and IL-11 first bind to their specific  $\alpha$ -receptor subunits (Yamasaki et al., 1988; Davis et al., 1991; Hilton et al., 1994). Subsequently, the  $\alpha$ -receptor-ligand complex associates with gp130, which then recruits either another gp130 molecule, in the case of IL-6 and IL-11, or the LIF receptor  $\beta$  (LIFR $\beta$ ) subunit in the case of CNTF (Murakami et al., 1993; Yin

et al., 1993; Davis et al., 1993a). For CT-1, LIF and human OSM (hOSM), receptor activation requires the heterodimerization between gp130 and LIFR $\beta$  without the involvement of an  $\alpha$ -component.[There is some evidence for the presence of such a component, yet unknown, in the case of CT-1 (Pennica et al., 1995b; Robledo et al., 1997)]. LIF and CT-1 first bind LIFR $\beta$  with low affinity and then to gp130, which increases the binding affinity but does not bind LIF or CT-1 by itself. In contrast, hOSM binds directly with low affinity to gp130 and the binding affinity is increased by LIFR $\beta$ , which does not bind hOSM by itself (Gearing et al., 1992; Liu et al., 1992; Pennica et al., 1995b). Recently, two additional OSM receptors have been described. The first, was isolated from human cells and composed of the hOSM receptor  $\beta$  (hOSMR $\beta$ ) and gp130 (Mosley et al., 1996). The second, was cloned from a mouse colon library and composed of mouse OSMR $\beta$  (mOSMR $\beta$ ) and gp130. Human and mouse OSMR $\beta$ s are believed to be orthologous (Lindberg et al., 1998). Interestingly, mOSM, in contrast to hOSM, cannot signal through the LIFR $\beta$ -gp130 complex (Ichihara et al., 1997; Lindberg et al., 1998).

Neurokine receptors (NRs) do not possess any intrinsic enzymatic activities. Their cytoplasmic domains contain no kinase or phosphatase domain and have regions of limited homology in the membrane-proximal part, often termed the box1 and box2 motifs (Murakami et al., 1991). Their extracellular domain displays characteristic structural motifs including four conserved cysteine residues in the amino-terminal part and the presence of a conserved WSXWS motif in the carboxy-terminal part (Bazan, 1990).

The key features of the transduction processes utilized by neurokines may be defined as follows. Upon ligand binding, dimerization of two gp130 or one gp130 and one LIFR $\beta$  occurs. This event leads to rapid phosphorylation of tyrosine residues on the  $\beta$ -receptor components and other cellular proteins. Since

NRs lack any intrinsic enzymatic activity, tyrosine phosphorylation is mediated by the JAK kinases (JAK). Four members of JAKs have been identified (Wilks et al., 1991; Harpur et al., 1992; Kawamura et al., 1994; Firnbach-Kraft et al., 1990) and both gp130 and LIFR $\beta$  can bind JAK1, JAK2, and Tyk2 with some specificity, depending on the cell type in which they are expressed (Stahl et al., 1994, 1995). It is now generally believed that JAK kinases are pre-associated with gp130 and LIFR $\beta$  prior to receptor dimerization, and that dimerization of  $\beta$  components result in activation of these kinases (Stahl et al., 1994). The phosphorylated receptors recruit SH2-bearing proteins through their tyrosine phosphorylated sites. These signal transducing proteins are then activated by the receptor-associated JAKs (for review see Stahl and Yancopoulos, 1994; Miyajima, 1997; Moutoussamy et al., 1998). It has been shown that substrates for JAK phosphorylation include: PLC $\gamma$ , PI3-kinase, PTP1D, Shc, GRB2, Raf-1 and the MAP kinases ERK1 and ERK2 (Boulton et al., 1994). Another target for tyrosine phosphorylation by activated JAK is the STAT (Signal Transducers and Activator of Transcription) family of transcription factors.

There are seven STATs, of which STAT1 and STAT3 are the preferential signal transducers for neurokines (Stahl et al., 1995; Lai et al., 1995; Lütticken et al., 1994). STATs bind to activated NRs by virtue of their SH2-containing domain. Following tyrosine phosphorylation by JAKs, STATs form either a homodimer or a heterodimer, before translocating to the nucleus where they bind DNA and regulate gene expression. STATs can also bind other transcription factors and thus modulate their function (for review see, Moutoussamy et al., 1998; Hoey and Schindler, 1998).

gp 130 and LIFR $\beta$  are widely distributed both in the periphery and nervous system. gp130 mRNA has been detected in brain, heart, thymus, kidney, lung, liver and spleen. Additionally, gp130 mRNA is expressed in ES cells,

embryos and in all cell lines tested (Saito et al., 1992; Hibi et al., 1990; Taga et al., 1989). At least two soluble variants of gp130, with the ability to bind both LIF and LIFR $\beta$ , have been identified (Zhang et al., 1998; Diamant et al., 1997). Recent evidence suggest that soluble gp130 is an antagonist of the effects of IL-6 on various cell types especially in the presence of soluble IL-6R $\alpha$  (sIL-6R $\alpha$ ) and plays a role in inflammation (Müller-Newen et al., 1998; Zhou et al., 1998; Narazaki et al., 1993). Northern blotting and *in situ* hybridization analysis of LIFR $\beta$  mRNA expression has revealed that it is expressed in liver, placenta, ovaries, testes, brain (especially in regions relevant to the motor and sensory systems), kidney, skeletal muscle, uterus, pancreas, ES cells and in all cell lines examined (Owczarek et al., 1996; Yamakuni et al., 1996; Hilton and Nicola, 1992). Like gp130, LIFR $\beta$  exists in both membrane-bound and soluble form (Layton et al., 1992; Zhang et al., 1998; Owczarek et al., 1996; Tomida, 1997). Although the role of sLIFR $\beta$  *in vivo* is presently unclear, *in vitro* experiments have demonstrated that sLIFR $\beta$  is able to block the activity of murine LIF (Layton et al., 1992; Tomida, 1995; Yamaguchi-Yamamoto et al., 1993).

The intracytoplasmic portion of IL-6R $\alpha$  is very short (Yamasaki et al., 1998) and is not required for signaling (Taga et al., 1989). IL-6R $\alpha$  mRNA is found in many myeloma and normal cell lines (Kishimoto et al., 1992; Navarro et al., 1991) as well as various regions of the brain (Yamasaki et al., 1998; Banning et al., 1998). In the nervous system, IL-6R $\alpha$  appears to colocalize with its ligand IL-6 and is expressed by both neuronal (including PNS sympathetic and sensory neurons) and glial cells (Schobitz et al., 1992, 1993; Gradient and Otten, 1994, 1996). The IL-6R $\alpha$  exists also in a soluble form (Lust et al., 1992) that is generated through either proteolytic shedding of the cognate receptor or differential mRNA splicing (Müllberg et al., 1993; Jones et al., 1998). Soluble IL-6R $\alpha$  s(IL-6R $\alpha$ ) is functional, and *in vitro* appears to enhance the effect of IL-6.

(Lust et al., 1995; Taga et al., 1989). *In vivo*, sIL-6R $\alpha$  has been implicated in diseases such as Alzheimer's (Angelis et al., 1998; Hampel et al., 1998), extramedullary hematopoiesis and plasmacytoma formation (Peters et al., 1997; Schirmacher et al., 1998). In addition sIL-6R $\alpha$  has been shown to prolong plasma IL-6 half-life (Peters et al., 1996).

A unique feature of CNTFR $\alpha$  is that it lacks a transmembrane domain. Instead, CNTFR $\alpha$  is anchored to the plasma membrane via a glycosyl-phosphatidylinositol (GPI) linkage that can be cleaved by phosphatidylinositol-specific phospholipase C (PIPLC), an enzyme that cleaves the anchors of GPI linked proteins (Davis et al., 1991). CNTFR $\alpha$  expression is largely restricted to the nervous system where it is localized to neuronal populations that respond to CNTF, including sympathetic, sensory and parasympathetic ganglia (Ip et al., 1993a). In the CNS, CNTFR $\alpha$  is a prominent marker for motor neurons and motor related brain areas, consistent with the role of CNTF in maintaining motor system function (Ip et al., 1993a). In addition, CNTFR $\alpha$  is widely expressed within other areas of the CNS, suggesting additional potential targets for CNTF action (Ip et al., 1993a). In contrast to CNTF, CNTFR $\alpha$  is expressed during embryonic development. In particular, CNTFR $\alpha$  is expressed by neuronal precursors in the neuroepithelium of the CNS and by neural crest-derived progenitors in the PNS. Embryonic expression of CNTFR $\alpha$  implies the existence of a still unknown second ligand for this receptor (Ip et al., 1993a). In the periphery, CNTFR $\alpha$  is expressed in skeletal muscle (Ip et al., 1993a), an observation that and this is consistent with the myotrophic actions of CNTF (Helgren et al., 1994). CNTFR $\alpha$  can also exist as a soluble protein (Davis et al., 1993b). Soluble CNTFR $\alpha$  (CNTFR $\alpha$ ) has been detected in cerebrospinal fluid and in skeletal muscle after nerve injury. This later observation suggests that soluble

CNTFR $\alpha$  may play a role in the physiological response to injury (Davis et al., 1993b).

The *in vivo* role of the NRs has been further examined by the use of knockout mice. Targeted inactivation of the gp130 gene results in a reduction in the total complement of haematopoietic cells and striking defects within the developing heart that lead to embryonic lethality (Yoshida et al., 1996).

LIFR $\beta$  deficient mice die perinatally (Ware et al., 1995) and display a significant reduction in the number of facial and spinal motor neurons (Li et al., 1995). In addition, they show decreased numbers and impaired differentiation of spinal brainstem and cord astrocytes (Ware et al., 1995; Koblar et al., 1998).

Ablation of the CNTFR $\alpha$  gene in mice results in a reduction in all motor neuron populations examined (DeChiara et al., 1995), as well as a reduction in neuronal number in other PNS and CNS populations examined (DeChiara and Yancopoulos, unpublished data). CNTFR $\alpha$  knockout mice do not initiate the feeding process and die shortly after birth (DeChiara et al., 1995).

#### **1.5.4. The superfamily of TGF- $\beta$ -related cytokines and their receptors**

The transforming growth factor (TGF)- $\beta$  superfamily is a large group of cytokines which are among the most versatile carriers of growth and differentiation signals. Members of this family participate in setting up the basic body plan during embryogenesis in mammals, frogs and flies; control formation of neural tube, limbs, cartilage, bone and sexual organs; suppress epithelial cell growth; promote wound repair; and influence important immune and endocrine functions. This superfamily, which currently includes around 40 members, has several groups. The main families are: TGF- $\beta$ s, *decapentaplegic*-Vg1-related (DVR), activins and glial cell line-derived neurotrophic factor (GDNF). Some members cannot be classified into a specific subgroup, however, and new ones are still being identified. With the exception of GDNF family, all members of the TGF- $\beta$  superfamily signal through a receptor complex formed by two distantly related types of serine/threonine kinase proteins.

#### Structure of the ligands

The various members of the TGF- $\beta$  superfamily are initially synthesized as larger precursor molecules with an amino-terminal signal sequence and a pro-domain of varying size. This precursor protein is usually cleaved at a dibasic or RXXR site to release a mature carboxy-terminal segment (Massagué, 1990). Although the pro-domain is poorly conserved across different superfamily members, it is often well conserved for a particular family member isolated from several different organisms. The pro-domain appears to be required for normal

synthesis and secretion of TGF- $\beta$  family members (Gray and Mason, 1990; Hammonds et al., 1991; Thomsen and Melton, 1993). The pro-domain can also remain associated with the carboxy-terminal signaling fragment to produce an inactive complex (Gentry and Nash, 1990). The mature TGF- $\beta$  proteins are much more highly conserved and contain most of the sequence landmarks by which new family members are usually recognized. Seven cysteine residues within the mature region are nearly invariant in members of the superfamily. Crystallography studies have shown that six of these cysteines are closely grouped to make a rigid structure called a "cysteine knot". This knot locks the base of several  $\beta$ -sheet strands together and probably accounts for the strong resistance of many TGF- $\beta$  superfamily members to heat, denaturants, and extreme pH. The remaining cysteine residue in each monomer forms an additional disulfide bond that links two monomers into a dimer (Daopin et al., 1992; Schlunegger and Grutter, 1992). Many hydrophobic contacts exist between two monomer subunits that could promote dimer formation even in the absence of a disulfide bond. Indeed this is the case for GDF-3 and GDF-9, proteins that lack the seventh cysteine (Schlunegger and Grütter, 1992; Daopin et al., 1992; Mcpherron and Lee, 1993).

### **The TGF- $\beta$ family**

The TGF- $\beta$  family comprises at least five distinct protein species which are more closely related to each other than to any other members of the TGF- $\beta$  superfamily (for review, see Kriegstein et al., 1995). These proteins are TGF- $\beta$ 1, - $\beta$ 2, - $\beta$ 3, - $\beta$ 4, and-  $\beta$ 5. TGF- $\beta$ 5 is an isoform which has been found in amphibians, and TGF- $\beta$ 4 is probably the chick homologue of mammalian TGF- $\beta$ 1 (Burt and Paton, 1992). All TGF- $\beta$  isoforms are 25 kDa dimers, with 9 conserved cysteine

residues and a more than 70% sequence identity at the amino acid level (Roberts et al., 1990).

TGF- $\beta$ s are widely distributed in developing and adult tissues representing all three primary embryonic germ layers. Tissues that express one or more TGF- $\beta$  proteins include cartilage, bone, teeth, muscle, heart, blood vessels, lung, kidneys, gut, liver, eye, ear, skin and brain (Pelton et al., 1991; Millan et al., 1991; Schmid et al., 1991). In the developing and adult nervous system TGF- $\beta$ 1 expression is restricted to meninges and choroid plexus. In contrast, TGF- $\beta$ 2 and TGF- $\beta$ 3 are co-expressed in astroglial and Schwann cells as well as in many populations of central and peripheral neurons. TGF $\beta$ -2 and TGF $\beta$ -3 appear to be expressed at particularly high levels in neurons that project over long distances (for review, see Kriegstein et al., 1995).

Current knowledge on the functions of TGF- $\beta$ s in the nervous system has been deduced from *in vitro* and *in vivo* lesioning studies. The available mouse mutants that are defective for either one of the isoforms (Shull et al., 1992; Kulkarni et al., 1993; Kaartinen et al., 1995; Proetzel et al., 1995; Sanford et al., 1997) have failed to reveal overt deficits in neural structure and function, possibly by virtue of compensatory effects of the remaining isoforms. TGF- $\beta$ s have been implicated in the regulation of neuronal survival of motoneurons (Martinou et al., 1998), sensory neurons (Chalazonitis et al., 1992) and midbrain dopaminergic neurons (Kriegstein and Unsicker, 1994; Poulsen et al., 1994). However, it is now emerging that the trophic effects of the TGF- $\beta$ s require the presence of other co-operative factors found in serum or produced by non-neuronal cells. Indeed it has been shown that highly enriched serum-free neuron cultures do not respond to TGF- $\beta$ s alone (Kriegstein and Unsicker, 1996; Kriegstein et al., 1998a). Many recent studies have emphasized the synergistic potential of the TGF- $\beta$ s. For example, TGF- $\beta$ 3 can significantly increase the survival of cultured DRG neurons

treated with NT-3 (Kriegstein and Unsicker, 1996). Similarly, TGF- $\beta$ 1 greatly enhances the survival effects of NGF on DRG neurons (Chalazonitis et al., 1992). Moreover, addition of TGF- $\beta$ 1 to cultures of spiral ganglion cells modulates bFGF receptor mRNA and increases FGF2-induced neuronal survival (Lefebvre et al., 1991). TGF- $\beta$ 1, - $\beta$ 2 or - $\beta$ 3 enhance ciliary neuron survival mediated by CNTF or FGF-2 and in combination with NT-3 or NGF promote it (Gouin et al., 1996; Kriegstein et al., 1998b) Recently, it has been reported that GDNF requires TGF $\beta$ s for its trophic effects on chicken sensory, sympathetic and parasympathetic neurons (Kriegstein et al., 1998a).

TGF- $\beta$ s also act on the non-neuronal cells of the nervous system. They are mitogens for Schwann cells (Ridley et al., 1989) and, together with FGF, synergistically regulate mitosis (Schubert, 1992). TGF- $\beta$ 1 is chemotactic for cultured astrocytes (Morganti-Kossman et al., 1992) and TGF- $\beta$ s have, in most contexts, antiproliferative effects on them (for review, see Kriegstein et al., 1995).

### **The GDNF family**

#### **Glial cell line-derived neurotrophic factor**

The observation that the rat glial cell line, B49 released a factor which exhibited relative specificity for dopaminergic neurons in dissociated cultures of the rat embryonic midbrain lead to identification of GDNF a new member of the neurotrophic growth factor family (Engele et al., 1993; Lin et al., 1993a, b, 1994). GDNF is a glycosylated, disulfide-bonded homodimer that is distantly related (less than 25% amino acid identity) to members of the TGF- $\beta$  superfamily. The

molecular weight of the GDNF homodimer is approximately 33-45 kDa while the monomer has a molecular weight of 16 kDa after glycosylation (Lin et al., 1993a, 1994). Sequence data suggests that GDNF is synthesized as a precursor of 211 amino acids that is cleaved and secreted as a mature protein of 134 amino acids (Bektesh et al., 1993; Lin et al., 1993a).

GDNF has a wide tissue distribution and is expressed both during development and adulthood. GDNF is expressed by both neuronal and non-neuronal cells in the brain. The highest levels of GDNF mRNA are found in pyramidal and granular cells of the hippocampus. A tense signal is also observed in Purkinje cells of the cerebellum, in dopaminergic neurons of the substantia nigra and in subpopulations of striatal neurons. In addition, cholinergic neurons of the medial septal nucleus, the diagonal band of Broca, the ventral pallidum and habenula show high levels of GDNF mRNA. Moreover, motoneurons of the facial nucleus, SCG and DRG also express GDNF mRNA. In the spinal cord, neuronal expression is found in Clarke's column and the dorsal and ventral horn. Additional regions where GDNF mRNA is expressed in the head region include the carotid body, retina, vibrissae, inner ear, ear canal, developing teeth and epithelium in the nasal cavity (Nosrat et al., 1996; Pochon et al., 1997; Trupp et al., 1995; Schaar et al., 1993; Golden et al., 1998; Henderson et al., 1994). In the periphery, GDNF mRNA is present in numerous organs and tissues. These include kidney, testis, ovary, stomach, skin, muscle, adrenal, liver, spleen, intestine, lung, and blood (Suter-Cazzolara and Unsicker, 1994; Choi-Lundberg and Bohn, 1995; Trupp et al., 1995).

GDNF was initially characterized on the basis of its ability to promote the survival and morphological differentiation of cultured dopaminergic neurons (Lin et al., 1993a). Subsequently, using animal models of Parkinson's disease, GDNF injected into the substantia nigra or the striatum has been shown to protect

lesioned dopaminergic neurons from the degenerative process induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Tomac et al., 1995), transection of the medial forebrain bundle (Beck et al., 1995) and 6-hydroxydopamine (Bowenkamp et al., 1995). The neurotrophic actions of GDNF are not confined to dopaminergic neurons. Many studies have confirmed the neurotrophic effects of GDNF on motoneurons (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995; Zurn et al., 1994, 1996; Houenou et al., 1996; Li et al., 1995; Trok et al., 1996; Arce et al., 1998; Junger and Varon, 1997). For example, GDNF is more effective than neurotrophins in promoting the *in vitro* survival of both cranial and spinal motor neurons with higher potency and longer lasting support than neurotrophins (Henderson et al., 1994). In addition, GDNF induces choline acetyl-transferase (ChAT) activity and neurite outgrowth in motor neurons (Zurn et al., 1994, 1996). In motoneuron lesion paradigms, GDNF prevents facial motoneuron death following axotomy in newborn rats (Yan et al., 1995), degeneration of adult spinal motoneurons after ventral root avulsion (Li et al., 1995), and the lesioned-induced decrease in ChAT immunoreactivity of adult facial motoneurons (Yan et al., 1995). Moreover, GDNF significantly improves the conduction velocity of motor axons of the tibial nerve in adult rats (Munson and McMahon, 1997). Other CNS neuronal populations that are supported by GDNF include noradrenergic neurons, cerebellar Purkinje cells, and basal forebrain cholinergic neurons (Arenas et al., 1995; Mount et al., 1995; Williams et al., 1996). In the peripheral nervous system, GDNF promotes the survival of chicken embryonic sympathetic, parasympathetic and most sensory neurons (Buj-Bello et al., 1995; Ebendal, 1995; Trupp et al., 1995; Forgie et al., 1999). GDNF does not support, with the exception of late submandibular (E17, E18) and early nodose (E12), the survival of mouse PNS neurons (Cacalano et al., 1998).

The physiological role of GDNF has been further analyzed by the use of transgenic models. Mice lacking the GDNF gene have deficits in dorsal root ganglion (23%), superior cervical ganglion (35%) and petrosal-nodose complex (23%), but not in hindbrain noradrenergic or midbrain dopaminergic neurons. Furthermore, these mice lack most of the enteric nerve plexus and display agenesis or severe dysgenesis of the kidneys (Moore et al., 1996; Pichel et al., 1996a, b; Sanchez et al., 1996; Granholm et al., 1997). In another transgenic model, mice in which muscle fibers overexpress GDNF, show increased numbers of motor axons innervating neuromuscular junctions (Nguyen et al., 1998).

### **Neurturin**

Neurturin (NTN) is a neurotrophic factor that was purified and cloned based on its ability to support the survival of rat SCG neurons *in vitro* (Kotzbauer et al., 1996). It is synthesized as a precursor protein of 195 amino acids that upon cleavage yields a 100-residue mature protein. NTN contains all seven conserved cysteine residues found in the same relative position across the entire TGF- $\beta$  superfamily. Sequence comparison has revealed that mature NTN shares 42% similarity with mature GDNF and less than 20% with other members of the superfamily. Biological active NTN is a homodimer with molecular mass of 25 kDa on non-reducing electrophoresis gels (Kotzbauer et al., 1996).

NTN mRNA is found both within and outside the nervous system. In the CNS, NTN mRNA is present in the lateral and medial magnocellular paraventricular nucleus and in the supraoptic nucleus of the hypothalamus, in anteromedial, anteroventral, ventrolateral and ventral anterior nuclei of the thalamus, in a number of areas of the hippocampal formation, including all three

fields of Ammon's horn (CA1-3) within the hippocampus proper and in the dentate gyrus, in the granule and Purkinje cell layers of the cerebellum, in the piriform cortex, in the striatum, in the neocortex and in the cingulate cortex (Golden et al., 1998). Additional areas of NTN mRNA expression in head region include the developing inner ear, olfactory mucosa, the vibrissae, the epithelial portion of salivary glands and developing teeth (Widenfalk et al., 1997). In the periphery, NTN mRNA is present in the developing buds of the metanephric kidney, smooth muscle layers of the developing ureter, lungs, heart, Sertoli cells, epithelium of the oviduct, blood and liver (Widenfalk et al., 1997; Kotzbauer et al., 1996).

The neurotrophic activities of NTN have been analyzed using cell culture and *in vivo* approaches. In the PNS, neurons responsive to NTN include embryonic chicken SCG, nodose, lumbar sympathetic, DRG, ciliary and DMTG (Kotzbauer et al., 1996; Forgie et al., 1999). Like GDNF, NTN supports the survival of only late submandibular and early nodose neurons in the mouse embryo (Cacalano et al., 1998). In the CNS, NTN promotes the *in vitro* survival of both developing and mature dopaminergic (DA) neurons and protects mature DA neurons from cell death induced by 6-OHDA, *in vivo* (Horger et al., 1998). Furthermore, NTN has been shown to support the survival of enteric neuron precursors and induce the proliferation of both these neurons and enteric glia (Heuckeroth et al., 1998).

Neurturin deficient mice are viable and fertile but have defects in the enteric nervous system, including reduced myenteric plexus innervation density and reduced gastrointestinal motility. Parasympathetic innervation of the lacrimal and submandibular salivary gland is dramatically reduced in NTN knockout mice, consistent with *in vitro* work showing that NTN is a neurotrophic factor for parasympathetic neurons. In addition, absence of NTN appears to result in the loss of cells from the trigeminal and dorsal root ganglia (Heuckeroth et al., 1999).

## **Persephin**

Persephin (PSP), the third member of the GDNF family to be identified, has recently been cloned based on homology to GDNF and NTN (Milbrandt et al., 1998). Persephin is synthesized as a precursor protein of 156 amino acids that upon cleavage at the RXXR site yields a 96 residues mature protein. PSP is approximately 40% identical to NTN and GDNF and displays the cysteine residue spacing characteristic of the TGF- $\beta$  superfamily members as well as regions that are only common to GDNF family members. Biological active PSP is a homodimer, with the monomer having a molecular weight of 10-12 kDa (Milbrandt et al., 1998).

Initially, PSP mRNA expression was analyzed using RNA blotting and *in situ* hybridization. This approach detected no PSP mRNA, indicating low abundance for PSP transcripts (Milbrandt et al., 1998). Subsequently, semi-quantitative RT-PCR was employed and revealed ubiquitous PSP mRNA expression in both neuronal and non-neuronal tissue. PSP mRNA appears to be present at similar levels in most tissues examined, with slightly higher levels in embryonic tissues. Further, in the CNS, PSP mRNA has been detected in both neurons and glial cells (Milbrandt et al., 1998; Jaszai et al., 1998).

Persephin, like GDNF and NTN, promotes the survival of ventral hindbrain dopaminergic neurons in culture and prevents their degeneration after 6-hydroxydopamine treatment *in vivo*. PSP supports the survival of motor neurons both *in vivo* following sciatic nerve axotomy and *in vitro* (Milbrandt et al., 1998). PSP, like GDNF, also promotes uteric bud branching (Milbrandt et al., 1998). However, in contrast to GDNF and NTN, PSP does not support the survival of any neurons of the PNS (Milbrandt et al., 1998; Forgie, unpublished observations; personal observations).

## **Artemin**

Artemin (ART) is the most recent member of the GDNF family to be isolated. It was identified from a HTGS database using the BLAST 2.0 algorithm with mature NTN protein sequence as a query (Baloh et al., 1998a). Artemin, like other family members contains a signal sequence for secretion and synthesized as a precursor protein that upon cleavage yields a 113 amino acids mature protein. Artemin shares 45% sequence identity to NTN and PSP and 36% to GDNF and possesses all seven conserved cysteine residues characteristic for the TGF- $\beta$  superfamily (Baloh et al., 1998a).

Northern blotting has revealed that ART mRNA is expressed at very low levels in the adult and fetal brain. Interestingly, low-levels of ART transcripts are present in structures of the basal ganglia (subthalamic nucleus, putamen, substantia nigra) and in the thalamus, suggesting that it may influence subcortical motor systems. ART mRNA has been detected in many adult non-neuronal tissue, albeit at low levels. Areas where ART mRNA is expressed include the pituitary gland, placenta, and trachea. Among fetal tissues, kidney and lung show the highest levels of ART mRNA expression (Baloh et al., 1998a). Like GDNF and NTN, artemin supports the *in vitro* survival of several peripheral neuron populations (DRG, SCG, trigeminal, nodose) and dopaminergic neurons of the ventral midbrain (Baloh et al., 1998a).

## TGF- $\beta$ superfamily receptors

Most members of the TGF- $\beta$  superfamily signal through a conserved family of transmembrane serine/threonine kinase receptors, all of which have a characteristic structure that includes a short cysteine-rich extracellular domain, a single transmembrane domain and an intracellular ser/thr kinase domain (for review, see Massagué, 1998). Many of these receptors have now been identified in a wide variety of vertebrates and invertebrates including, humans, mouse, *Xenopus*, *C.elegans* and *Drosophila*. Functional characterization and comparison of their primary amino acid sequence indicates that these receptors can be divided into two classes, the type I and the type II receptors. The type I receptor class is distinguished by a highly conserved sequence known as the "GS domain", which contains a repetitive glycine-serine motif and is located between the transmembrane and kinase domains. The type II receptor has a C-terminal extension which is rich in serine and threonine but appears to have no role in type II receptor function (Wieser et al., 1993).

In addition to these signaling receptors, TGF- $\beta$  subfamily members bind to two other transmembrane proteins, betaglycan (or type III receptor) and endoglin. Betaglycan binds all three TGF- $\beta$  isoforms with high affinity (Segarini et al., 1989; Cheifetz and Massagué, 1991) and facilitates TGF- $\beta$  binding to the type II receptor (Wang et al., 1991; López-Casillas et al., 1993). Endoglin binds TGF- $\beta$ 1 and TGF- $\beta$ 3, but not TGF- $\beta$ 2 (Cheifetz et al., 1989). As with betaglycan, complexes between endoglin and TGF- $\beta$  receptors have been observed (Yamashita et al., 1994). However, the role of endoglin in TGF- $\beta$  binding to signaling receptors is unclear. Some evidence suggest that endoglin over-expression diminishes TGF- $\beta$  responses (Lastres et al., 1996).

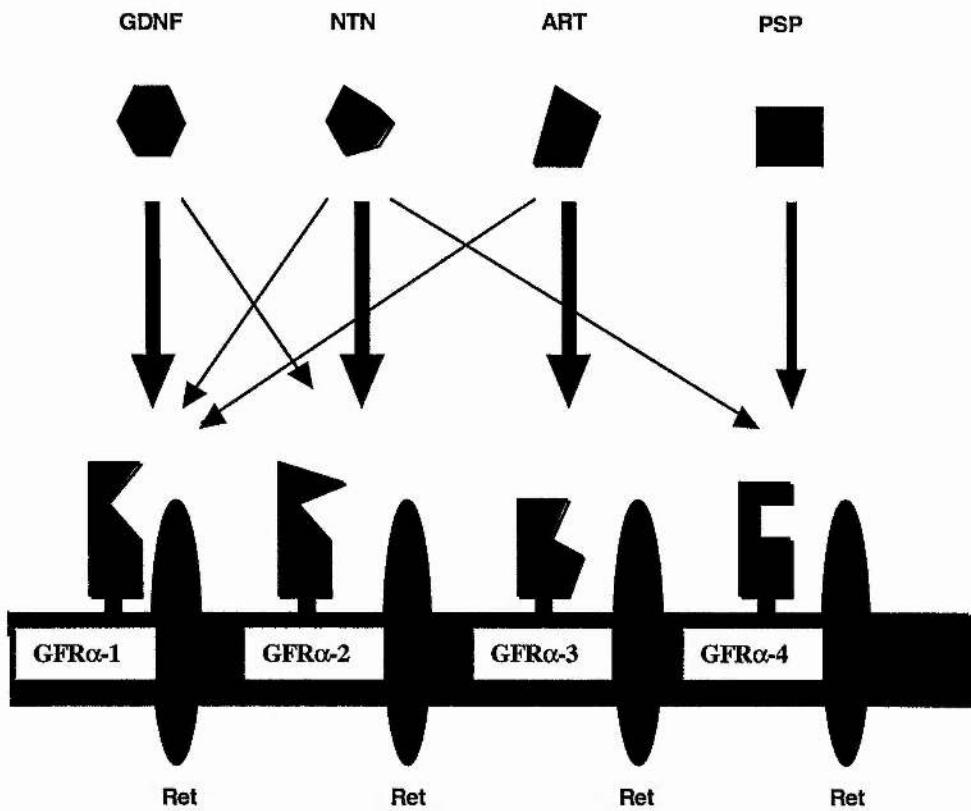
| Ligands     | receptor type II | receptor type I | receptor-regulated Smad | common Smad |
|-------------|------------------|-----------------|-------------------------|-------------|
| Activin     | ActRII           | ActRIB          | Smad2                   |             |
|             |                  |                 | Smad3                   | Smad4       |
|             | ActRIIB          |                 | Smad2                   |             |
| TGF $\beta$ | T $\beta$ RII    | T $\beta$ RI    | Smad2                   |             |
|             |                  |                 | Smad3                   | Smad4       |
| BMP2/4      | BMPRII           | BMPRI           | Smad1                   |             |
|             | ActRII           | BMPRIIB         | Smad5                   | Smad4       |
|             |                  |                 | Smad8                   |             |
| Dpp         | Punt             | Tkv             |                         | Medea       |
|             |                  | Sax             |                         |             |

**Table 1.1.** Molecular components of TGF- $\beta$  superfamily signal transduction.

TGF- $\beta$ s and related factors activate signaling by binding to and bringing together pairs of type I and II receptors (Table 1.1). Two general modes of binding ligands have been observed (for review, see Massagué, 1998). The first involves direct binding of ligand to the type II receptor and subsequent interaction of this complex with the type I receptor, which in effect becomes recruited into the complex. This binding mode is characteristic of TGF- $\beta$  and activin receptors. The second binding mode is typical of BMP receptors and involves a co-operative, interaction between type I and II receptors that bind ligand with high affinity when expressed together but low affinity when expressed separately. Upon heterotetrameric complex formation, receptor II, which is a constitutively active kinase, phosphorylates receptor I on serine and threonine residues within the 'GS domain'. Once activated, receptor I phosphorylates Smad (for *Sma* and *Mad*) proteins that carry the signal to the nucleus.

A total of nine vertebrate Smads have been identified to date, as well as two in *Drosophila* and five in nematodes (for review see Massagué, 1998; Whitman, 1998). Smads are classified into three distinct categories. The first category comprises Smads that are directly phosphorylated by type I receptors and when overexpressed mimic specific signaling pathways ("receptor-regulated Smads", e.g. vertebrate Smad1, Smad2, Smad3, Smad5, and Smad8, *Drosophila* Mad). The second group of Smads appears to be shared among multiple signaling pathways (e.g. vertebrate Smad4 and *Drosophila* Medea). The third category of Smads are those that when overexpressed inhibit TGF- $\beta$  superfamily signaling ["inhibitory Smads", e.g. vertebrate Smad6, Smad7, and *Drosophila* Daughter against Dpp (Dad)]. There remain some Smads that have not been categorized in the above framework (e.g. daf-3). Examination of the primary sequence of Smads has revealed extensive sequence homology in two distinct regions. The first is an amino-terminal domain designated the Mad homology 1 (MH1) domain and the second a carboxy terminal domain designated MH2. The region between the MH1 and MH2 domains is referred to as the linker region and is poorly conserved among Smads.

In the basal state, Smads exist as homotrimers that reside in the cytoplasm. Upon ligand activation of the receptor complex, the type I receptor kinase phosphorylates the receptor regulated Smads which then form a heterohexameric complex with Smad4 and move into the nucleus. Three types of mechanisms by which Smads direct specific transcriptional responses have been identified. These are: transcriptional transactivation (a function intrinsic to the MH2 domain), specific association with a nuclear transcriptional factor and DNA-binding activity intrinsic to the MH1 domain (for review, see Whitman, 1998; Massagué, 1998).



**Figure 1.5.** Schematic diagram of ligand-receptor interactions in the GDNF ligand family deduced from multiple experimental paradigms *in vitro*.

Large arrows indicate preferred ligand-receptor interactions, whereas smaller arrows indicate alternative receptor interactions. GFR $\alpha$ -1/Ret is the most promiscuous member of the receptor family, with the ability to interact with three of the four ligands, whereas GFR $\alpha$ -3/Ret is the least, in that it can only interact with artemin.

The GDNF family signal transduction is unique for two reasons. First, they are the only TGF- $\beta$ -like factors that signal through a tyrosine kinase. Second, this is the first example of tyrosine kinase receptor that requires a glycolipid-anchored component ( $\alpha$ -component) to bind ligand. GDNF family ligands signal through a common signal transducing component, named Ret (Durbec et al., 1996a; Trupp

et al., 1996; Vega et al., 1996; Worby et al., 1996) in conjunction with one of a family of GPI-linked receptors (GFR $\alpha$ -1 to 4) that confers ligand specificity (Baloh et al., 1988a; Buj-Bello et al., 1997; Jing et al., 1996, 1997; Klein et al., 1997; Naveilham et al., 1998; Thompson et al., 1998; Treanor et al., 1996; Trupp et al., 1998; Worby et al., 1998). Studies of ligand binding, Ret phosphorylation, and the responses of cells expressing these receptors have indicated that GFR $\alpha$ -1/Ret is the preferred receptor for GDNF (Jing et al., 1996; Treanor et al., 1996), GFR $\alpha$ -2/Ret 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; Sanicola et al., 1997; Suvanto et al., 1997; Trupp et al., 1998; Widenfalk et al., 1997), GFR $\alpha$ -3/Ret is the receptor for artemin (Baloh et al., 1998a) and GFR $\alpha$ -4/Ret is the receptor for persephin (Enokido et al., 1998) (see Figure 1.5).

Ret (rearranged during transfection) is a proto-oncogene that was first identified by transfection of NIH 3T3 cells with human T-cell lymphoma DNA (Takahashi et al., 1985). Full length human ret cDNA was subsequently cloned, sequenced and identified as a receptor tyrosine kinase (RTK) protein (Takahashi et al., 1988, 1989). The homologous mouse, chicken and zebrafish Ret proto-oncogenes have also been cloned (Iwamoto et al., 1993; Schuchardt et al., 1995; Marcos-Gutierrez et al., 1997). Two Ret protein isoforms, which differ in their carboxy-terminal sequence, have been conserved between mammals and birds. The 53 C-terminal amino acids of the long isoform are absent from the short isoform, and are replaced by nine different amino acids (Tahira et al., 1990; Iwamoto et al., 1993; Schuchardt et al., 1995). In addition to the two isoforms, a third with 43 carboxyl terminal amino acids has been identified in mice (Myers et al., 1995). The Ret protein contains a large extracellular domain with no similarity to any other RTK. This domain includes a cadherin-like region distal to the membrane and a closer cysteine-rich region. The extracellular domain is followed

by a single transmembrane domain, and an intracellular tyrosine kinase domain that is separated into two by a small insertion sequence.

Mutations in the human Ret gene are involved in the pathogenesis of at least five diseases (for review see, Pasini et al., 1996; Edery et al., 1997; Kusafuka and Puri, 1997; Goodfellow and Wells, 1995; Mak and Ponder, 1996). Somatic rearrangements of Ret are responsible for a variable proportion of papillary thyroid carcinomas (Grieco et al., 1990; Bongarzone et al., 1994; Santoro et al., 1994), while germline Ret mutations are associated with the three variants of the inherited cancer syndrome known as multiple endocrine neoplasia type 2: MEN2A, MEN2B and familial medullary thyroid carcinoma (FMTC) (Donis-Keller et al., 1993; Mulligan et al., 1995; Hofstra et al., 1994; Carlson et al., 1994a, b). Finally, germline Ret mutations have also been associated with an autosomal dominant form of the Hirschsprung disease (HSCR) (aganglionic megacolon) (Angrist et al., 1993; Attié et al., 1995; Romeo et al., 1994; Seri et al., 1997), a congenital disorder of the enteric nervous system characterized by a marked genetic heterogeneity (Chakravarti, 1996; Edery et al., 1996; Hofstra et al., 1996; Ivanchuk et al., 1996). Hirschsprung disease is likely the result of loss-of-function mutations in Ret and the cancer syndromes seem to be gain-of-function mutations. Interestingly, all of these lesions affect neural crest derivatives.

The requirement for Ret in the formation of the enteric nervous system has been confirmed by a transgenic mouse mutant in which targeted insertional mutagenesis was used to disrupt the Ret kinase domain. Mice homozygous for the mutant allele lack myenteric neurons from the small and large intestine, the oesophagus and stomach. These mice are also characterized by the absence of the superior cervical ganglion and either rudimentary kidneys or the absence of kidneys. The phenotype of the Ret mutant mouse bears a striking resemblance to that of the GDNF knockout (Schuchardt et al., 1994, 1996; Durbec et al., 1996b).

It is currently believed that GDNF and like ligands interact with Ret and one of a family of GPI-linked receptors (GFR $\alpha$ -1 to 4) to transduce their signal. GFR $\alpha$ -1, is a 468 amino acid protein that was initially isolated by virtue of its ability to bind GDNF in a screen of cDNA expression libraries derived from enriched populations of GDNF-responsive rat retinal photoreceptors (Jing et al., 1996) and rat midbrain dopaminergic neurons (Treanor et al., 1996). Both human and chicken GFR $\alpha$ -1 have subsequently been cloned and share 93% and 80% amino acid identity with their rat homologue, respectively (Jing et al., 1996; Buj-Bello et al., 1997). GFR $\alpha$ -1 is a glycoprotein that contains 31 conserved cysteine residues, a putative signal peptide at the amino terminus, and a stretch of 23 hydrophobic amino acids at the carboxy-terminus which mediate its binding to the cell membrane via a GPI-linkage (Treanor et al., 1996).

A BLAST search of the Genbank Expressed Sequence Tag (EST) database for sequences similar to rat GFR $\alpha$ -1 resulted in the identification of GFR $\alpha$ -2 and GFR $\alpha$ -3 (Trupp et al., 1998; Nomoto et al., 1998; Klein et al., 1997; Baloh et al., 1997; Jing et al., 1997; Worby et al., 1998). Rat GFR $\alpha$ -2 is a 464 amino acid residue polypeptide that has 48% and 33% identity with GFR $\alpha$ -1 and GFR $\alpha$ -3, respectively. Both human and chicken GFR $\alpha$ -2 have also been cloned (Sanicola et al., 1997; Suvanto et al., 1997; Buj-Bello et al., 1997).

Rat GFR $\alpha$ -3 is 397 amino acids long and has 35% identity to GFR $\alpha$ -1 protein. It appears to be the most divergent member of the GFR $\alpha$  receptor family (Jing et al., 1997; Naveilhan et al., 1998; Nomoto et al., 1998; Worby et al., 1998).

GFR $\alpha$ -4 was isolated by screening an E10 chicken brain cDNA library with a mouse GFR $\alpha$ -1 probe. The nucleotide sequence of chicken GFR $\alpha$ -4 predicts a 431 amino acid protein that has approximately 40% identity to both mouse and chicken GFR $\alpha$ -1 and GFR $\alpha$ -2, but only 27% identity with mouse GFR $\alpha$ -3 (Thompson et al., 1998). A common feature among all four receptors is the

conservation of 28 cysteine residues, indicating that they have similar three-dimensional structures. Several potential N-glycosylation sites exist in the GFR $\alpha$  but are not found at the same position in all receptors.

The physiological relevance of the GPI-linked receptors has been examined by the use of knockout mice. GFR $\alpha$ -1 deficient mice demonstrate absence of enteric neurons and agenesis of the kidney, characteristics that are reminiscent of both GDNF and Ret deficient mice (Cacalano et al., 1998; Enomoto et al., 1998). Midbrain dopaminergic and motor neurons are largely unaffected in GFR $\alpha$ -1 null mice (Cacalano et al., 1998; Enomoto et al., 1998). Minimal or no neuronal losses are also observed in a number of peripheral ganglia, including the superior cervical and nodose, which are severely affected in both Ret and GDNF deficient mice (Cacalano et al., 1998; Enomoto et al., 1998). These observations suggest that while stringent physiological pairing exists between GFR $\alpha$ -1 and GDNF in renal and enteric nervous system development, significant cross-talk between GDNF and GFR $\alpha$  receptors must occur in other neuronal populations (Cacalano et al., 1998; Enomoto et al., 1998).

Unlike GFR $\alpha$ -1 knockout mice which die soon after birth, mice lacking GFR $\alpha$ -2 are viable and fertile. However, they have dry eyes and grow poorly after weaning, presumably due to malnutrition. GFR $\alpha$ -2 deficient mice display deficits in the enteric and parasympathetic nervous system that are strikingly similar to those of NTN deficient mice. The cholinergic innervation of the lacrimal and salivary gland is almost absent and is severely reduced in the small bowel. In contrast, sympathetic innervation appears normal (Rossi et al., 1999).

At present, all evidence indicate that the GPI components are required for activation of the Ret receptor tyrosine kinase by GDNF-like ligands. However, it is still unclear whether GDNF first binds to GPI-linked components and subsequently this complex interacts with Ret (Jing et al., 1996; Treanor et al.,

1996), or whether GDNF ligands stabilize existing complexes between Ret and GPI anchored receptors at the cell membrane. In fact, both GFR $\alpha$ -1 and GFR $\alpha$ -2 have been detected in Ret immunoprecipitates from unstimulated cells co-expressing both receptors (Klein et al., 1997; Treanor et al., 1996) and soluble Ret extracellular domain is able to interact with soluble GFR $\alpha$ -1 *in vitro* (Sanicola et al., 1997). These data suggest that complexes between Ret and GPI-linked receptors are present at the cell membrane in the absence of ligand.

Details of the intracellular signaling pathway triggered by activated Ret have recently emerged. It has been shown that Ret can bind and phosphorylate the SH2 domain of adaptor proteins such as Shc, She, Grb2, and Grb10 (Borello et al., 1994; Pandey et al., 1995; Arighi et al., 1997; Lorenzo et al., 1997). Further, GDNF and NTN have been shown to stimulate PI-3 kinase activity and activate the Ras-MAPK-ERK pathway (Creedon et al., 1997; van Weering and Bos, 1997; Ohiwa et al., 1997; Worby et al., 1996). Moreover, Ret is able to activate JNK, in a pathway divergent from that of ERK, mediated by Rho/Rac related small GTPases and, particularly, by Cdc42 (Chiariello et al., 1998).

## **1.6. Research objectives**

The aims of this project were four-fold.

My first objective was to determine the mRNA distribution of the newly discovered GFR $\alpha$ -4 receptor and provide a comparative expression study between this receptor and other family members at successive stages of tissue development.

My second objective was to analyze the distribution of GDNF family receptors in very early hindbrain tissues.

My third objective was to examine whether there is a correlation between receptor expression and the change in responsiveness of peripheral neurons to GDNF and NTN during development.

My last objective was to investigate the regulation of GDNF receptor expression in developing PNS neurons.

## **Chapter 2**

### **Expression of GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNAs during development**

#### **2.1. Introduction**

GDNF (Lin et al., 1993a) and neurturin (Kotzbauer et al., 1996), together with the recently identified persephin (Milbrandt et al., 1998) and artemin (Baloh et al., 1998a), comprise a group of closely related, secreted proteins within the TGF- $\beta$  superfamily that promote the survival of various kinds of neurons of the peripheral and/or central nervous system. This protein family utilizes multi-component receptors that consist of a common signaling component, the Ret receptor tyrosine kinase, plus one of a family of GPI-linked receptors (GFR $\alpha$ -1 to 4) that confers specificity.

Northern blotting and *in situ* hybridization have revealed widespread expression of ret and GFR $\alpha$  mRNAs, with both overlapping and complementary patterns, in the body and nervous system. In the adult CNS, GFR $\alpha$ -1 mRNA is widely distributed and found in almost all areas of the mesencephalon and pons, the lateral and medial habenular, zona incerta, dorsal lateral geniculate and subgeniculate nuclei of the thalamus, the dentate gyrus and pyramidal cells of the hippocampus, the hypothalamic nuclei, the amygdala, the cerebellum, layers IV-V

of the cerebral cortex and ventral horn of spinal cord (Trupp et al., 1997, 1998; Yu et al., 1998; Glazner et al., 1998; Golden et al., 1998). Outside the CNS, GFR $\alpha$ -1 mRNA expression has been detected in the inner ear, olfactory epithelium, vomeronasal organ, developing tongue papillae and teeth, liver, uterine buds of kidney, heart, in embryonic smooth and striated muscles around the enteric nervous system in the oesophagus, gut and stomach. In the PNS, GFR $\alpha$ -1 mRNA has been detected in trigeminal (TG), superior cervical (SCG) and dorsal root (DRG) ganglia (Treanor et al., 1996; Nosrat et al., 1997; Yu et al., 1998; Sanicola et al., 1997).

GFR $\alpha$ -2 mRNA is also widely distributed, but not quite to the same extent as GFR $\alpha$ -1 mRNA. In the CNS, GFR $\alpha$ -2 mRNA is expressed in the glomerular, mitral and granule cell layers of the olfactory bulb, inferior and superior colliculi, pineal gland, lateral septum, cortex, striatum, olfactory tubercle, hippocampus, amygdala, both reticular and ventral medial thalamus, ventral tegmental area, lateral fields of the substantia nigra and ventral horn of the spinal cord (Trupp et al., 1998; Yu et al., 1998; Golden et al., 1998; Widenfalk et al., 1997). Outside the CNS, GFR $\alpha$ -2 mRNA expression has been detected in lung, spleen, kidney, heart, developing teeth, testis, intestine and kidney. Within the PNS, GFR $\alpha$ -2 mRNA has been found in dorsal root, trigeminal and superior cervical ganglia (Widenfalk et al., 1997; Jing et al., 1997; Yu et al., 1998; Suvanto et al., 1997).

Unlike GFR $\alpha$ -1 and GFR $\alpha$ -2 mRNAs, GFR $\alpha$ -3 mRNA could not be found in RNA samples extracted from total postnatal or adult rat brain. Low levels of GFR $\alpha$ -3 mRNA have, however, been detected in samples of embryonic rat brain

(Trupp et al., 1998; Nomoto et al., 1998). GFR $\alpha$ -3 distribution in neuronal tissue is very limited. By *in situ* hybridization, GFR $\alpha$ -3 mRNA has been detected in the thalamic parafascicular nucleus, medial preoptic nucleus of the hypothalamus and amygdalohippocampal anterolateral nucleus (Trupp et al., 1998). High levels of GFR $\alpha$ -3 mRNA are expressed in the PNS ganglia including the TG, SCG and DRG and lower levels are expressed in the spinal cord. GFR $\alpha$ -3 mRNA shows a widespread pattern of expression in non-neuronal tissues including embryonic, but not adult, pancreas and skeletal muscle; adult spleen, ovary, and heart; late postnatal salivary gland, liver, lung and kidney (Trupp et al., 1998; Nomoto et al., 1998; Naveilham et al., 1998; Jing et al., 1997).

GFR $\alpha$ -4 mRNA appears to be widely expressed within the chick CNS. Northern analysis shows high levels of expression in the spinal cord and lower levels of expression in the medulla oblongata, pons, cerebellum, and midbrain. *In situ* hybridization has revealed GFR $\alpha$ -4 transcripts in both the dorsal and ventral gray column of the spinal cord as well as in Purkinje cells and granule cells and neurons of the deep nuclei of the cerebellum (Thompson et al., 1998).

Finally, several studies have confirmed the broad distribution of ret transcripts in the CNS. In the CNS, ret mRNA can be found in almost all areas of mesencephalon and pons, the reticular thalamic, subthalamic, lateral habenular and medial habenular nuclei of the thalamus, the glomerular layer of the olfactory bulb, the Purkinje layer, molecular layer and deep cerebellar nuclei of the cerebellum and the ventral horn of the spinal cord (Trupp et al., 1998; Yu et al., 1998; Glazner et al., 1998; Golden et al., 1998). Outside the CNS, ret mRNA has been

detected in kidney, intestine, lung, salivary gland, thymus, spleen and lymph node. Within the PNS, ret mRNA can be found in dorsal root, trigeminal and superior cervical ganglia (Yu et al., 1998; Tsuzuki et al., 1995).

The diverse actions of GDNF and NTN in the nervous system, the severity of GDNF, GFR $\alpha$ -1, and ret knockouts and the widespread distribution of both ligands and receptors in developing and adult tissues indicate very important roles for the GDNF family of neurotrophic factors in the development and maintenance of body and brain (Moore et al., 1996; Pichel et al., 1996a, b; Cacalano et al., 1998; Schuchardt et al., 1994, 1996; Suter-Cazzolara and Unsicker, 1994; Choi-Lundberg and Bohn, 1995; Widenfalk et al., 1997; Kotzbauer et al., 1996; Milbrandt et al., 1998; Baloh et al., 1998a; Trupp et al., 1997, 1998; Jing et al., 1997; Nomoto et al., 1998; Thompson et al., 1998).

I have sought to further characterize these roles in three ways. First, I analyzed the mRNA expression of the GFR $\alpha$  and ret receptors in very early embryos especially in the hindbrain region. Second, I determined the distribution of the mRNA for the recently discovered receptor GFR $\alpha$ -4 in the body and undertook a comparative study of GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNA expression (GFR $\alpha$ -3 remains to be found in the chicken) at successive stages of organ development. Finally, I attempted to link the responsiveness of PNS neurons to GDNF and NTN with the levels of receptor mRNAs expressed by the neurons.

The method I employed to assay the levels of receptor mRNAs in early hindbrain and PNS neurons was competitive RT-PCR, while for the distribution of the receptors in different organs I used a semi-quantitative RT-PCR assay.

## **2.2. Methods**

### **I. Introduction**

RT-PCR is an extremely sensitive method of detecting mRNAs and is the method of choice when analyzing the expression of rare mRNA species. RT-PCR is at least an order of magnitude more sensitive than Northern blotting and RNase protection assays, allowing the detection of fewer target mRNA molecules from lower starting amounts of RNA extracted from smaller amounts of tissues. RT-PCR has not routinely been used in the past for quantitative or semi-quantitative assays because of the difficulty in standardizing the efficiency of individual reactions. If two identical RT-PCR reactions were set up side by side, in identical tubes with the same amount of starting mRNA template, one would expect both tubes to contain the same amount of the specific cDNA product at the end of the RT-PCR reaction. However, because of the enormous amplification of reverse transcribed mRNAs that occurs in each RT-PCR reaction during multiple rounds of thermocycling, very small differences between the efficiency of the two reactions, either in the reverse transcription step or in individual PCR cycles (due, for example, to variations in microfuge tube thickness, pipetting or thermocycler block temperature), can result in large differences in the amount of the final DNA product. When RT-PCR is being used to measure large differences in the initial amounts of target mRNAs between two samples, for example when measuring viral loads in serum samples, such small differences in reaction efficiencies are insignificant and meaningful data can be obtained, as long as the PCR reaction is stopped whilst it is in the log phase, either without any form of

calibration or with an external calibration curve. However, when RT-PCR is being used to measure small differences in the levels of rare mRNAs between different RNA samples some form of internal control or calibration is required within each RT-PCR reaction to ensure accurate results. One way to calibrate individual reactions is to include known amounts of a so-called competitor DNA species in each RT-PCR reaction. The competitor is amplified with the same primers as the target cDNA and so theoretically the ratio between competitor PCR products from individual PCR reactions can be used as a measure of the relative efficiency of amplification of the target cDNA species in each reaction. This information can be used to adjust quantitative data obtained from the RT-PCR assay to allow for differences in the efficiency of the PCR between different reactions. The most commonly used competitor species are synthetic DNAs that bear little resemblance to the cDNA being assayed apart from the primer binding sequences. Because the size and sequence of such competitors are often very different to the target cDNA species being assayed, they have very different amplification kinetics from the target cDNA and therefore the relative efficiency of competitor amplification between individual reactions does not accurately reflect the differences in the efficiency of amplification of the target cDNA between these reactions. In addition, the use of cDNA competitors does not allow differences in the efficiency of reverse transcription between individual RT-PCR reactions to be assessed. In short, the use of such competitor cDNA species does not allow accurate calibration of RT-PCR reactions and therefore does not allow accurate quantification of the levels of specific mRNAs within individual RNA samples.

The quantitative RT-PCR assay used to obtain the majority of the expression data presented in this thesis used a different specific cRNA competitor species to calibrate the RT-PCR assay for each mRNA under investigation. Since the competitor was added as cRNA to the initial RT reaction, differences in the efficiency of reverse transcription between individual reactions were taken into account. The cRNA competitor was transcribed *in vitro* from a cDNA competitor construct that was made from a cDNA clone of each of the mRNAs being assayed. The cDNA competitor construct was identical to the cDNA of the mRNA under assay except that it was 3-4% longer than the native cDNA between the PCR assay primer binding sites. The extra nucleotide base pairs were simply added to the cDNA clone by cutting it with a restriction enzyme that produces cohesive ends with a 5' overhang, filling the cohesive ends with Klenow polymerase to make blunt ends and religating the blunt ends. Because the competitor cRNA contains the same primer bindings sites and is almost identical in sequence to the target mRNA, it is reverse transcribed and amplified with virtually identical kinetics to the target mRNA. Therefore, if the initial amount of competitor cRNA added to each reaction is known, the ratio between the RT-PCR products of the competitor cRNA and the target mRNA can be used to calculate an accurate value for the amount of target mRNA initially present.

The products of the RT-PCR assay were small, in the region of 100 base pairs, which greatly increased the efficiency of the reverse transcription and PCR steps. The small size of the products, however, has two drawbacks. First, because of the rapid annealing of short DNA strands, it encourages the formation of heteroduplexes between competitor and target DNA strands as the concentrations

of PCR products build up (observed as higher molecular weight bands on autoradiographs). Heteroduplex formation reduces the accuracy of the RT-PCR assay and so must be avoided by limiting the number of PCR cycles performed. Second, the small size of the RT-PCR products means that they cannot be visualized by ethidium bromide staining when the PCR reaction is still in the log phase. To solve this problem, and also to increase the sensitivity of the assay, the PCR reaction was performed with  $^{32}\text{P}$  end-labelled primers and the products were visualized by autoradiography. The use of end-labelled primers, as opposed to incorporation of radiolabelled nucleotides, greatly increases the percent labelling of PCR products when the PCR products are small. In addition, it prevents the high background, resulting from mispriming by short cDNA fragments that is often seen on autoradiographs following radioactive incorporation. The RT-PCR products of the target mRNA and competitor cRNA were separated by electrophoresis on 8% polyacrylamide gels and, following autoradiography, the amount of each product was quantified by densitometry.

The response of X-ray film to  $^{32}\text{P}$  is only linear over a narrow range. Therefore, to obtain accurate results from the assay it was important that the intensity of the RT-PCR products of the target mRNA and competitor cRNA did not differ by more than three-fold. Initial RNA/competitor titrations were performed to establish the correct level of competitor for each RNA sample. In most cases reverse transcription reactions were performed containing the appropriate amount of competitor cRNAs for several different mRNA species, including the mRNA for the housekeeping protein L27. Small aliquots of the cDNA products from each reaction were subsequently amplified in separate

reactions with primers specific to each cDNA being assayed. This approach not only saved time, but also reduced the amount of valuable total RNA samples needed for analysis of the expression of several mRNAs and increased the accuracy of data when the level of receptor mRNA expression was calculated relative to L27 mRNA.

The competitive RT-PCR technique is extremely accurate, but is relatively time consuming to perform. When analyzing the expression of GDNF family receptors in a range of organs and tissues throughout development (this chapter), I was not interested in the fine details of developmental expression of each mRNA, in each organ, at each time point. Rather, I was looking for broad trends in expression patterns, both between different tissues and organs and also in a developmental context. For this reason, mRNA levels were not assayed using competitive RT-PCR, but by using a semi-quantitative RT-PCR assay that did not attempt to calibrate individual RT-PCR reactions. In short, RNA samples were appropriately diluted so that they contained the same approximate concentration of total RNA (following pilot RT-PCR reactions with L27 primers to assess approximate RNA concentrations). An equal amount of each total RNA sample was reverse transcribed in a separate reaction. Aliquots of cDNA from each completed RT-reaction were then amplified in separate reactions with  $^{32}\text{P}$  end-labelled primers specific for each receptor cDNA, in addition to L27 cDNA. RT-PCR products were then analyzed by electrophoresis on polyacrylamide gels, followed by autoradiography. In order to obtain as accurate data as possible on the relative expression of each receptor mRNA, in each tissue, at each

developmental stage, it was necessary to ensure that: (i) the PCR reaction remained in the mid-log phase (determined by analyzing RT-PCR products from individual reactions after different numbers of cycles), (ii) the volumes of RNA and cDNAs pipetted at each stage were accurate, (iii) exactly the same volume of RT-PCR products from each reaction were analyzed by electrophoresis.

An account of the techniques used to study the expression of the GDNF family receptors is given below. First, tissues from chicken embryos were isolated. Then, RNA isolation was performed, and finally RT-PCR was carried out. The detailed methodology that led to the construction of the competitor RNAs is also provided.

## **II. Protocols**

### **Dissection of Embryonic Tissue**

White Leghorn chicken eggs were incubated at 38°C in a forced-draft incubator. The various organs, rhombomeres, and PNS ganglia were dissected from chicken embryos at the required age (More details are given in the methods section of chapter 3). For the studies using purified neurons, dissected ganglia were incubated in 0.1% trypsin/calcium-and magnesium-free Hank's balanced salt solution (HBSS) for 10, 15, 20, 22, and 25 minutes at 37°C for E8, E10, E12, E14, and E16 ganglia, respectively. The trypsinized tissue was washed twice in

Hank's F14 medium containing 10% heat inactivated horse serum (HIHS) and once in HBSS before being mechanically dissociated by gentle trituration, in ~1ml of HBSS, using a fine-polished Pasteur pipette.

Non-neuronal cells were removed by differential sedimentation (Davies, 1988c). Dissected tissues and >95% pure neurons were stored at -80°C until required. The dissections were performed under standard sterile conditions in a laminar flow hood under a stereomicroscope. All dissections were done in L15 media.

#### **RNA isolation and purification**

The acidic guanidinium thiocyanate RNA extraction method described by Chomczynski and Sacchi, (1987) provides a pure preparation of undegraded RNA in high yields. Cells were lysed in 500µl of solution D {50ml of stock solution (250g guanidinium thiocyanate; 293ml DEPC-treated H<sub>2</sub>O; 17.6ml 0.75M sodium citrate pH7; 24.6ml 10% N-laryl sarcosyl) plus 360µl 2-mercaptoethanol} by passing the neuronal suspension through a 25 gauge needle fitted to a 1ml syringe. The following solutions were added sequentially: 2µl of 5mg/ml *E. coli* tRNA (SIGMA); 50µl of 2M sodium acetate pH4.4; 500µl of water-saturated acidic phenol (SIGMA); 150µl of 25:1 chloroform: isoamyl alcohol (SIGMA). The mixture was shaken vigorously, incubated on ice for 5-15 minutes and centrifuged at 13,000rpm for 5-30 minutes. The upper aqueous phase was transferred to a fresh microfuge tube and RNA was precipitated by the addition of two volumes of ethanol, followed by incubation at -20°C overnight. RNA was recovered from the

ethanolic mixture by centrifuging at 13,000rpm for 30 minutes. The resulting RNA pellet was washed thoroughly with 70% ethanol before being allowed to air dry.

After resuspending the RNA pellet in 50 $\mu$ l of 10mM Tris pH7.5, 6mM MgCl<sub>2</sub> (Promega), 20mM VRC (vanadyl-ribonucleoside complex, RNAase inhibitor, SIGMA), 5 $\mu$ l of 7,500units/ml RNAase-free DNAase I (Pharmacia), the RNA was incubated at 37°C for 90-120 minutes. The RNA was further purified using the RNaid kit from BIO 101. In brief, 180 $\mu$ l (~3 volumes) of 3M NaClO<sub>3</sub> were added to the RNA followed by 10-30 $\mu$ l of RNA binding (RNAID) matrix (the exact volume was dependent on the amount of RNA as judged from the starting tissue). After thorough mixing, the slurry was incubated at room temperature for 15-30 minutes with periodic mixing to allow the RNA to adsorb to the matrix. The matrix was sedimented by brief centrifugation and was washed with 700 $\mu$ l of the RNaid wash solution. This wash procedure was repeated one more time, followed by a final sedimentation of the matrix and complete removal of the wash solution. RNA was eluted from the matrix material by resuspending in 40-100 $\mu$ l of water (preferably DEPC-treated water), followed by incubating at 60°C for 2-3 minutes. Following centrifugation for 1-2 minutes, the supernatant containing the RNA was transferred to a fresh tube and stored at -80°C until required.

## **Construction of RNA competitor species**

### **General plan**

The first step in the construction of the competitor RNA was the isolation of a fragment of the required cDNA by RT-PCR. For this purpose, primers 600-800bp apart were used to amplify first strand cDNA from chicken brain. The PCR products were then run out next to a DNA ladder on an agarose gel and the band of interest excised and purified. The cDNA was then ligated into a transcription plasmid vector and the resulting construct was used to transform competent cells. The transformed cells were plated out onto agar and left to grow until visible colonies appeared. To determine which of the transformed colonies bore the desired recombinant plasmid, DNA was purified from several colonies using a mini-prep kit. Aliquots of plasmid DNA were digested with different restriction enzymes, and constructs containing the correct insert were identified by electrophoresis of digested DNA. Sufficient quantities of the correct plasmid DNA, to allow construction of the competitor cDNA, were purified by a miniprep plasmid purification kit.

The next step in the construction of the competitor RNA was the modification of the insert cDNA so that it would be slightly different in size from the native template. To do this, plasmid DNA was digested with a restriction enzyme that produces 4bp 5' overhangs. The overhangs were filled-in with Klenow polymerase and the blunt ends were re-ligated. The result was a control template 4bp longer than the native template. Before transformation into competent bacteria, the recombinant plasmid was digested with the same

restriction enzyme used to cut it before the fill-in reaction. Plasmid containing a filled-in site will not cut, whereas unfilled plasmid will. Since transformation efficiency of linear DNA is a lot smaller than that of a circular DNA, very few plasmid DNA molecules which were not filled-in with the Klenow polymerase find their way into competent cells. Following transformation, the cells were grown on agar plates until colonies appear. To confirm that the fill-in reaction had worked and find the orientation of the receptor cDNA fragment in the vector, several colonies were picked, grown in LB broth and the plasmid DNA was purified using a kit. Aliquots of plasmid DNA were digested with different enzymes and the digested DNA was resolved by electrophoresis. The digests were compared to a DNA ladder and recombinant plasmids containing the proper insert were determined. Since it was possible that 1,2 or 3 rather than 4bp were filled-in, PCR was performed against an unfilled plasmid DNA and products were resolved by acrylamide gel electrophoresis. To further confirm the quality of the control cDNA, an aliquot was sent to an in-house sequencing service for sequencing.

The last step in the construction of the competitor RNA was the transcription of the filled cDNA clone. In order to produce run-off transcripts of defined length, the plasmid DNA was linearized with an appropriate restriction enzyme that cuts at the 3' end of the insert. Special care was taken to avoid the use of an enzyme, which produced 3' protruding ends. Extraneous transcripts have been reported to appear in addition to the expected transcript when such template is transcribed (Schenborn and Mierendorf, 1985). The extraneous transcripts can contain sequences complementary to the expected transcript as well as sequences corresponding to the vector DNA. Moreover, the restriction

enzyme chosen to cut the competitor, had its cleavage site as far as possible from the fill-in site. In this way, multiple points along the 3' end of the RNA transcript could serve as templates for random hexamers to prime, improving the efficiency of the reverse transcription reaction. Following digestion, the plasmid was run out on an agarose gel and the band corresponding to the linearized plasmid was excised. Linearized DNA was then purified and *in vitro* transcribed. Plasmid DNA was removed from the RNA by DNase treatment. An aliquot of purified RNA was checked for integrity and size on an agarose gel and its concentration was spectrophotometrically determined. The remaining RNA was diluted, ethanol precipitated and stored until required.

#### **A, Cloning the cDNA competitor templates by RT-PCR**

Total RNA from E13 chick brain (known to express high levels of the GDNF family receptors) was extracted by the Chomczynski and Sacchi (1987) method. 5 $\mu$ l of RNA was reverse transcribed in 50 $\mu$ l reaction mixture containing 10 $\mu$ l of 5xRT Superscript buffer (GIBCO BRL), 5 $\mu$ l of 5mM dNTPs (MBI Fermentas), 5 $\mu$ l of 100 $\mu$ M random hexanucleotides (Pharmacia) and 5 $\mu$ l of 0.1M DTT (GIBCO BRL). The sample was heated for 3 minutes at 90°C to denature any secondary structure within the RNAs. Following this, 1-2 $\mu$ l of Superscript reverse transcriptase was added to the mixture, which was then incubated at 37°C for 1 hour. The reaction was stopped, and the RNA template degraded, by heating the sample for 6 minutes at 95°C.

PCR primers for isolating GDNF family receptor cDNAs were designed based on published sequence information. Care was taken to choose primers that lay in regions of the receptor sequences that contained little homology with other GDNF receptor family members (see Table 2.1, 2.2 and 2.3). For each receptor, a region of about 600-800bp was amplified. The PCR reaction mixture consisted of:

32.75 $\mu$ l H<sub>2</sub>O

5 $\mu$ l RT product

5 $\mu$ l 10x buffer (Promega)

3 $\mu$ l 25mM MgCl<sub>2</sub> (Promega)

2 $\mu$ l 5mM dNTPs

1+1 $\mu$ l 50pmoles primers

0.25 $\mu$ l of 3u/ $\mu$ l *Taq* DNA polymerase (Promega)

After mixing, the reaction was overlaid with ~40 $\mu$ l of mineral oil (SIGMA). The tube was transferred to the heating block of a PCR machine and amplification was carried out for 42 cycles using the following parameters: denaturation at 95°C for 60 seconds, annealing at 50-65°C (exact annealing temperature was dependent on primer sequence) for 60 seconds and DNA synthesis at 72°C for 90 seconds. The PCR reaction was completed with a 10 minute extension at 72°C. The PCR products were run out on a 1% agarose gel (GIBCO BRL) and the band of interest was excised with a scalpel. The DNA was purified from agarose with the Geneclean®II kit. Purified DNA was recovered in 20 $\mu$ l of water. The purified DNA was ligated into the pGEM-T vector (Promega) according to manufacturer's recommendations. The ligation mixture was used directly to transform highly competent *E.coli* XL1 cells (Maniatis, 1989).

**Table 2.1.** Forward isolation primer sequences for the GDNF family receptors.

| <b>Receptor</b> | <b>Forward isolation primers</b> |
|-----------------|----------------------------------|
| GFR $\alpha$ -1 | 5'-GCCACATATCCTCGGAGAATTTC-3'    |
| GFR $\alpha$ -2 | 5'-CCAGAACGACCTTGTGGATCAG-3'     |
| GFR $\alpha$ -4 | 5'-TCATGCAGTGTATCGCAG-3'         |
| Ret             | 5'-GAGAACGTCTACATTGACC-3'        |

**Table 2.2.** Reverse isolation primer sequences for the GDNF family receptors.

| <b>Receptor</b> | <b>Reverse isolation primers</b> |
|-----------------|----------------------------------|
| GFR $\alpha$ -1 | 5'-TAGAATGGCTTCTCAGCAGG-3'       |
| GFR $\alpha$ -2 | 5'-GACAGCCTCACACCGTTGAAT-3'      |
| GFR $\alpha$ -4 | 5'-GCATAACGCGACCTACAGACG-3'      |
| Ret             | 5'-GCATCATACACAGTTAGCG-3'        |

**Table 2.3.** Sizes of the GDNF family receptor cDNAs cloned by RT-PCR.

| <b>Receptor</b> | <b>Size of receptor</b> |
|-----------------|-------------------------|
|                 | <b>cDNA cloned bp</b>   |
| GFR $\alpha$ -1 | 687                     |
| GFR $\alpha$ -2 | 627                     |
| GFR $\alpha$ -4 | 622                     |
| Ret             | 785                     |

## **B, Transformation of competent cells**

Competent cells (*E. coli* XL1 Blue MRF<sup>r</sup>) were stored at -80°C. They were removed from the freezer prior to transformation and defrosted on ice. 5-10µl (<1/10 of competent cell volume) of the ligation mixture was added to 100µl of competent cells and mixed by gently flicking the tube. Following incubation on ice for 40-60 minutes, the cells were transferred to a water bath at 42°C for 90 seconds (heat shock). The cells were returned to ice for 5 minutes after which 800µl of LB-broth was added and the whole suspension was transferred to a 7ml bijou. The cells were incubated at 37°C, for 40-60 minutes, before being plated onto LB agar (GIBCO BRL) containing 100mg/ml ampicillin (SIGMA).

## **C, Analysis of transformants to verify the presence of insert cDNA**

After 16-20 hours at 37°C, ampicillin resistant colonies were picked and used to inoculate 3ml of LB medium containing ampicillin (100 mg/ml). Following 10-16 hours incubation at 37°C, plasmid DNA was extracted from the resulting bacterial suspension using a QIAprep Spin Miniprep kit. The DNA was recovered in 60µl of H<sub>2</sub>O. To confirm the presence of the insert cDNA, I performed a restriction analysis of the plasmid DNA. 10µl aliquots of plasmid DNA were digested with different enzymes in reaction volumes of 20µl. The mixtures were incubated at the required temperature for at least an hour. DNA fragments

resulting from the restriction enzyme digest were analyzed by electrophoresis in a 1% agarose gel (NA-grade agarose, GIBCO BRL).

## **D, Modification of cloned DNA to make competitor cDNA**

### **I. Purification of plasmid DNA**

50ml LB media containing 100mg/ml ampicillin were inoculated with 5µl of a bacterial suspension that contained vector DNA with an appropriately sized insert. Following overnight incubation at 37°C, the QIAprep Spin Miniprep kit was used to purify plasmid DNA from 3ml of the bacterial suspension. Plasmid DNA was recovered in 60µl of H<sub>2</sub>O.

### **II. Restriction digestion to produce 5' protruding ends**

40µl of each plasmid DNA (~10µg) was digested overnight with an appropriate restriction enzyme in the manufacturers recommended buffer at the required temperature. The restriction enzyme chosen cut the cloned cDNA at a single internal site to leave 4bp 5' overhangs (see Table 2.4).

**Table 2.4.** Restriction sites in the GDNF receptor family filled to make the competitors.

| Receptor | Restriction site filled-in |
|----------|----------------------------|
| GFRα-1   | Bst98 I                    |
| GFRα-2   | BamH I                     |
| GFRα-4   | Kas I                      |
| Ret      | Xho I                      |

### **III. Fill-in of DNA ends with Klenow polymerase**

Following digestion with restriction enzymes, plasmid DNA was purified using the Geneclean®II kit. Klenow DNA polymerase was used to fill-in the recessed 3' ends (to produce blunt ends) in the following way. A microfuge tube containing 11μl H<sub>2</sub>O, 3μl of 10x reaction buffer (NEB), 5μl of 5mM dNTPs (MBI Fermentas), 10μl (0.2-4μg) plasmid was heated for 5 minutes at 68°C before being placed on ice for 1 minute (this step denatures double-stranded structures that may have formed from protruding single-stranded termini). 1-5 units of Klenow polymerase (NEB or Promega) per microgram of DNA were then added and the reaction was allowed to proceed for 15 minutes at 25°C. After this period, the reaction was stopped by heating at 75°C for 10 minutes.

#### **IV. Purification of filled cDNA**

Following the fill-in reaction, DNA was purified away from dNTPs and Klenow enzyme using the Geneclean®II kit. The DNA was recovered in 17µl H<sub>2</sub>O.

#### **V. Ligation of newly generated blunt-ends**

The following reagents were added to a microfuge tube and mixed thoroughly:

2µl 10x T4 ligase buffer (NEB, containing ATP)

16µl plasmid DNA

2µl of 400u/µl T4 DNA ligase (NEB)

The ligation reaction was performed overnight at a temperature gradient starting at 12°C and finishing at 4°C.

#### **VI. Purification**

Following ligation DNA ligase and ATP were removed from plasmid DNA with the Geneclean®II kit.

#### **VII. Restriction enzyme digestion to reduce false positive clones**

In order to reduce the number of false positive colonies (i.e. colonies which contain plasmid DNA in which the 5' overhangs have not been filled with Klenow), plasmid DNA was digested prior to transformation with the same restriction enzyme that was used to generate the 5' overhangs for the fill-in (for 2 hours, followed by heat inactivation).

### **VIII. Transformation of filled, ligated cDNAs**

7 $\mu$ l of digested plasmid were used for the transformation of 100 $\mu$ l of competent cells as detailed above.

### **IX. Restriction enzyme analysis of transformants**

After 16-20 hours at 37°C, ampicillin resistant colonies were picked and used to inoculate 3ml of LB medium containing ampicillin (100 mg/ml). These cultures were incubated for 10-16 hours at 37°C to produce enough bacteria for miniprep plasmid DNA extraction. A QIAprep Spin Miniprep kit was then used to purify the plasmid DNA. The DNA pellet was recovered in 60 $\mu$ l of H<sub>2</sub>O. To confirm that the fill-in reaction had worked, and to determine the orientation of the receptor cDNA fragment in the vector, a series of restriction enzyme digests were performed on the miniprep DNA. 10 $\mu$ l aliquots of plasmid DNA were digested with different enzymes in reaction volumes of 20 $\mu$ l. The mixtures were incubated at the appropriate temperature for at least an hour. DNA fragments resulting from the restriction digest were analyzed by electrophoresis on 1.5-2% agarose gels (NA-grade agarose, GIBCO BRL).

### **X. PCR analysis of transformants**

Since restriction digestion could not determine whether all 4 bases were filled-in by Klenow, PCR was used to compare the different plasmids with an unfilled plasmid. PCR reactions were carried out in a volume of 50 $\mu$ l. For each PCR reaction, 45 $\mu$ l of master mix solution was added to the 5 $\mu$ l of 0.1 $\mu$ g/ml plasmid DNA. The composition of the master mix was (n:= number of tubes): n x

36.33 $\mu$ l of H<sub>2</sub>O, n x 5 $\mu$ l of 10xPCR buffer supplemented with MgCl<sub>2</sub> (Helena Biosciences), n x 1 $\mu$ l of 5mM dNTP (MBI Fermentas), n x 0.17 $\mu$ l of 5u/ $\mu$ l Taq DNA polymerase (Helena Biosciences) and n x 2.5 $\mu$ l labelled primers. After mixing, the reaction was overlaid with 40 $\mu$ l of mineral oil. The tubes were transferred to the heating block of a PCR machine and amplification was carried out for 22 cycles using the following parameters: denaturation at 95°C for 60 seconds, annealing at 50-65°C (exact annealing temperature was dependent on primer sequence) for 60 seconds and DNA synthesis at 68°C for 60 seconds. The PCR reaction was completed with a 10 minute extension at 68°C. The PCR products were run out on a 8% non-denaturing polyacrylamide gel and the preferred plasmid detected. To further confirm the sequence quality of the control cDNA, a sample was sent out for sequencing.

## **XI. Restriction enzyme digestion to produce cDNA of defined length**

In order to produce run-off RNA transcripts of a defined length from the cloned cDNA, plasmid DNA (~10 $\mu$ g) was linearized at the 3' end of the receptor cDNA insert by overnight digestion with an appropriate restriction enzyme. Special care was taken to choose an enzyme that produces blunt-end and cuts as far away as possible from the fill-in site.

## **XII. Purification of linearized plasmid**

Linearized plasmid was purified away from any tracers of uncut plasmid in the following manner. The digested plasmid was run on a 1% low melting agarose

gel and the band corresponding to linearized plasmid DNA was cut out with a razor blade. DNA was recovered from the gel slice with the Geneclean®II kit.

#### **E, *In vitro* transcription**

##### **Synthesis of competitor RNA**

Competitor RNA transcripts were transcribed in 2x50µl reaction mixtures containing the following:

3µl H<sub>2</sub>O

10µl 5x transcription buffer (Promega)

4µl 100mM dTT (Promega)

4µl 10mM ATP (Promega)

4µl 10mM GTP (Promega)

4µl 10mM UTP (Promega)

5µl 32u/µl RNA guard (Pharmacia)

10µl plasmid (1-5µg)

1µl of 30-50u T7/SP6 RNA polymerase (Promega)

It is important that all materials are at room temperature and are added in the sequence shown above. The reaction components were thoroughly mixed before and after the addition of the RNA polymerase. Following incubation at 37°C for 1 hour, a further 1µl of Sp6/T7 RNA polymerase was added to each reaction, and the reactions were allowed to proceed at 37°C for another 60 minutes. At the end

of this period, the two reactions were pooled in one tube. At this point, 5 $\mu$ l VRC, 20 $\mu$ l of 25mM MgCl<sub>2</sub> and 5 $\mu$ l of 7,500u/ml DNAase I were added and the plasmid DNA was degraded by incubation at 37°C for 90-120 minutes. The RNA transcripts were purified with the RNAid kit as described above with the exception that the washing step was repeated three rather than two times, so that traces of VRC would be totally removed. (VRC are a potent inhibitor of reverse transcriptase and can also interfere with the measurement of RNA concentration). Purified RNA was recovered in 70 $\mu$ l of DEPC-treated H<sub>2</sub>O. A 10 $\mu$ l aliquot of the RNA was used to determine the RNA concentration spectrophotometrically. Another 10 $\mu$ l was run out on a 1% DEPC-treated agarose gel to check the size and integrity of the RNA product. The remaining RNA was diluted and precipitated by the addition of 0.1 volumes of 3M sodium acetate, 3 volumes of ethanol and *E. coli* tRNA, such that the final ethanolic mixture contained 1ng of transcript RNA and 20ng of *E.coli* tRNA per microlitre. The ethanol precipitated transcripts were stored at -20°C until required.

### **Labeling primers**

Primers were labelled with <sup>32</sup>P on their 5' ends in a 30 $\mu$ l reaction containing the following: 6 $\mu$ l H<sub>2</sub>O, 3 $\mu$ l of 10x T4 PNK forward buffer A (MBI Fermentas), 3 $\mu$ l of 1 $\mu$ g/ $\mu$ l primer, 15 $\mu$ l  $\gamma$ <sup>32</sup>P ATP (Amersham) and 3 $\mu$ l of 10u/ $\mu$ l T4 polynucleotide kinase (MBI Fermentas). Following thorough mixing, the

labelling reaction was carried out at 37°C for 60-120 minutes. Primers were purified using the Mermaid oligonucleotide purification kit (BIO 101). Labelled oligos were eluted from the mermaid matrix in 400µl of H<sub>2</sub>O by incubating at 65°C for 2-5 minutes. Following brief (2 minutes) centrifugation at 13,000 rpm, the primers were transferred into fresh microfuge tubes and stored at -20°C until required.

### **RT-PCR assay**

#### **A, Reverse transcription**

Reverse transcription reactions were carried out in a volume of 30µl. Each reaction mixture contained the following components: 6µl of 5x reverse transcription buffer, 3µl of 5mM dNTPs, 3µl of 100µM random hexanucleotides, 3µl of 0.1M DTT, 1µl of each of the receptor competitor RNAs at an appropriate concentration (to compare the relative levels of the receptors in different samples, the cRNA competitor template for ubiquitous, constitutively expressed L27 ribosomal protein was also included in the reverse transcription reaction), 0.1-10µl of total RNA, water up to a volume of 29µl. The reverse transcription mix was made as a master mix for each batch of RT reactions being carried out. The master mix contained all components except total RNA and Superscript Reverse Transcriptase. The use of a master mix ensured uniformity of conditions in all reactions carried out using that mix. A negative control, where all different RNAs

were added together but no reverse transcriptase, was used to determine the purity of the RNA samples (i.e. no DNA contamination). The tubes were transferred to a PCR block and heated for 2-3 minutes at 90°C, followed by 45-90 minutes at 37°C and concluding with 6 minutes at 95°C. Reverse transcriptase was added to each reaction, except the no RT control at the beginning of the second step.

### **B, Polymerase chain reaction (PCR) assay**

PCR reactions were carried out in a volume of 50 $\mu$ l. For each PCR reaction 45 $\mu$ l of master mix solution was added to the 5 $\mu$ l of cDNAs. The composition of the master mix was (n:= number of tubes): n x 36.33 $\mu$ l of H<sub>2</sub>O, n x 5 $\mu$ l of 10xPCR buffer supplemented with MgCl<sub>2</sub> (Helena Biosciences), n x 1 $\mu$ l of 5mM dNTP (MBI Fermentas), n x 0.17 $\mu$ l of 5 $\mu$ / $\mu$ l Taq DNA polymerase (Helena Biosciences) and n x 2.5 $\mu$ l labelled primers (see Tables 2.5, 2.6, and 2.7). After mixing, each reaction was overlaid with ~40 $\mu$ l of mineral oil. The denaturation, annealing and elongation conditions for each template are shown in Table 2.8. Each step of the cycle lasted 1 minute. The number of cycles were dependent on the nature and concentration of the cDNA template to be amplified and largely had to be determined empirically.

**Table 2.5.** Forward assay primer sequences for the GDNF family receptors.

| <b>Receptor</b> | <b>Forward assay primers</b> |
|-----------------|------------------------------|
| GFR $\alpha$ -1 | 5'-ACCTGAGAAGGAGGATGG-3'     |
| GFR $\alpha$ -2 | 5'-CCTTGATCAGAAGGC-3'        |
| GFR $\alpha$ -4 | 5'-CGTGGACGAGATGTGCCAACG-3'  |
| Ret             | 5'-CACTTCATCTGTGCCAG-3'      |
| L27             | 5'-GGCTGTCATCGTGAAGAACATC-3' |

**Table 2.6.** Reverse assay primer sequences for the GDNF family receptors.

| <b>Receptor</b> | <b>Reverse assay primers</b> |
|-----------------|------------------------------|
| GFR $\alpha$ -1 | 5'-TGACATCCTTGATAATCT-3'     |
| GFR $\alpha$ -2 | 5'-AGCTTCAGCAGCACAAATGG-3'   |
| GFR $\alpha$ -4 | 5'-GCACGCGGTCGAAGAACTTGC-3'  |
| Ret             | 5'-AGTCTTCTCTATCTAGGC-3'     |
| L27             | 5'-CTTCGCTATCTTCTTCTGCC-3'   |

**Table 2.7.** Size of RT-PCR products.

| Receptor        | Native PCR<br>product bp | PCR product of<br>control template bp |
|-----------------|--------------------------|---------------------------------------|
| GFR $\alpha$ -1 | 99                       | 103                                   |
| GFR $\alpha$ -2 | 82                       | 86                                    |
| GFR $\alpha$ -4 | 129                      | 133                                   |
| Ret             | 122                      | 126                                   |
| L27             | 127                      | 131                                   |

**Table 2.8.** PCR cycling conditions.

| Receptor        | Denaturation (°C) | Annealing (°C) | Elongation (°C) |
|-----------------|-------------------|----------------|-----------------|
| GFR $\alpha$ -1 | 95                | 50             | 68              |
| GFR $\alpha$ -2 | 95                | 50             | 68              |
| GFR $\alpha$ -4 | 95                | 50             | 68              |
| Ret             | 95                | 50             | 68              |
| L27             | 95                | 50             | 68              |

### **C, Electrophoresis of PCR products**

The PCR products of the control and native cDNA templates were resolved on 8% non-denaturing polyacrylamide gels. The gels were run at constant 430V. Following electrophoresis, the gel was transferred to Whatman 3MM paper and dried for ~15 minutes at 80°C on a slab gel dryer. After the gel was thoroughly dried, it was autoradiographed. The autoradiographs were scanned with a densitometer and the intensity of the respective signals ascertained using ImageQuant software.

### **D, Semi-quantitative RT-PCR assay**

Semi-quantitative RT-PCR was used to determine the expression pattern of GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNAs in various embryonic tissues. The method employed is very similar to the one reported earlier for the competitive PCR. However, it differs in two points. First, the RT reaction did not include competitor RNAs. Second, to ascertain the reproducibility of the results, each PCR was repeated twice for two different numbers of cycles. In general, these were 22-24 cycles for GFR $\alpha$ -1, 21-23 cycles for GFR $\alpha$ -2, 25-27 cycles for GFR $\alpha$ -4, 25-27 cycles for ret, and 19 cycles for L27. The cycling conditions were optimal for the amplification of each transcript so that the rate of reaction did not plateau.

## **2.3. Results**

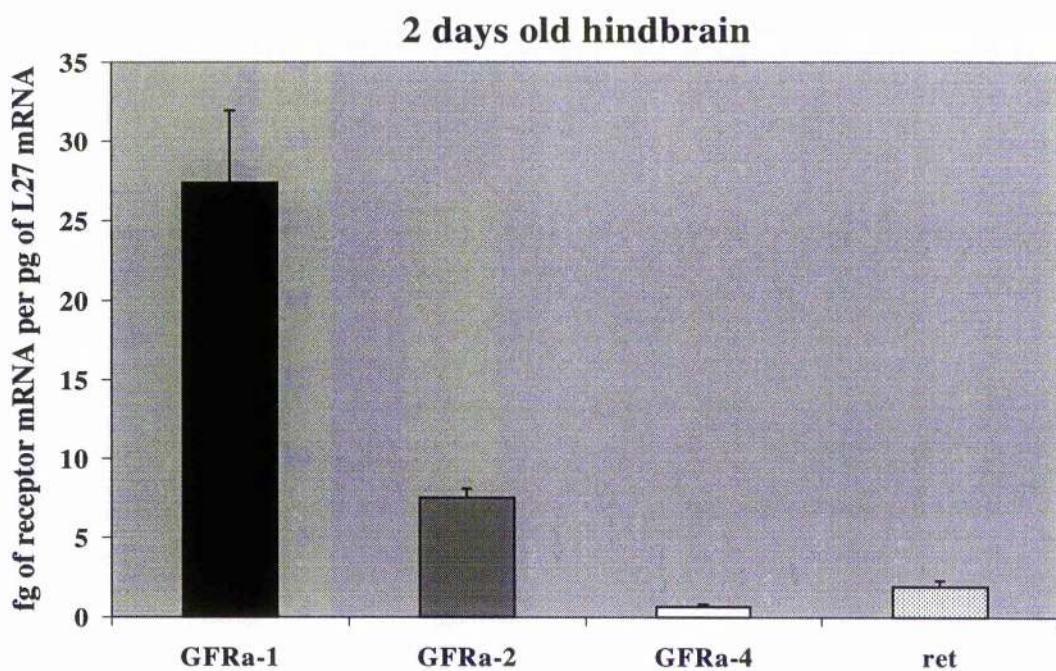
### **I. Early expression of GDNF family receptors in chicken rhombomeres**

Using the very sensitive, quantitative method of competitive RT-PCR, I performed an analysis on hindbrains from E2, E3, and E4 chicken embryos. At E2, all four receptors could be detected in total hindbrain (see Figure 2.1). Moderate GFR $\alpha$ -1, low GFR $\alpha$ -2 and weak ret and GFR $\alpha$ -4 mRNA expression levels were observed.

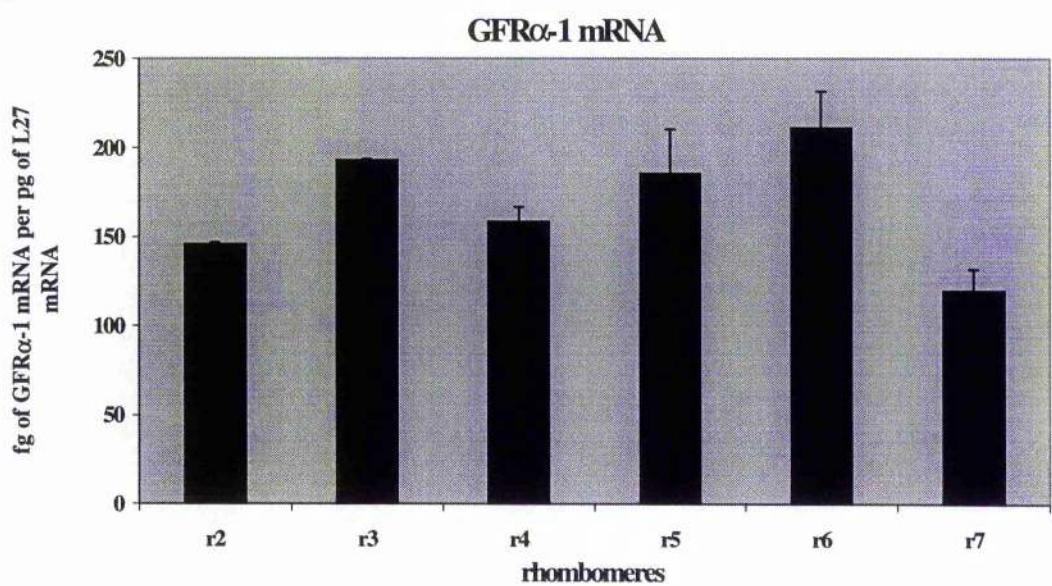
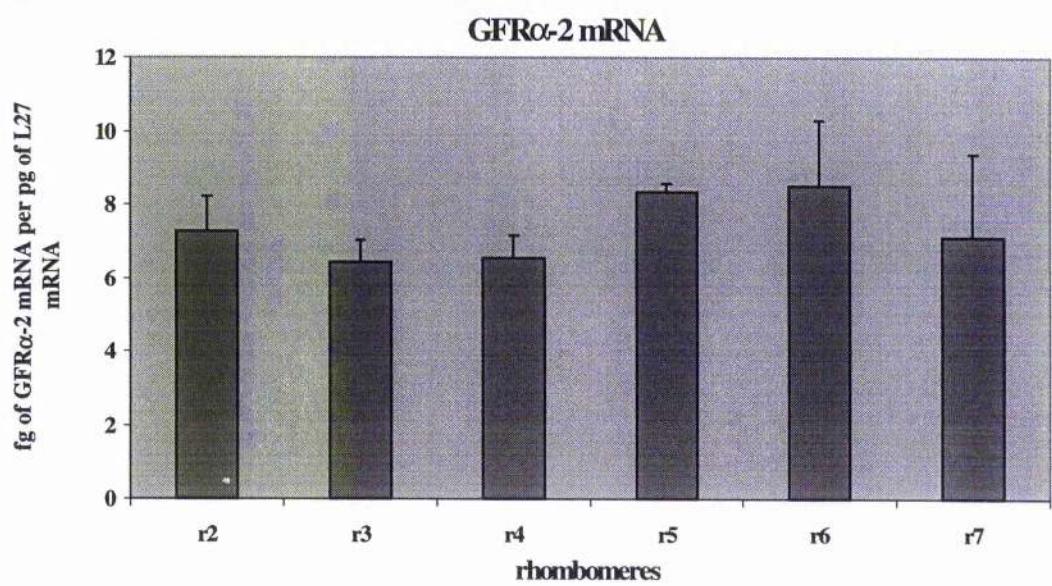
24 hours later (day 3), when the rhombomeres have increased in size and their dissection is possible, receptor expression is widespread. All rhombomeres examined appear to express receptor components with approximately the same intensity (see Figures 2.2A, B, C, D). Interestingly, in a similar study by Pachnis and colleagues, ret expression could only be localized in rhombomere 4 of the mouse embryo at this stage of development (Pachnis et al., 1993). Figure 2.2D shows that rhombomere 4 is actually the one with the lowest ret expression. Nevertheless, the trend changes at day 4, with r4 being the rhombomere with the highest expression (Figure 2.3D).

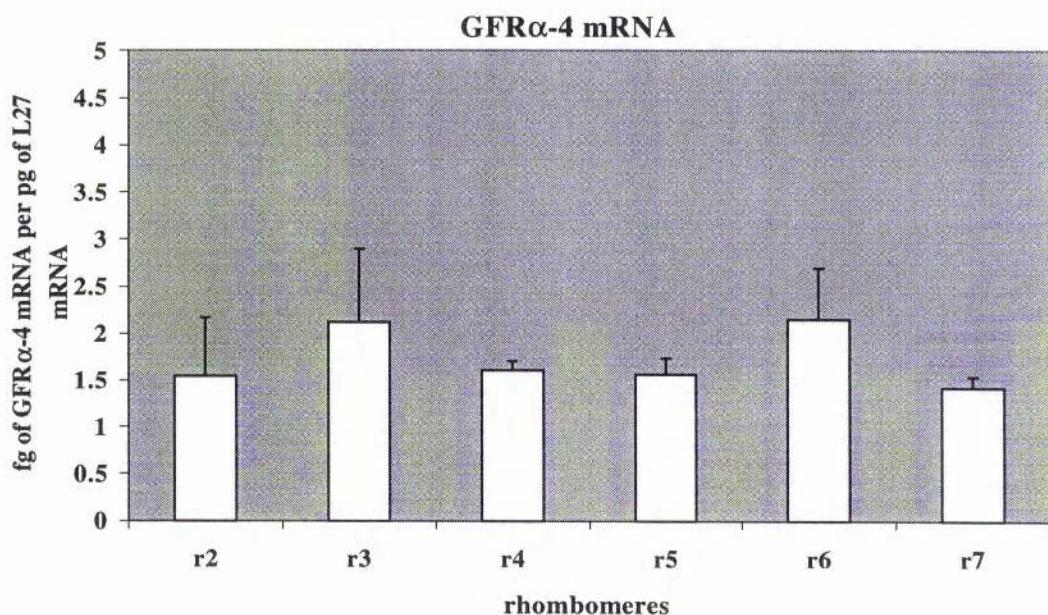
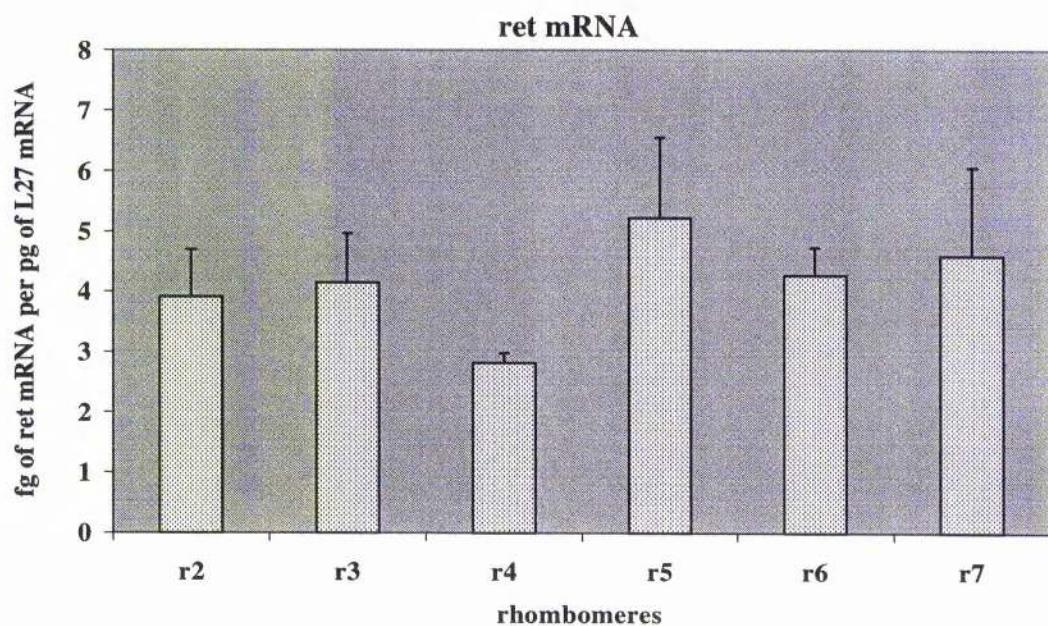
In addition to the widespread, uniform expression of the receptors, there was a 6.5-fold increase in the levels of GFR $\alpha$ -1 mRNA between days 2 and 3, and a more moderate 2.5-fold increase in the levels of GFR $\alpha$ -4 and ret mRNAs. GFR $\alpha$ -2 mRNA expression remained at the same low level as day 2.

Between day 3 and 4 there was a small increase in GFR $\alpha$ -1 and ret mRNA levels in the hindbrain whereas, GFR $\alpha$ -2 and GFR $\alpha$ -4 mRNA levels remained the same (see Figures 2.3A, B, C, D). Again, there was no dramatic difference in the expression of each receptor between individual rhombomeres, although there was some minor variation, namely in r2 which had a 2-fold increase in GFR $\alpha$ -4 levels from the previous day, and r3 which had a small decrease in ret expression (Figure 2.3C, D). At day 4 rhombomere 7 cannot be clearly delineated and therefore only data for rhombomeres 2 to 6 are shown.



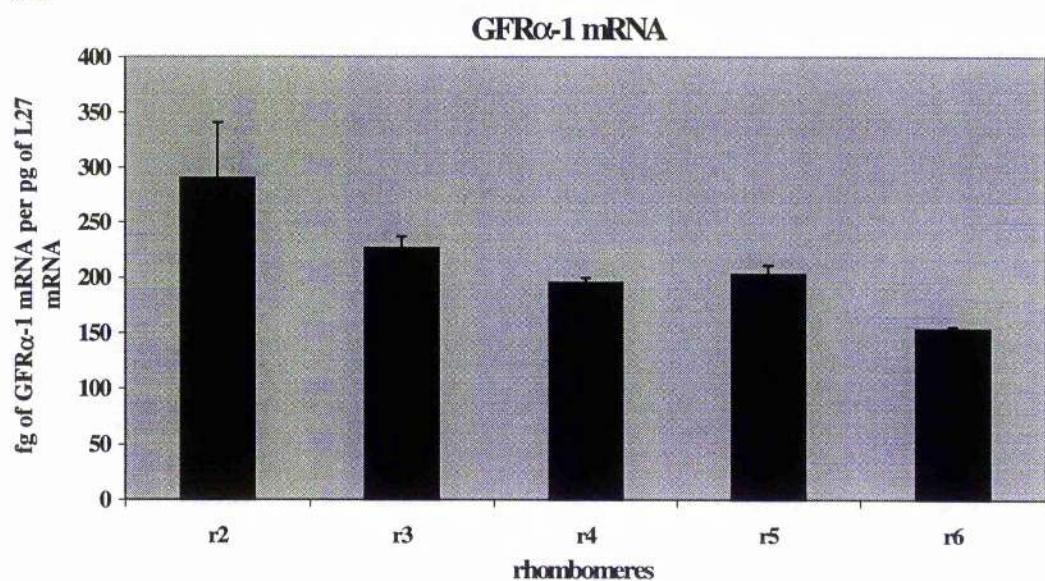
**Figure 2.1.** Bar chart of the levels of GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNAs relative to L27 mRNA in total RNA extracted from E2 chicken hindbrains. The mean  $\pm$  standard error of 2 separate assays are represented by each bar.

**A****B**

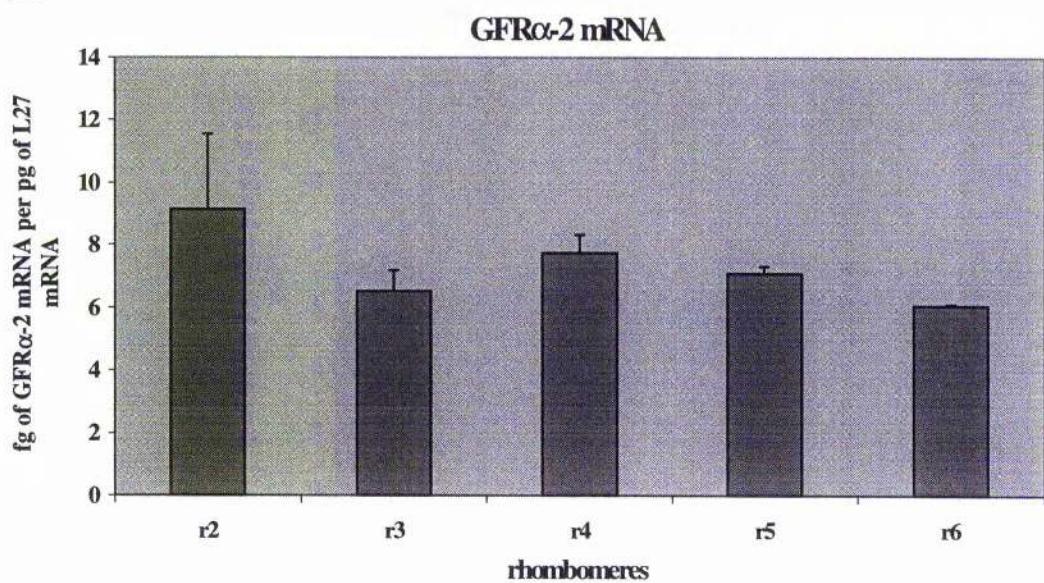
**C****D**

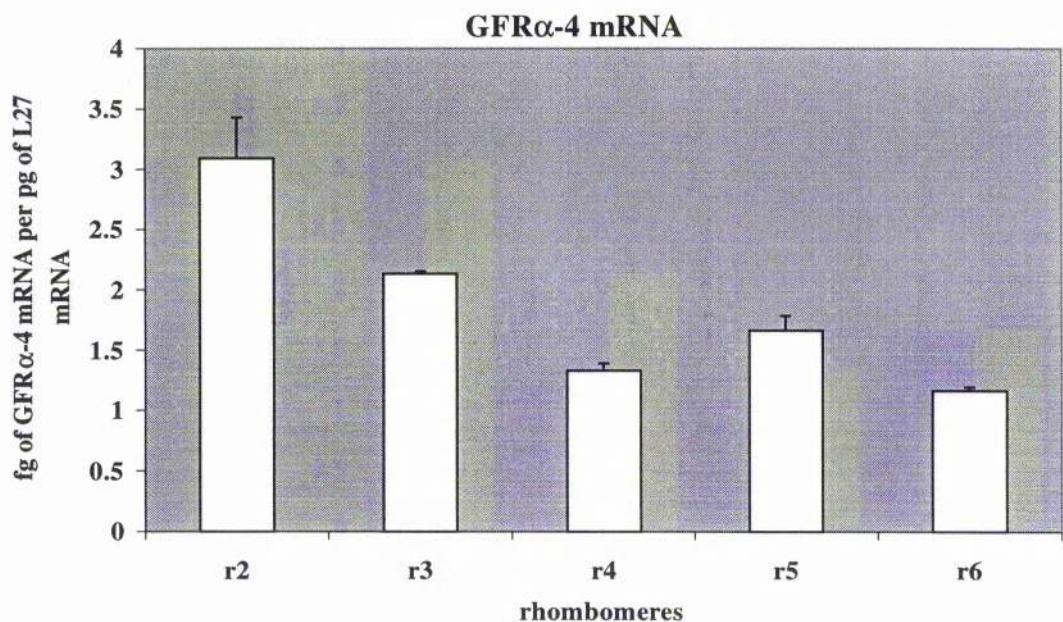
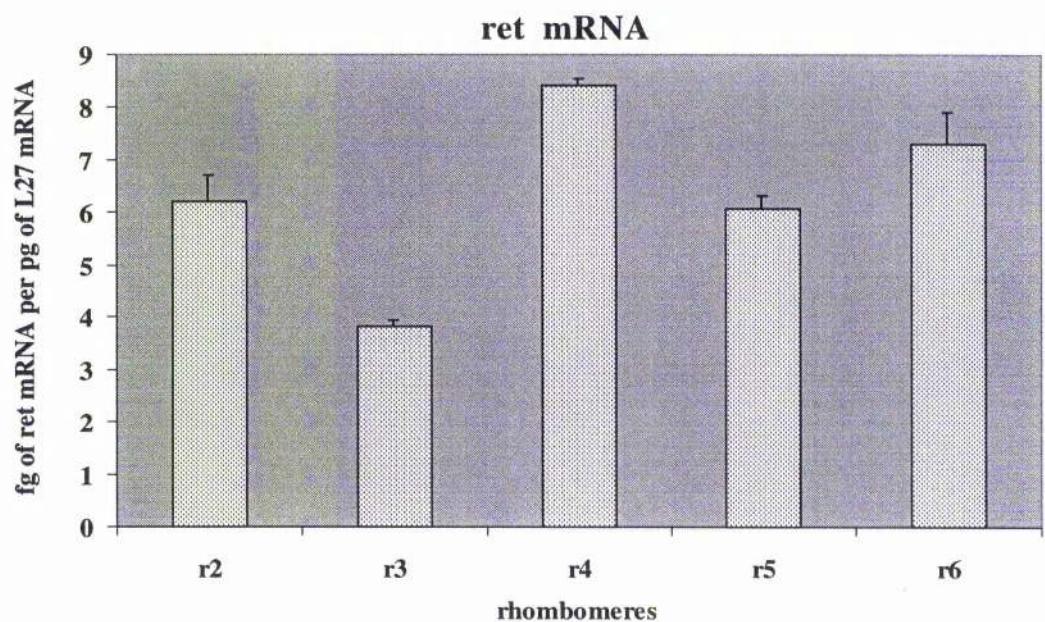
**Figure 2.2.** Bar charts of the levels of GFR $\alpha$ -1 (A), GFR $\alpha$ -2 (B), GFR $\alpha$ -4 (C) and ret (D) mRNAs relative to L27 mRNA in total RNA extracted from rhombomeres 2 to 7 of E3 chicken hindbrains. The mean  $\pm$  standard error of 2 separate assays are represented by each bar.

A



B



**C****D**

**Figure 2.3.** Bar charts of the levels of GFR $\alpha$ -1 (A), GFR $\alpha$ -2 (B), GFR $\alpha$ -4 (C) and ret (D) mRNAs relative to L27 mRNA in total RNA extracted from rhombomeres 2 to 6 of E4 chicken hindbrains. The mean  $\pm$  standard error of 2 separate assays are represented by each bar.

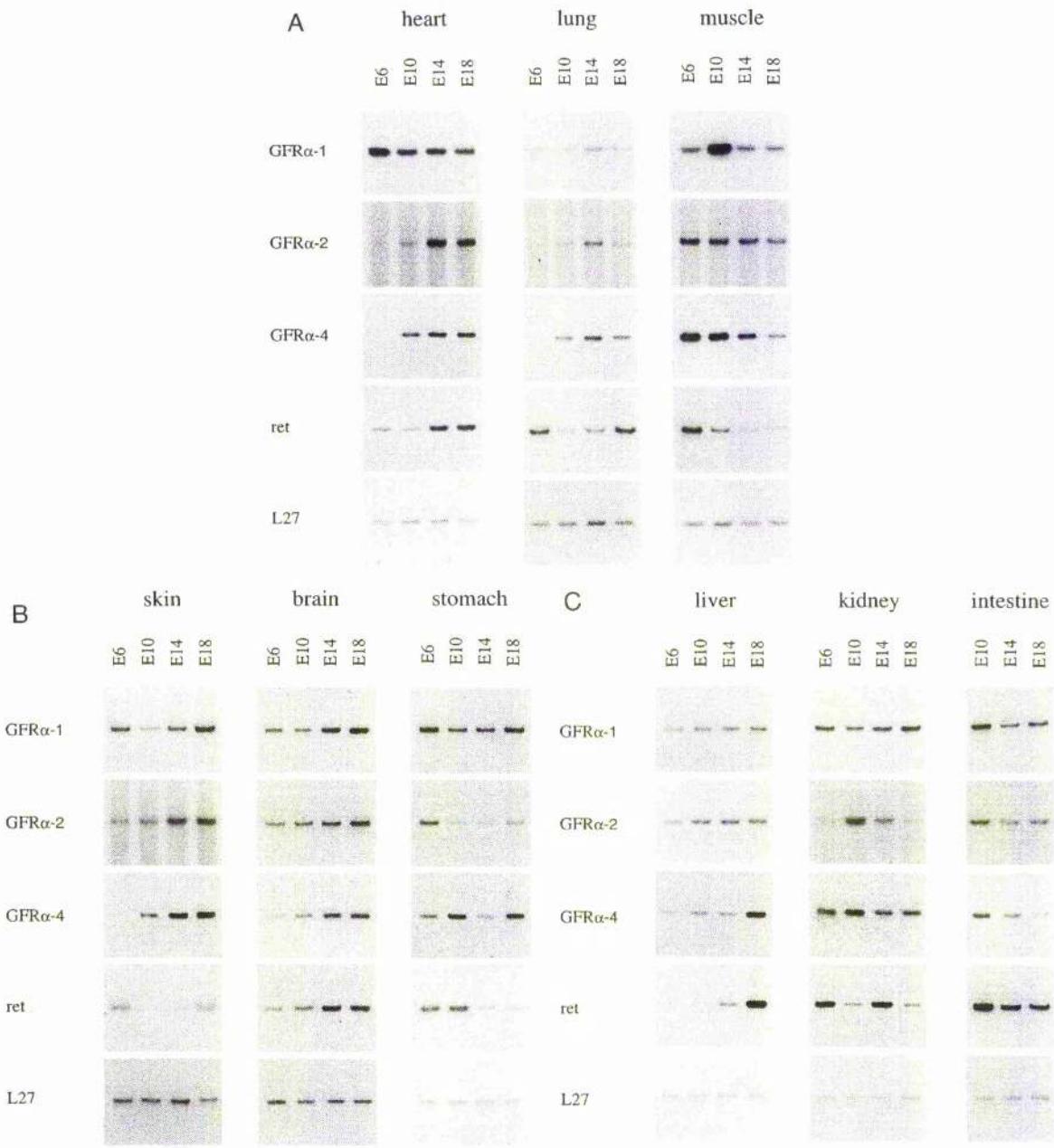
## **II. Expression of GDNF family receptor components during development**

To understand if and how GDNF family members are involved in the formation of any particular tissue or organ of the body, it is essential to study the expression of the receptors at successive stages of development. Previous studies have indicated that all organs express one or more of the GDNF family receptor components to some extent. Therefore, I decided to map the developmental expression of all receptor components in the major tissues of the chicken embryo at four day intervals. Figure 2.4 shows that the developmental pattern of expression of receptor mRNAs varies widely across different tissues. In some tissues, there are similar developmental changes in the expression of GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4, and ret mRNAs. For example, between E6 and E18 the levels of these mRNAs increase in brain and decrease in muscle and intestine. In some tissues, the relative levels of some of these mRNAs, such as GFR $\alpha$ -1 and GFR $\alpha$ -4 in the case of kidney, and GFR $\alpha$ -1 and GFR $\alpha$ -2 in the case of liver, remain fairly constant during development. In other tissues, these mRNAs display different temporal patterns of expression. For example, whereas the level of GFR $\alpha$ -1 mRNA in heart gradually decrease between E6 and E14, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNAs exhibit a clear increase over the same period.

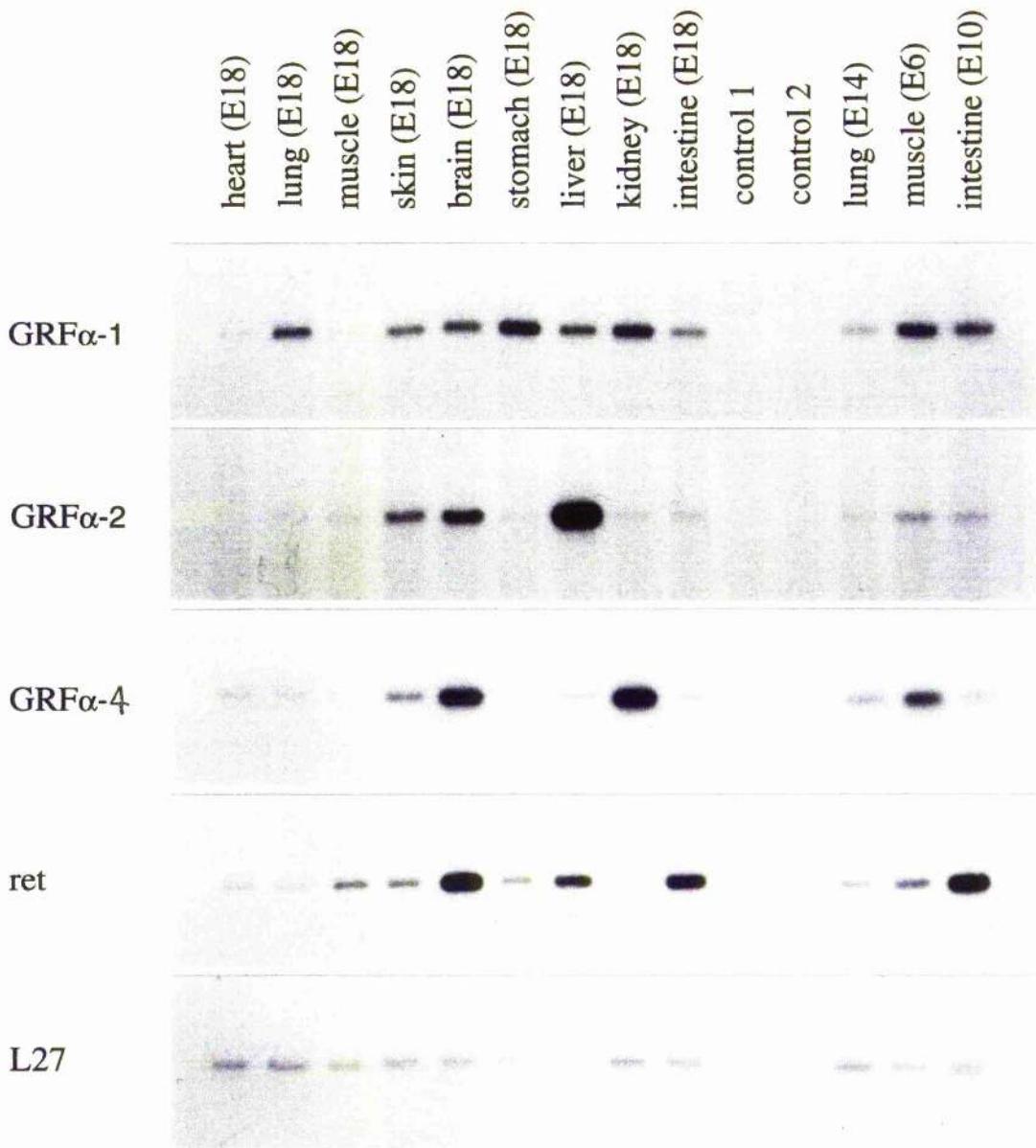
Figure 2.4 indicates that with the exception of lung, muscle, intestine and kidney, GFR $\alpha$ -4 mRNA is expressed highest at later ages. I have, therefore, directly compared the relative levels of GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNAs in a variety of tissues and organs at E18 by the same method (semi-

quantitative RT/PCR). Figure 2.5 reveals that GFR $\alpha$ -4 is expressed in high levels in the kidney and brain, lower levels in the skin, and very low levels in liver, heart, muscle, stomach and intestine. For the same age and tissues, GFR $\alpha$ -1 mRNA is expressed at similar levels in most organs except the heart and muscle where the levels are much lower; GFR $\alpha$ -2 mRNA levels are high in the liver, lower in the brain and skin, and very low elsewhere. Ret mRNA is expressed at higher levels in the brain, liver and intestine than in other tissues.

Taken together, the above study demonstrates that GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNAs have overlapping, though distinct, patterns of expression in the chicken embryo during development, indicating different physiological roles in organ- and tissue- genesis.



**Figure 2.4.** Autoradiograms showing the RT/PCR products for GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret at E6, E10, E14, and E18 in heart, lung, and skeletal muscle (A), skin, brain, and stomach (B), and liver, kidney and intestine (C). The same mRNA preparations were used for all reactions with each set of primers and were diluted appropriately to contain very similar levels of L27 mRNA, as shown by the similar levels of the RT/PCR products for L27 mRNA in the bottom autoradiograms in the age set for each tissue. Control reactions from which the reverse transcriptase step was omitted (not shown) were carried out for each reaction and were negative in all cases, demonstrating that there was no detectable contamination by genomic DNA in any of the preparations. The autoradiograms were developed for different lengths of time so that very weak bands could be observed. Very similar results were obtained from a separate set of tissues.



**Figure 2.5.** Autoradiograms showing the RT/PCR products for GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret in a variety of tissues at E18 and in lung, skeletal muscle, and intestine at E14, E6, and E10, respectively, the ages at which GFR $\alpha$ -4 mRNA expression was highest in these tissues. The same mRNA preparations were used for all reactions with each set of primers and were diluted appropriately to contain very similar levels of L27 mRNA, as shown by the similar levels of the RT/PCR products for L27 mRNA in the bottom autoradiogram. Control reactions from which the reverse transcriptase step was omitted (controls 1 and 2 which contain extracted RNA from 6 of the 12 tissues) show that there was no detectable contamination by genomic DNA.

Very similar results were obtained from a separate set of tissues.

### **III. Expression of GFR $\alpha$ -1, GFR $\alpha$ -2 and ret mRNAs in the PNS neurons**

Several studies have indicated that GDNF and NTN are trophic factors for PNS neurons and that their receptors are expressed in various ganglia at some developmental stages. However, no detailed developmental studies of receptor expression in different ganglia have been reported and there has been no systematic comparison of receptor expression with the responsiveness of neurons to GDNF and NTN.

Here, I describe the expression of the receptors in representative populations of avian parasympathetic, sympathetic and sensory neurons throughout embryonic development.

#### **Parasympathetic neurons: ciliary ganglion**

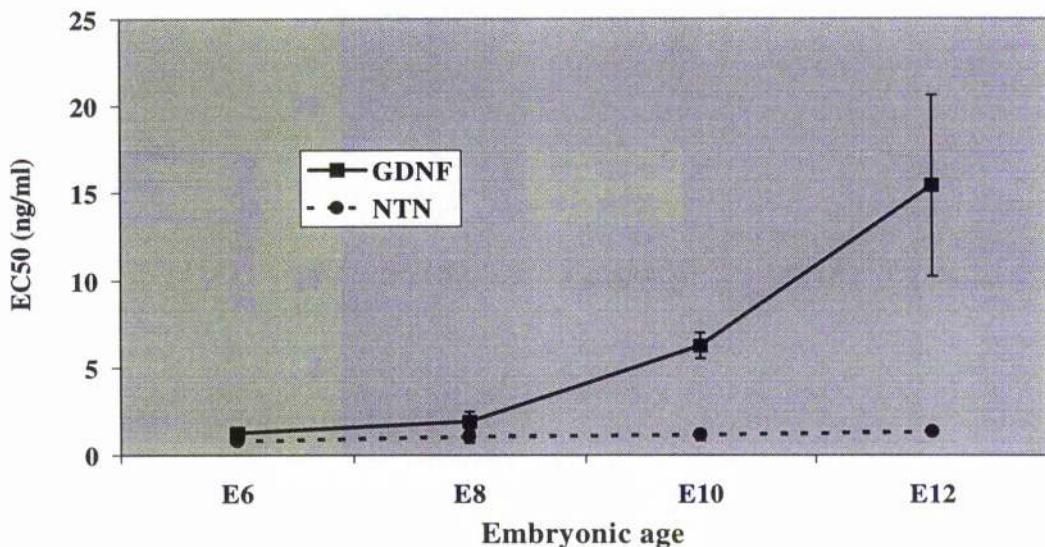
The ciliary ganglion is comprised of parasympathetic neurons that innervate the iris, ciliary body and choroid and are supported by CNTF, GDNF and NTN in culture but not by neurotrophins (Barbin et al., 1984; Buj-Bello et al., 1995; Allsopp et al. 1993, Forgie et al., 1999). A colleague, Alison Forgie, has shown that ciliary neurons are equally responsive to GDNF and NTN early in development but later on (E8 onwards) they rapidly lose responsiveness to GDNF whilst retaining sensitivity to NTN (Figure 2.6A). Figures 2.7A and B show the developmental expression of GFR $\alpha$ -1, GFR $\alpha$ -2 and ret mRNAs in intact ciliary ganglia with embryonic age. In agreement with the survival data, GFR $\alpha$ -2 mRNA is expressed at higher levels than GFR $\alpha$ -1 mRNA. However, the dramatic loss of responsiveness to GDNF between E8 and E12 is not accompanied by a

decrease in the level of GFR $\alpha$ -1 mRNA. Rather, the level of GFR $\alpha$ -1 mRNA remains the same from E8 onwards.

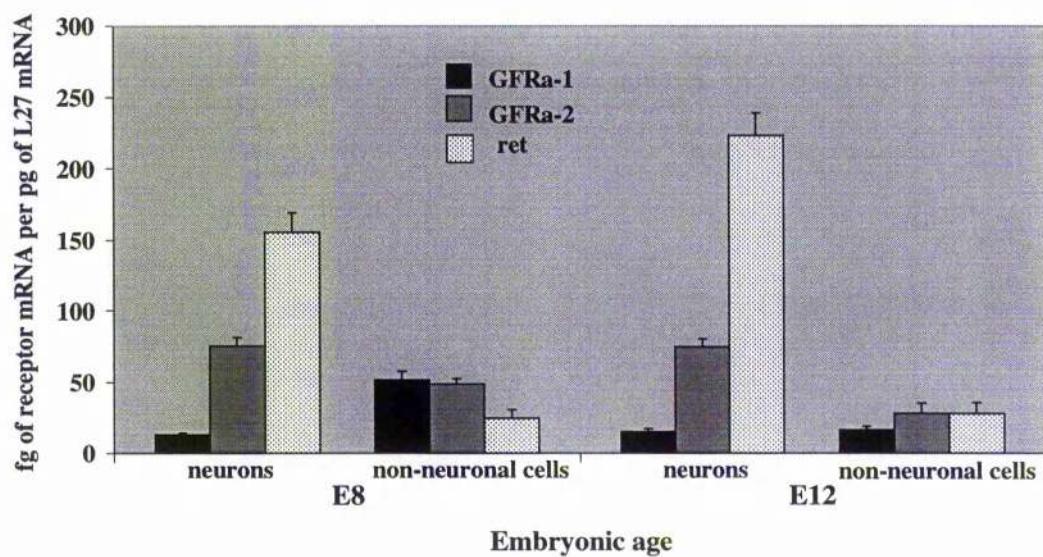
As mentioned earlier, GFR $\alpha$  and Ret distribution is wide, therefore I have examined the extent to which non-neuronal cells in peripheral ganglia express GDNF family receptors. Using the method of differential sedimentation (Davies, 1988c), I prepared purified neurons and non-neuronal cells. To my surprise, GFR $\alpha$ -1 and GFR $\alpha$ -2 mRNAs were expressed at approximately the same levels in both neurons and non-neuronal cells in all ganglia examined. Interestingly, the expression of ret mRNA was marginal in the case of non-neuronal cells (data for ciliary non-neuronal cells are shown in Figure 2.6B).

Since expression of GFR $\alpha$  mRNA by non-neuronal cells may explain the lack of correlation between GFR $\alpha$  expression and ciliary neuron survival, I re-examined receptor expression in purified neurons at E8 and E12. Again, as in the case of the intact ganglia, GFR $\alpha$ -2 mRNA levels were higher than those of GFR $\alpha$ -1 mRNA and the switch in responsiveness to GDNF was not accompanied by a decrease in the GFR $\alpha$ -1 mRNA levels (Figure 2.6B).

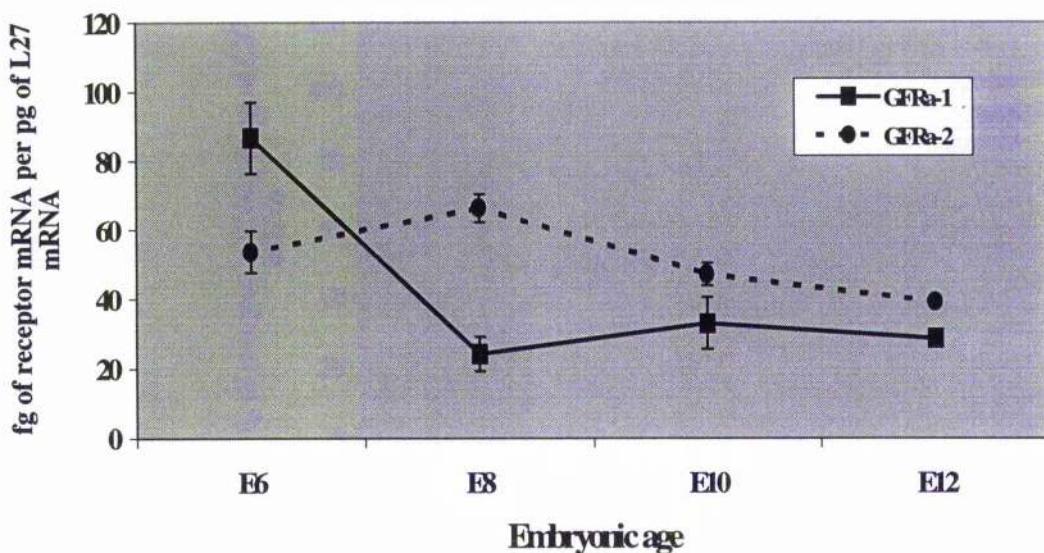
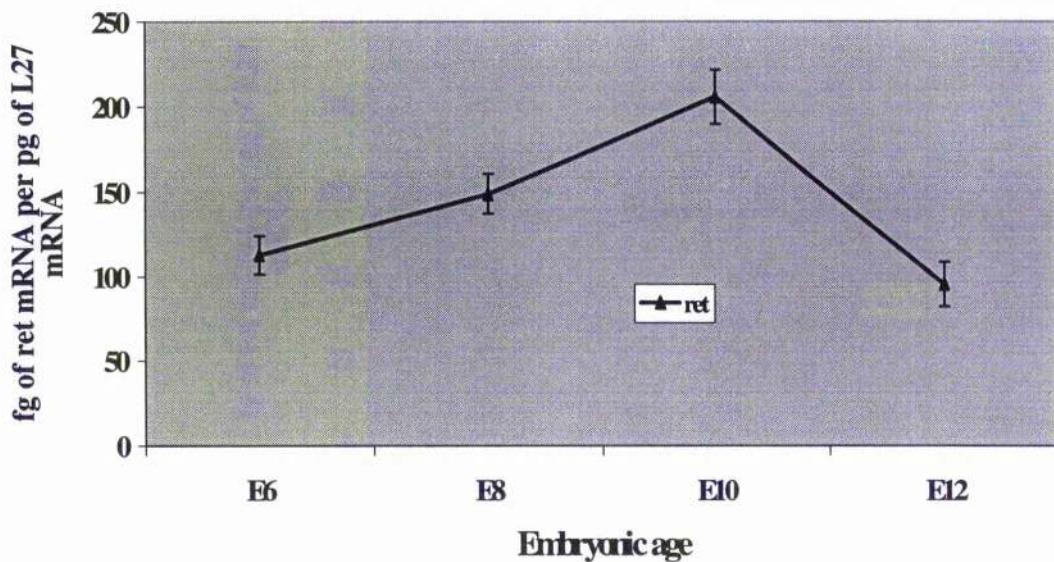
## A Ciliary neurons



## B



**Figure 2.6.** (A) Graph of the EC50 values of E6, E8, E10 and E12 ciliary ganglion neurons grown with GDNF or NTN over a range of concentrations (Adapted from Forgie et al., 1999). (B) Bar chart of the levels of GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNAs relative to L27 mRNA in total RNA extracted from purified ciliary ganglion neuronal and non-neuronal cells of E8 and E12 chicken embryos. The mean  $\pm$  standard error of 3 separate assays are represented by each bar.

**A****Ciliary ganglia****B**

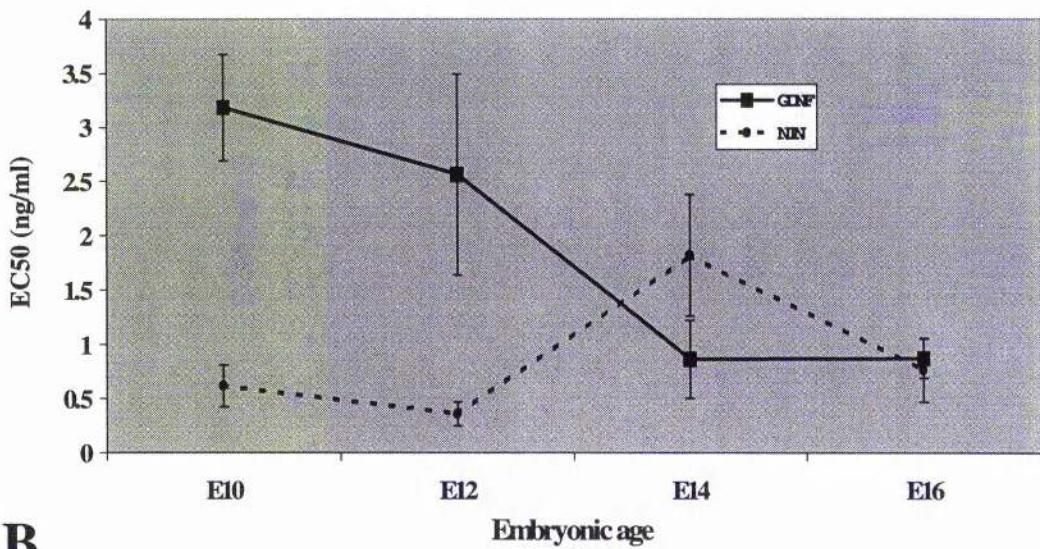
**Figure 2.7.** Graph of the levels of GFR $\alpha$ -1 (A), GFR $\alpha$ -2 (B) and ret (C) mRNAs relative to L27 mRNA in total RNA extracted from intact ciliary ganglia of E6, E8, E10 and E12 chicken embryos. The mean  $\pm$  standard error of 2 separate assays are represented by each bar.

### **Sympathetic neurons: lumbar sympathetic chain**

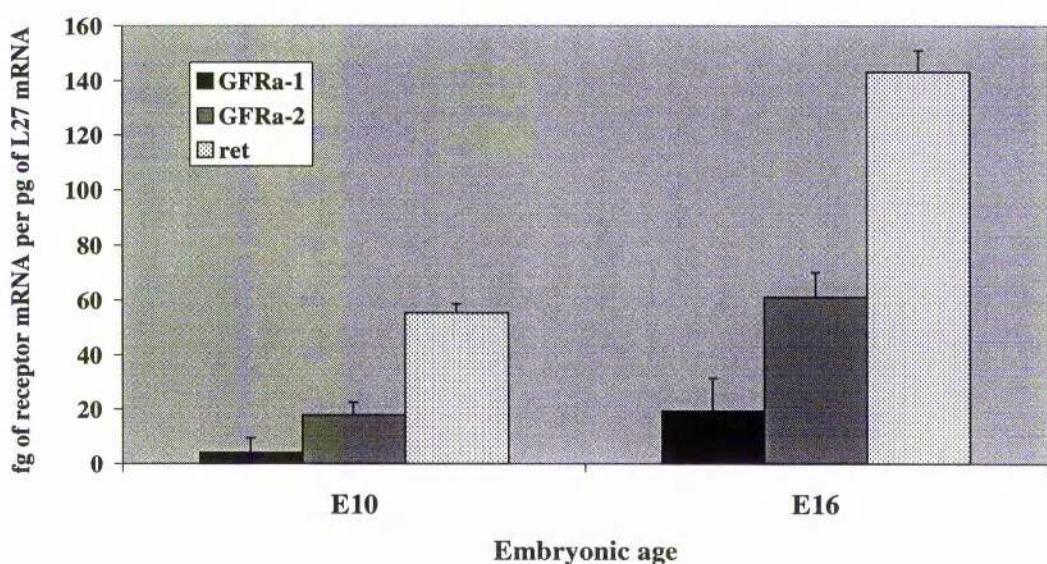
The sympathetic neurons of the embryonic paravertebral sympathetic chain are supported by NGF, CNTF, GDNF and NTN in culture (Chun and Patterson, 1977; Barbin et al., 1984; Buj-Bello et al., 1995; Forgie et al., 1999; personal data not shown). Alison Forgie has shown that sympathetic neurons are far more responsive to NTN early in development and equally responsive to GDNF and NTN later on (Figure 2.8A). Figures 2.9A and B show the expression of GFR $\alpha$ -1, GFR $\alpha$ -2 and ret mRNAs in the intact ganglia. As in the case of the ciliary ganglia, changes in responsiveness to GDNF and NTN are not reflected by changes in the expression of receptor mRNAs in intact ganglia; GFR $\alpha$ -1 mRNA levels remain more or less constant throughout development, while GFR $\alpha$ -2 and ret mRNA levels appear to slightly increase with age. In purified neurons (Figure 2.8B), GFR $\alpha$ -1 mRNA levels do go up with time as might be expected from the increase in responsiveness of the neurons to GDNF from E10 to E16. However, GFR $\alpha$ -2 mRNA expression also increases markedly between E10 and E16, although responsiveness to NTN does not significantly change.

## Sympathetic neurons

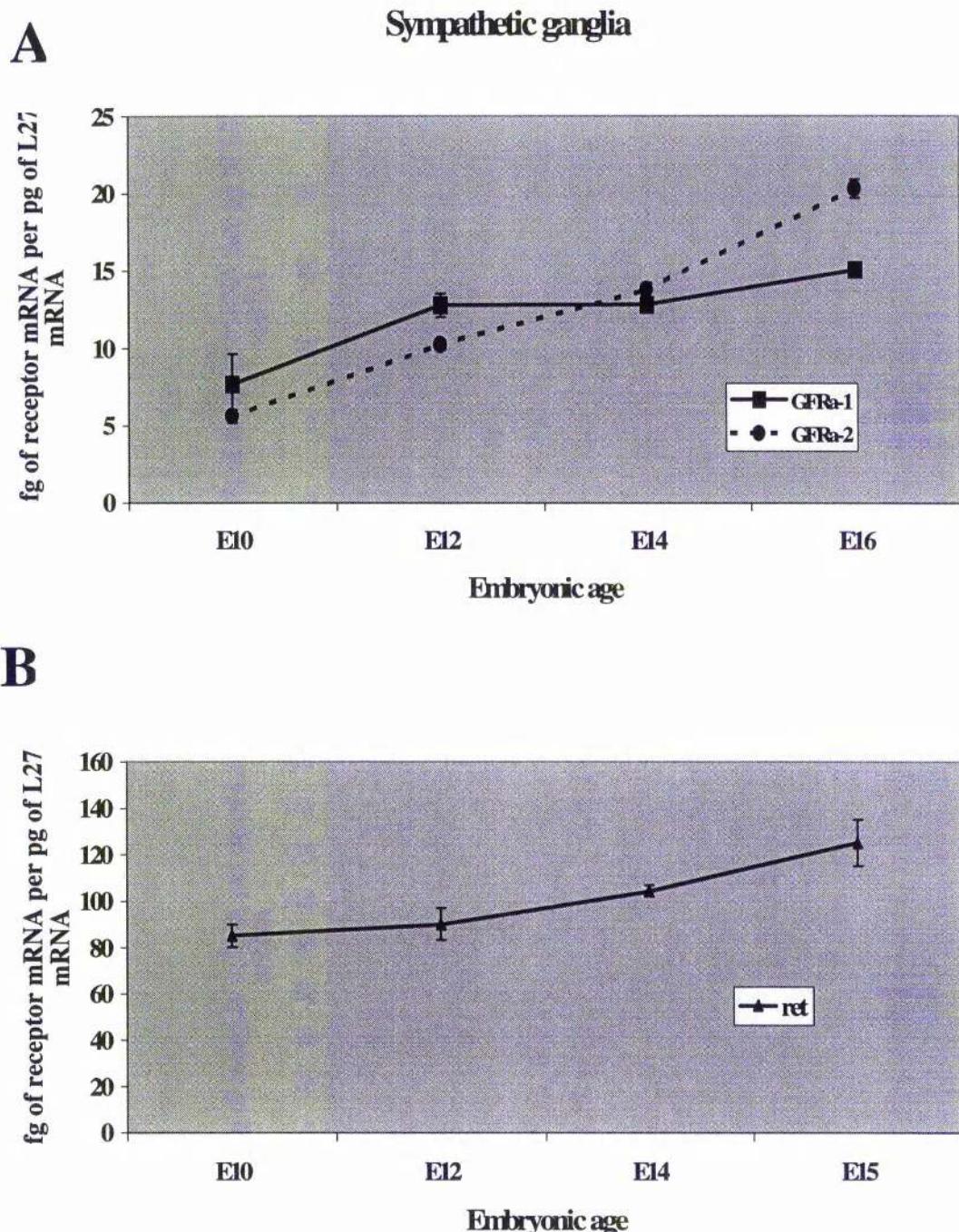
**A**



**B**



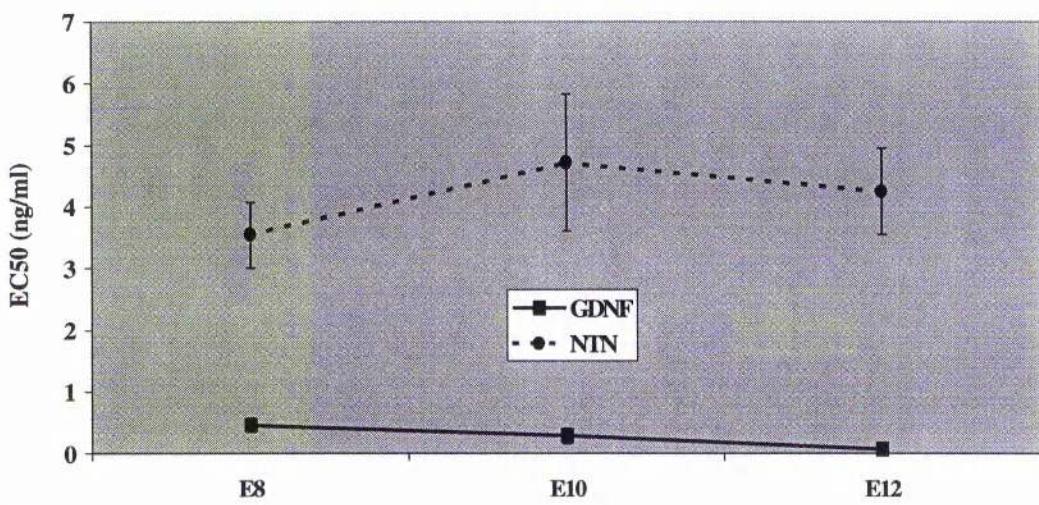
**Figure 2.8.** (A) Graph of the EC50 values of E10, E12, E14 and E16 sympathetic chain neurons grown with GDNF or NTN over a range of concentrations (Adapted from Forgie et al., 1999). (B) Bar chart of the levels of GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNAs relative to L27 mRNA in total RNA extracted from purified ciliary ganglion neuronal and non-neuronal cells of E10 and E16 chicken embryos. The mean  $\pm$  standard error of 3 separate assays are represented by each bar.



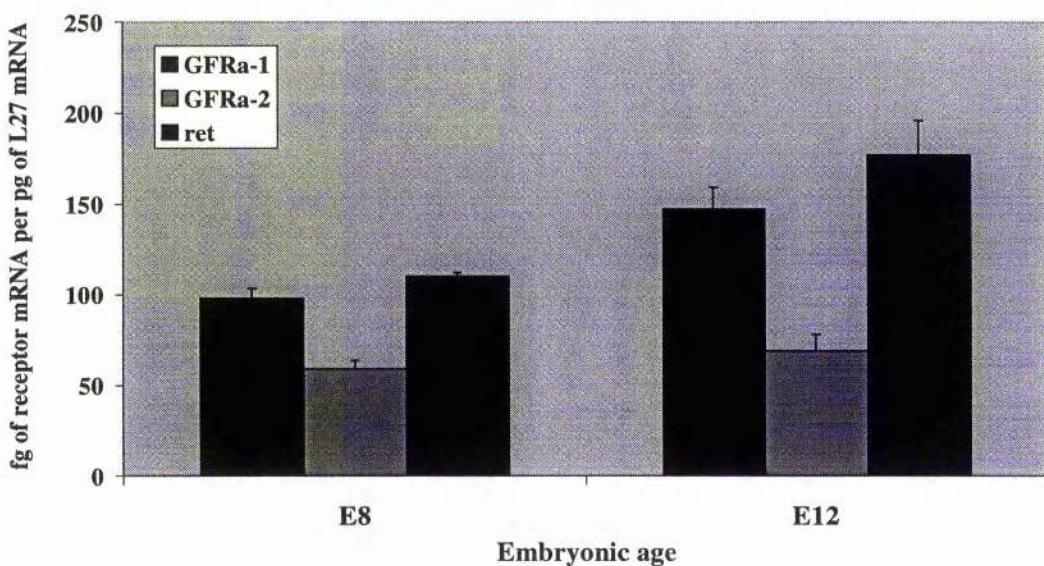
**Figure 2.9.** Graph of the levels of GFR $\alpha$ -1 (A), GFR $\alpha$ -2 (B) and ret (C) mRNAs relative to L27 mRNA in total RNA extracted from intact paravertebral sympathetic ganglia of E10, E12, E14 and E16 chicken embryos. The mean  $\pm$  standard error of 2 separate assays are represented by each bar.

### **Sensory neurons: nodose ganglia**

The sensory neurons of the nodose ganglion innervate the thoracic and abdominal viscera and consist of neurons that respond to BDNF, GDNF and NTN (Lindsay et al., 1985; Davies et al., 1986; Buj-Bello et al., 1995; Forgie et al., 1999). Alison Forgie has shown that nodose neurons are more sensitive to GDNF than NTN and become increasingly responsive to GDNF from E8 to E12 (Figure 2.10A). Figures 2.11A and B shows the developmental expression of the receptors in the intact ganglia. In agreement with the survival data, GFR $\alpha$ -1 mRNA levels are higher than GFR $\alpha$ -2 mRNA levels throughout development. GFR $\alpha$ -1 mRNA expression in intact ganglia does not appear to reflect the increasing responsiveness of nodose neurons to GDNF between E8 and E12. However, in the case of purified neurons (Figure 2.10B) GFR $\alpha$ -1 mRNA expression is higher than GFR $\alpha$ -2 mRNA and increases with age. In contrast, GFR $\alpha$ -2 mRNA expression in purified neurons remains constant throughout development.

**A****Nodose neurons****B**

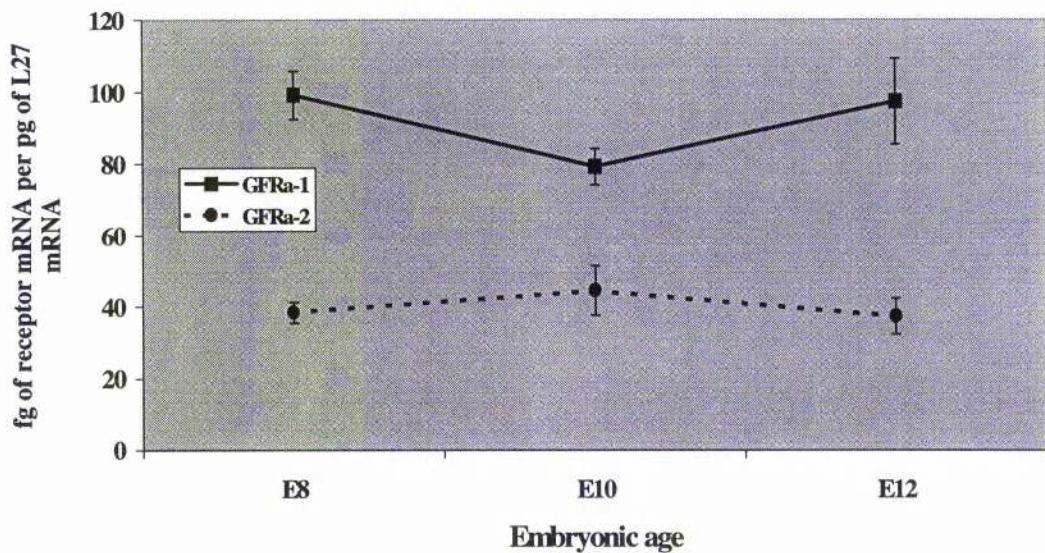
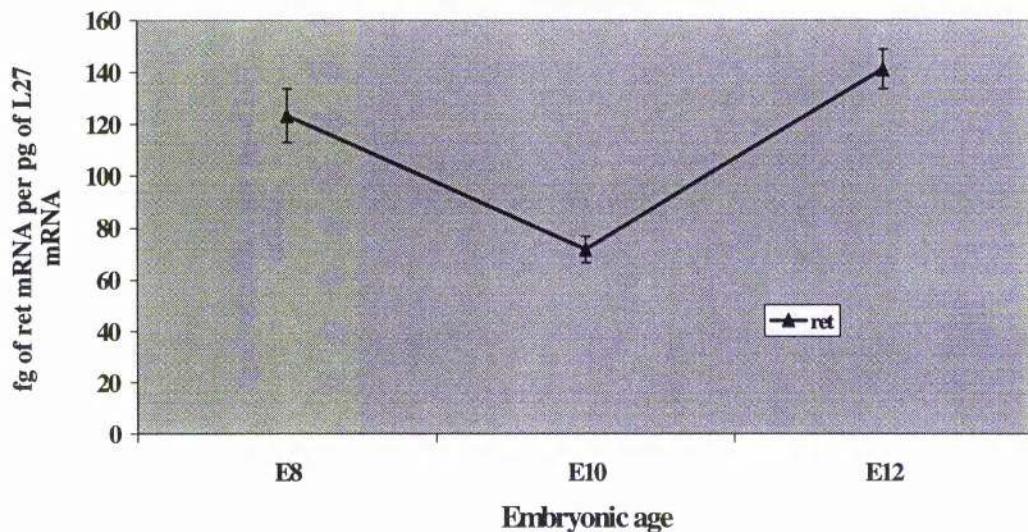
## Embryonic age



**Figure 2.10.** (A) Graph of the EC50 values of E8, E10 and E12 nodose neurons grown with GDNF or NTN over a range of concentrations (Adapted from Forgie et al., 1999). (B) Bar chart of the levels of GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNAs relative to L27 mRNA in total RNA extracted from purified nodose ganglion neuronal and non-neuronal cells of E8 and E12 chicken embryos. The mean  $\pm$  standard error of 4 separate assays are represented by each bar.

**A**

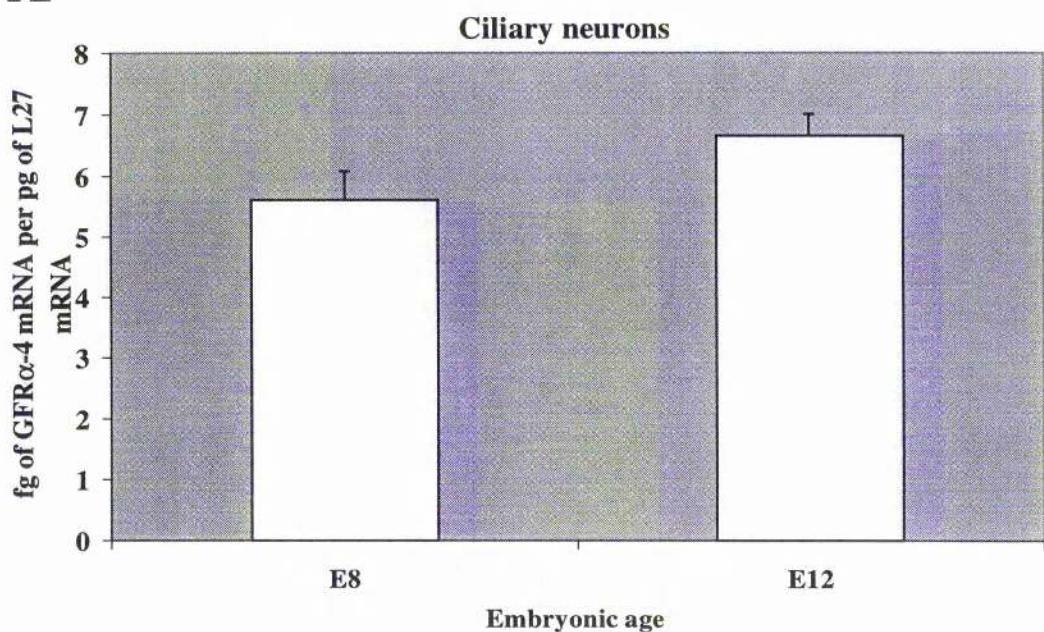
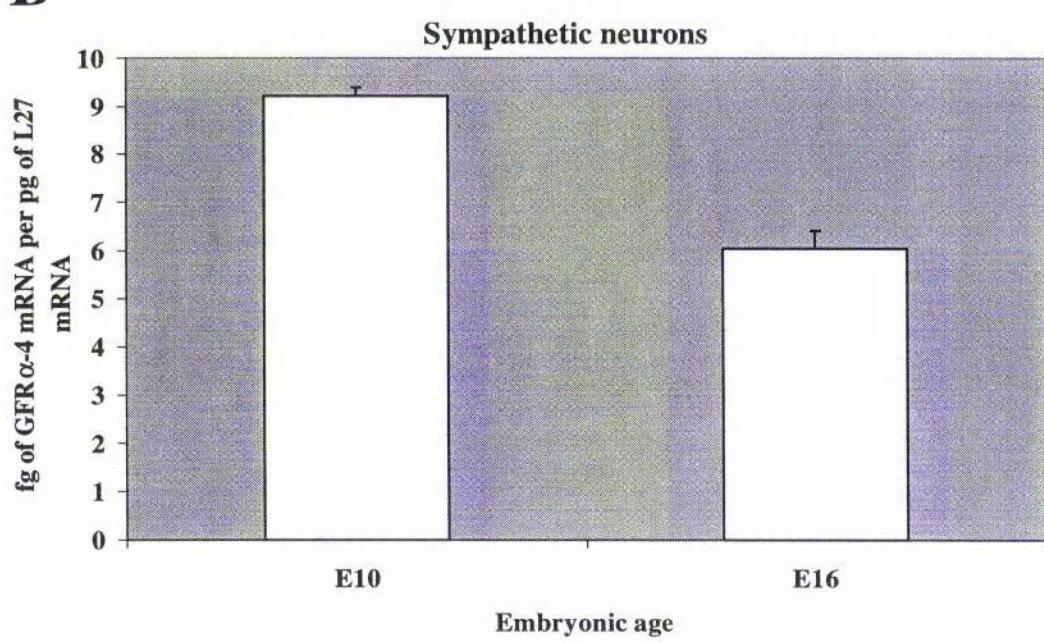
## Nodose ganglia

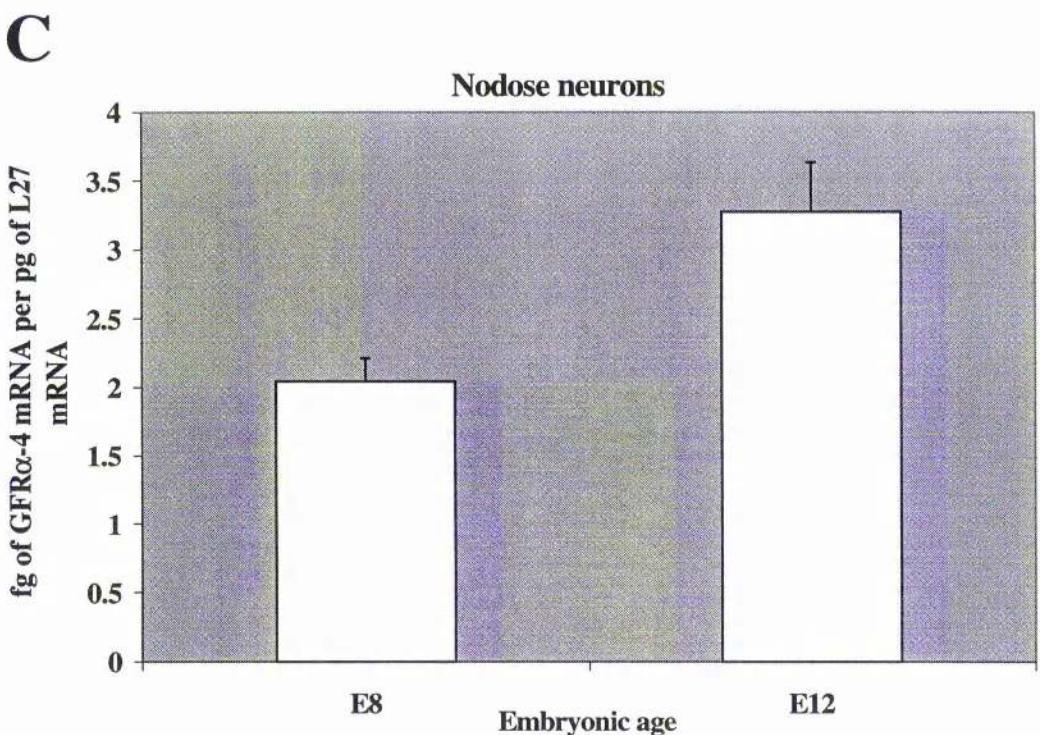
**B**

**Figure 2.11.** Graph of the levels of GFR $\alpha$ -1 (A), GFR $\alpha$ -2 (B) and ret (C) mRNAs relative to L27 mRNA in total RNA extracted from intact nodose ganglia of E8, E10 and E12 chicken embryos. The mean  $\pm$  standard error of 2 separate assays are represented by each bar.

#### **IV. Expression of GFR $\alpha$ -4 mRNA in PNS neurons**

Several studies have indicated that PSP is not a trophic factor for PNS neurons (Milbrandt et al., 1998; Forgie, unpublished observations, personal data not shown). To find out whether this is due to lack of GFR $\alpha$ -4 expression, I examined the mRNA levels of the receptor in these neurons. All neurons examined were found to express, albeit in low levels, GFR $\alpha$ -4 mRNA (results from ciliary, paravertebral sympathetic and nodose neurons are shown in Figure 2.12). In all cases, with the exception of paravertebral sympathetic neurons, the levels of GFR $\alpha$ -4 mRNA increased with age. The highest expression of GFR $\alpha$ -4 mRNA was detected in early paravertebral sympathetic neurons.

**A****B**



**Figure 2.12.** Bar charts of the levels of GFR $\alpha$ -4 mRNA relative to L27 mRNA in total RNA extracted from purified ciliary (A), paravertebral sympathetic (B) and nodose (C) ganglion 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**

In this chapter, I have examined the distribution of the GDNF-like receptors during embryogenesis, with particular emphasis on the PNS.

### **Hindbrain**

At E2, a developmental stage that coincides with the migration of neural crest all GFR $\alpha$  and ret receptor mRNAs are detectable in the hindbrain (a brief review of the development of the hindbrain is provided in appendix I). GFR $\alpha$ -1 mRNA shows the highest expression, followed by GFR $\alpha$ -2, ret and GFR $\alpha$ -4 mRNAs. Between E2 and E3, where dissection of the individual rhombomeres is feasible, there is a considerable increase in GFR $\alpha$ -1 expression, a moderate increase in GFR $\alpha$ -4 and ret expression and no change in GFR $\alpha$ -2 expression. Interestingly, all different rhombomeres express the various receptor components at similar levels. This is in contrast with the observation that ret mRNA expression is restricted to r4 in mouse at this stage of development (Pachnis et al., 1993). It is worth noting, that some other differences in the pattern of ret mRNA expression have also been observed between mouse and chicken. These differences include the trigeminal ganglion (where ret mRNA is expressed earlier in chicken development than in mouse development), the ventral roots of the spinal cord, the mesonephric tubules and the mesenchymal cells of the branchial arches (expression only in chicken) and testes (strong expression in chick versus a barely detectable signal in mouse) (Pachnis et al., 1993; Schuchardt et al., 1995; Iwamoto et al., 1993). These evolutionary differences come as a surprise taking into account the

importance of Ret as a transducing receptor for at least four neurotrophic factors. Several alternative explanations may be proposed. One is that after the divergence of birds and mammals, the function of Ret was taken over in some mammalian cell types by a different receptor. Alternatively Ret may have taken on an additional function in the avian lineage. Another possibility is that the sites where Ret is expressed in chick but not mouse may represent sites where the Ret gene is expressed but Ret protein is not functional or expressed, eliminating the evolutionary pressure to either maintain or silence expression. This issue may be addressed by further analyses of mouse embryos homozygous for the ret mutation, or through studies of ret function during avian embryogenesis.

It has been suggested that the patterning information for neuronal components of each branchial arch is contained within the neural crest that populates that arch and that this information has been imprinted on the neural crest according to where it originated along the anteroposterior axis of the brain neuroepithelium (Noden, 1983; 1988). Although the results we present here do not describe the expression pattern of the receptors in individual rhombomeres at day 2, when most migration takes place, the very low levels of all receptors at this age and the uniform expression of the receptors in all rhombomeres at days 3 and 4 suggest that the GDNF family receptors are not involved in specifying the migration pathway of neural crest cells, and the initial patterning of neural crest derivatives. Further studies will be required to establish whether the switch-on in the expression of the GDNF receptor in neural crest-derived ganglia later in development is due to intrinsic information acquired by their rhombomeric origin and/or local cues. At present, several studies suggest, that for at least the

patterning of neural crest migration, both inherited information from rhombomeres and pathway- or target- derived cues (e.g. from tissues such as the otic vesicle) are required (Noden, 1983; Hunt et al., 1991; Kurati and Eichele, 1993; Sechrist et al., 1994).

It is also worth noting the significant upregulation of GFR $\alpha$ -1 mRNA from day 2 to days 3 and 4. This very high GFR $\alpha$ -1 mRNA expression implies potentially important role for this receptor in the development of the hindbrain. Interestingly, ret mRNA in the hindbrain remains very low at all three ages studied when compared to its expression in neural crest derivatives later in development. These findings suggest that ret is not functional in hindbrain at this stage and that GFR $\alpha$ -1 may either use another signal transducing receptor to exert its role or alternatively may merely serve to regulate the availability of GDNF to other cell types. One caveat to this interpretation is that the relationship between mRNA and protein levels is not known for the GFR $\alpha$  and Ret receptors in developing chickens.

### **Organogenesis**

GDNF family ligands and receptors have a wide distribution in the periphery both in embryos and adults, suggesting important roles in organ development and maintenance. To get a better understanding of these roles in organogenesis, the expression of the receptors was analyzed at successive stages of development.

The results show a complex and varied pattern of receptor mRNAs expression. In some tissues, the developmental changes in the expression of

GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4, and ret mRNA are similar (e.g. brain, intestine, muscle). In others, some of the receptors show a decrease in expression while the remaining receptors show an increase in expression over the same period (e.g. heart). In some tissues, some receptors display constant expression throughout development, while the rest display a distinct temporal pattern (e.g. kidney).

To address the role of specific signaling by the  $\alpha$ -components it is necessary to compare their expression to that of ret. In general, it appears as if ret mRNA expression follows the major changes in the expression of GFR $\alpha$  mRNAs. For example, in liver between E6 and E14 the levels of GFR $\alpha$  mRNAs remain constant while between E14 and E18 GFR $\alpha$ -1 and GFR $\alpha$ -2 are maintained but there is a massive upregulation of GFR $\alpha$ -4 mRNA (Figure 2.4). Accordingly, ret mRNA is expressed at very low levels between E6 and E14 but is upregulated between E14 and E18. Similarly, in intestine and brain all GFR $\alpha$  mRNAs have a common expression pattern and ret mRNA appears to mirror this pattern. Furthermore, in heart all three GFR $\alpha$  mRNAs and ret mRNA are highly expressed between E14 and E18. In muscle, GFR $\alpha$  mRNA expression decrease with age, ret mRNA follows this decrease.

There are, however, some patterns that do not fit to the above assumption. For example, ret mRNA shows high level expression in E6 lung, compared to E10 and E14, but GFR $\alpha$  mRNAs are expressed at very low levels (Figure 2.4). In addition, ret mRNA expression is very low in E18 kidney compared to other tissues while both GFR $\alpha$ -4 and GFR $\alpha$ -1 mRNAs display among their highest expression in the

body at this age (Figure 2.5). Further, ret mRNA is expressed at high levels in E18 intestine, but GFR $\alpha$  mRNA expression is not significant (Figure 2.5).

These observations indicate a very complex mode of interactions, which at present is difficult to interpret. However, several possible explanations could account for the expression patterns we see. The presence of high levels of some of these GFR $\alpha$  mRNAs in tissues such as E18 kidney and stomach (Figure 2.5), which have no appreciable amounts of ret mRNA, suggest that the  $\alpha$ -components may associate with additional, still unknown, Ret-like signal transducing molecules. Alternatively, membrane bound or possibly truncated soluble forms of GFR $\alpha$  may act to regulate the availability of ligands in the extracellular space in the absence of ret signaling.

It appears as if ret mRNA can be expressed at high levels in some tissues whilst GFR $\alpha$  mRNAs are only marginally expressed (e.g. intestine) (Figure 2.5). This could be explained in various ways. Ret has been shown to be constitutively active in the absence of ligands and GFR $\alpha$ s (Trupp et al., 1998). Ret therefore may function in a ligand-independent signal transduction pathway when expressed alone. Another possibility is that Ret expression may be regulated at the protein, and not the mRNA level. Finally, it is possible that other, still unknown, GFR $\alpha$ s may also signal through Ret. These GFR $\alpha$ s could have complementary patterns with the ones we already know.

Taken together, this study has demonstrated that GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNAs have both complementary and overlapping, though distinctive, patterns of expression in the avian embryo during development.

## **Peripheral nervous system**

Neurons of the peripheral nervous system have been invaluable for understanding fundamental aspects of neurotrophic factor biology and for clarifying the functions of neurotrophic factor receptors (Davies, 1994b; Henderson, 1996; Davies, 1997a,b). I have, therefore, studied the relationship between responsiveness of PNS neurons to GDNF and NTN and the expression of mRNAs encoding receptors for these factors. Whereas all of the populations of neurons express relative high levels of ret mRNA, which appears to be essential for both GDNF and NTN signaling, there are differences in the relative levels of GFR $\alpha$ -1 and GFR $\alpha$ -2 mRNAs. Generally, neurons that are more responsive to GDNF express relatively higher levels of GFR $\alpha$ -1 mRNA, and neurons that are more responsive to NTN express relatively higher levels of GFR $\alpha$ -2 mRNA. This is consistent with data supporting the idea that GFR $\alpha$ -1 is the preferred receptor for GDNF and GFR $\alpha$ -2 is the preferred receptor for NTN and suggests that differences in responsiveness of neurons to GDNF and NTN is governed in part by the relative levels of expression of members of the GFR $\alpha$  family receptors. However, developmental changes in responsiveness to these factors are 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 is 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 are very similar in both ages (Figure 2.6B). Although it is possible that there are changes in the relative levels of GFR $\alpha$ -1 and GFR $\alpha$ -2 proteins in

PNS 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 such as expression of components of the signal transducing machinery could affect the sensitivity of neurons to GDNF and NTN.

An interesting characteristic of the PNS ganglia is that non-neuronal cells contained within them express similar levels of GFR $\alpha$  mRNAs to neurons, but do not express appreciable levels of ret mRNA (data for ciliary non-neuronal cells are shown in Figure 2.6B). In general, GFR $\alpha$ -1 is expressed at either similar or higher levels in non-neuronal cells than in neurons. GFR $\alpha$ -2 is expressed at similar or lower levels in non-neuronal cells compared to neurons. Whilst ret mRNA expression is only marginal in non-neuronal cells it is possible that even this low ret mRNA expression is the result of contamination (~ 5%) of the non-neuronal fraction with small neurons during the differential sedimentation procedure. The finding that non-neuronal cells express the GFR $\alpha$  mRNAs but not ret mRNA is similar to the situation where p75, but not Trks, are expressed by Schwann cells in developing or lesioned peripheral nerves. In this case it is assumed that the Schwann cells make neurotrophins and present them on the cell surface bound to p75. It is possible that Schwann cells, or other glial cells or fibroblasts, within peripheral ganglia synthesize GDNF-like ligands which they then present on their surface bound to GFR $\alpha$ . Alternatively, GFR $\alpha$  could function as reservoirs to capture ligands diffusing through the extracellular milieu. In both cases, neurons expressing ret could then be trans-activated by the ligand-GFR $\alpha$  complex.

Persephin is a recently isolated member of the GDNF family. Unlike the other members, PSP does not support the survival of PNS neurons, either alone or in combination with other growth factors (GDNF, TGF $\beta$ -1, and TGF $\beta$ -2) or depolarization (Milbrandt et al., 1998; personal data). One possible explanation is that GFR $\alpha$ -4, the receptor for PSP, is absent from the ganglia. Therefore, I examined whether GFR $\alpha$ -4 mRNA is expressed in purified PNS neurons. All neurons from SCG, VLTG, vestibular, paravertebral sympathetic, nodose, DMTG, and ciliary ganglia were found to express, albeit in low levels, GFR $\alpha$ -4 mRNA (data for ciliary, paravertebral sympathetic and nodose are shown in Figure 2.12). In all cases, with the exception of paravertebral sympathetic neurons, the levels of GFR $\alpha$ -4 mRNA increase with age. This developmental trend is similar to the one observed for organs and tissues, indicating that GFR $\alpha$ -4 acts late in embryogenesis.

The observation that PNS neurons do not respond to PSP but express GFR $\alpha$ -4 mRNA can be explained in various ways. One possibility is that PSP requires a still unknown cofactor in order to exert its neurotrophic potential as was observed for GDNF (Kriegstein et al., 1998a). Another possibility is that PSP is not a survival factor for peripheral neurons but a modulator of neural phenotype. Furthermore, it is possible that GFR $\alpha$ -4 expression may be regulated at the protein, and not the mRNA, level. Finally, GFR $\alpha$ -4 mRNA is dramatically (>5-fold) down-regulated (see Figure 3.55) when neurons are put in culture which could account for the lack of responsiveness of neurons to PSP.

Taken together, these results illustrate the growing complexity of trophic interactions governing the development of organs and ganglia at different stages of development. Further studies will be required to address the physiological importance of these interactions.

# **Chapter 3**

## **Regulation of GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNA expression in the peripheral nervous system**

### **3.1. Introduction**

Neurotrophic factors are a heterogeneous group of signaling molecules that orchestrate multiple aspects of the development and maintenance of the central and peripheral nervous systems. The GDNF family of ligands, consisting of glial cell line-derived neurotrophic factor (GDNF; Lin et al., 1993a), neurturin (NTN; Kotzbauer et al., 1996), persephin (PSP; Milbrandt et al., 1998), and artemin (ART; Baloh et al., 1998a) comprise a subfamily of ligands within the TGF- $\beta$  superfamily of signaling molecules that have neurotrophic properties. Interest in the GDNF family was initially fuelled largely by the ability of all known members to support the survival of dopaminergic midbrain neurons and spinal and facial motor neurons in both *in vitro* survival and *in vivo* injury paradigms, identifying them as potential therapeutic agents in the treatment of neurodegenerative diseases (Lin et al., 1993a; Henderson et al., 1994; Oppenheim et al., 1995; Horger et al., 1998; Milbrandt et al., 1998; Baloh et al., 1998a; reviewed by Grondin and Gash, 1998).

However, the GDNF ligands also influence a broad spectrum of other neuronal populations in both the CNS and PNS. GDNF, NTN and ART support the survival of many peripheral neurons in culture, including sympathetic, parasympathetic, sensory and enteric neurons (Buj-Bello et al., 1995; Ebendal et al., 1995; Trupp et al., 1995; Kotzbauer et al., 1996; Heuckeroth et al., 1998; Baloh et al., 1998a). In contrast, PSP does not share any of these peripheral

activities but does support the survival of dopaminergic midbrain neurons and motor neurons (Milbrandt et al., 1998).

Although members of the TGF- $\beta$  superfamily, the GDNF ligands utilize a receptor system that is more similar to that observed for many cytokines, in that a common signaling component, namely the Ret tyrosine kinase (Durbec et al., 1996a; Trupp et al., 1996; Vega et al., 1996; Worby et al., 1996) plus one of a family of GPI-linked receptors (GFR $\alpha$ -1 to 4) that confers specificity (Baloh et al., 1998a; Buj-Bello et al., 1997; Jing et al., 1996; 1997; Klein et al., 1997; Naveilhan et al., 1998; Thompson et al., 1998; Treanor et al., 1996; Trupp et al., 1998; Worby et al., 1998).

Results from ligand binding and Ret phosphorylation indicate that GFR $\alpha$ -1/Ret is the preferred receptor for GDNF (Jing et al., 1996; Treanor et al., 1996), GFR $\alpha$ -2/Ret is the preferred receptor for NTN (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; Trupp et al., 1998; Widenfalk et al., 1997), GFR $\alpha$ -3/Ret is the receptor for ART (Baloh et al., 1998a) and GFR $\alpha$ -4/Ret is the receptor for PSP (Enokido et al., 1998). These *in vitro* studies together with the finding that more neurons are lost in GDNF-/- mice than in GFR $\alpha$ -1-/- mice (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996a, b; Sanchez et al., 1996) have revealed that some degree of cross-talk can occur between receptors.

Ret and the GFR $\alpha$  receptors have distinctive patterns of expression that in general accord with the known responses of neurons and other cells to their ligands (Baloh et al., 1997, 1998b; Buj-Bello et al., 1997; Creedon et al., 1997; Jing et al., 1996, 1997; Klein et al., 1997; Molliver et al., 1997; Naveilhan et al., 1998; Pachnis et al., 1993; Sanicola et al., 1997; Schuchardt et al., 1995; Suvanto et al., 1997; Treanor et al., 1996; Trupp et al., 1996; Tsuzuki et al., 1995; Vega et

al., 1996; Widenfalk et al., 1997; Worby et al., 1996, 1998). However, nothing is known about what regulates the regional and dynamic changes in the expression of these receptors in the developing nervous system.

To begin to address this issue, I have used an *in vitro* approach involving neuronal cultures and competitive RT-PCR to study the steady-state levels of mRNAs encoding ret, GFR $\alpha$ -1, GFR $\alpha$ -2 and GFR $\alpha$ -4 in several populations of PNS neurons grown under different experimental conditions.

## **3.2. Methods**

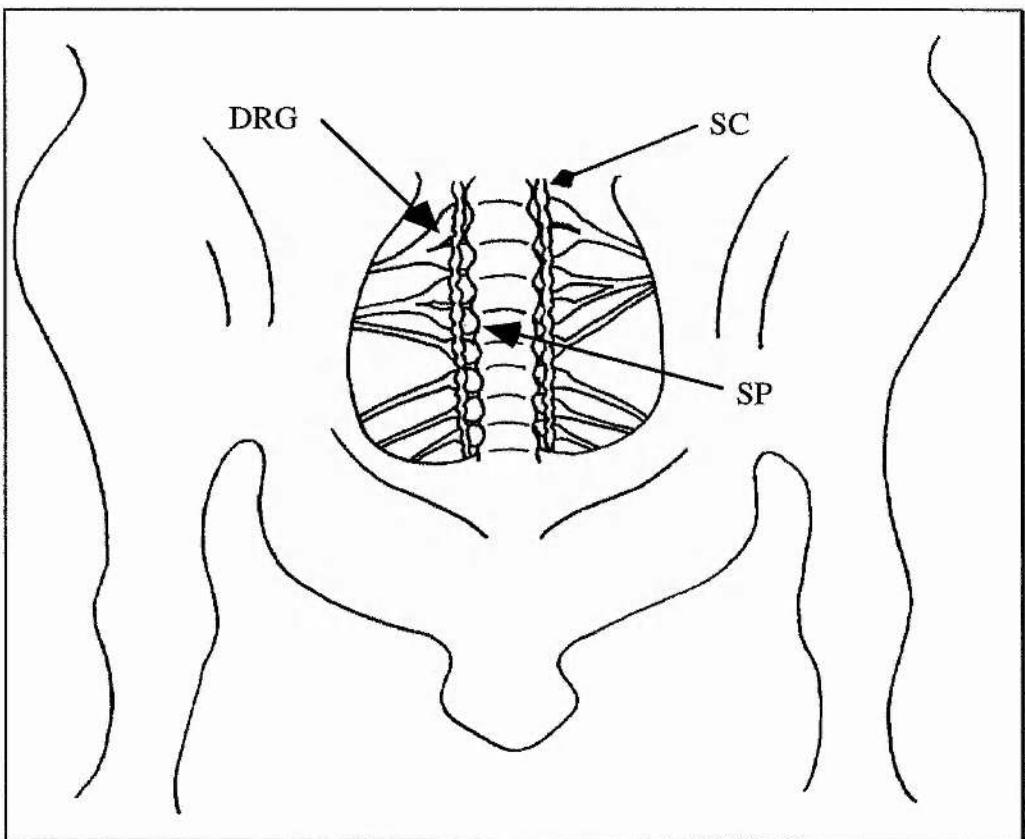
### **Neuronal cultures**

White Leghorn chicken eggs were incubated at 38°C in a forced-draft incubator. The sympathetic chain was dissected from E8, E10, E12, E14, and E16 embryos (Figure 3.1) and the nodose ciliary, and dorsomedial (DMTG) ganglia from E8 embryos (Figures 3.2 and 3.3) (Davies, 1995). The dissections were performed with electrolytically sharpened needles under sterile conditions, in a laminar flow hood, using under a stereo microscope. All dissections were done in L15 medium.

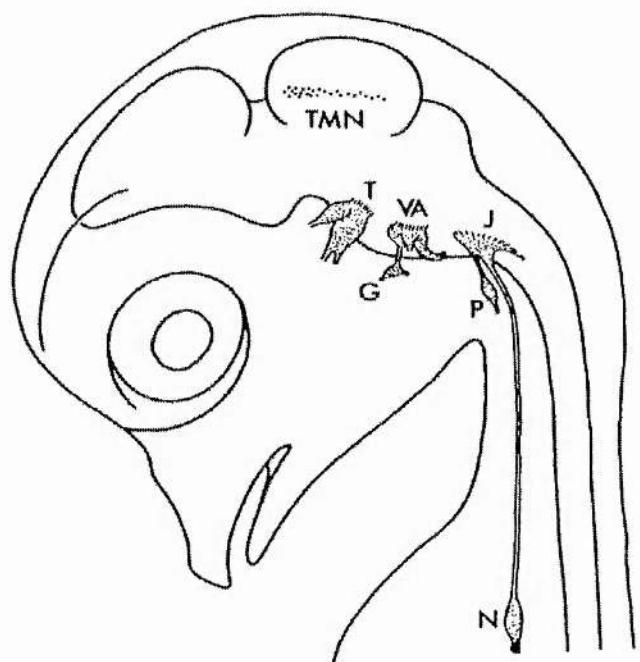
The dissected ganglia were incubated at 37°C in 0.1% trypsin/calcium-and magnesium-free Hank's balanced salt solution (HBSS) for 10, 15, 20, 22, and 25 minutes for E8, E10, E12, E14, and E16 ganglia, respectively. The trypsinized tissue was washed twice in Hank's F14 medium containing 10% heat inactivated horse serum (HIHS) and once in HBSS, after which it was mechanically dissociated by gentle trituration, in ~1ml of HBSS, using a fine-polished Pasteur pipette.

Non-neuronal cells were removed by differential sedimentation (Davies, 1988c) and the neurons (>95% pure) were plated in 35mm plastic tissue culture dishes (Nunclon) that had been pre-coated with poly-DL-ornithine (0.5mg/ml, in 0.15M borate buffer, overnight at RT) and laminin (20µg/ml, in F-14 medium for 4-12 hours in a humidified CO<sub>2</sub> incubator at 37°C). Neurons were plated at a density of 500-2,000 neurons per dish. The neurons were cultured in 2ml of Ham's F14 medium plus 10% HIHS, either with or without neurotrophic factors or different levels of KCl, in a humidified 4% CO<sub>2</sub> incubator at 37°C.

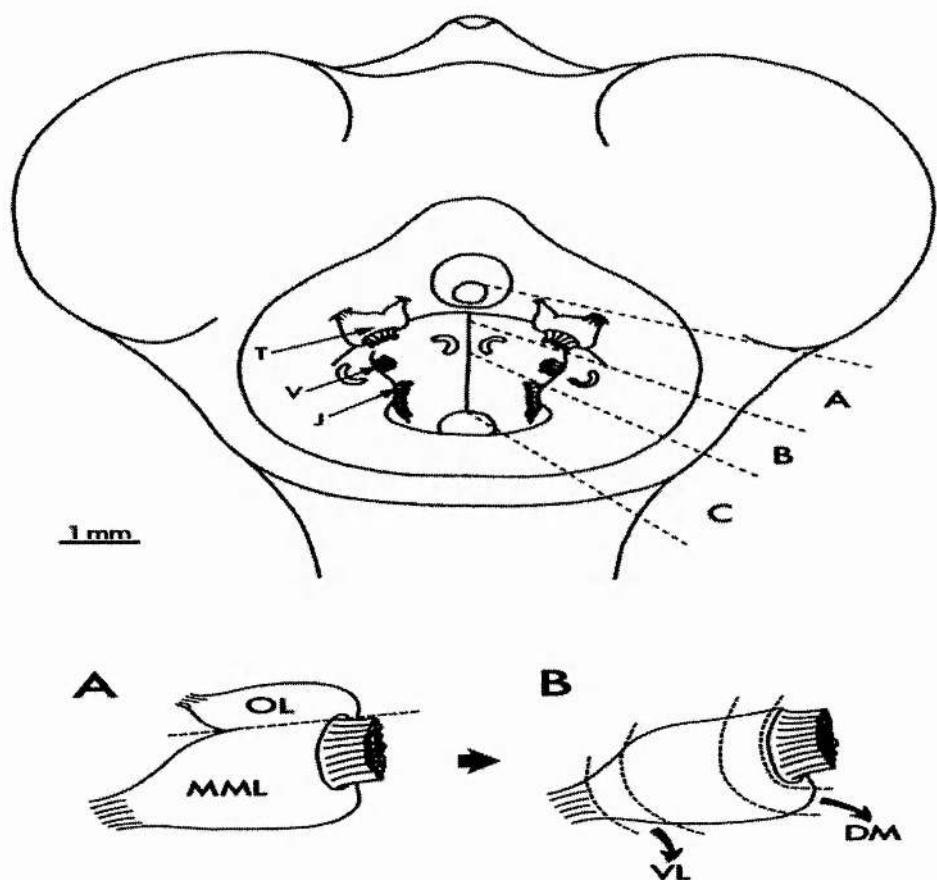
Three to six hours after plating, the number of attached neurons within a 12x12mm square in the centre of each dish was counted using an inverted phase-contrast microscope. The number of surviving cells in this same area was counted 24 and 48 hours after plating and survival was expressed as a percentage of the number of attached neurons at 3 to 6 hours.



**Figure 3.1.** Camera lucida drawing of the ventral aspect of the lumbosacral region of an E10 chick embryo after evisceration showing the location of the sympathetic ganglia. DRG: dorsal root ganglia; SC: sympathetic chain; SP: spinal cord. (Adapted from Davies, 1988c)



**Figure 3.2.** Schematic illustration of an E10 chick embryo showing the locations of populations of cranial sensory neurons. TMN: trigeminal mesencephalic nucleus; T: trigeminal ganglion; J: jugular ganglion; N: nodose ganglion; VA: vestibular acoustic ganglion; G: geniculate ganglion; P: petrosal ganglion. (Adapted from Davies and Lindsay, 1985).



**Figure 3.3.** Dorsal aspect of the cranial base after removal of the brain showing the lines for subdissecting the tissue into blocks that contain the trigeminal ganglion (T) (block A), the vestibulo-acoustic ganglion (V) and geniculate ganglion (block B), and the Jugular (J) and petrosal ganglia (block C). Subdissection of the trigeminal ganglion is achieved by separating the ophthalmic lobe (OL) from the maxillomandibular lobe (MML) along the interrupted line (A). The dorsomedial portion (DM) can be separated from the ventrolateral as shown (B). (Modified from Davies, 1988c).

### **Measurement of GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNA levels**

A competitive reverse transcription/polymerase chain reaction (RT/PCR) technique was used to measure the levels of mRNAs encoding GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret in total RNA extracted from freshly purified neurons and neurons that had been cultured under different experimental conditions. To compare the relative levels of the receptors in different samples, the cRNA competitor template for the ubiquitous, constitutively expressed L27 ribosomal protein was also included in the reverse transcription reactions. RNA extraction, RT and amplification were carried out as described in chapter 2. The PCR products were resolved on 8% polyacrylamide gels, autoradiographed and quantified as described previously (chapter 2).

## Results

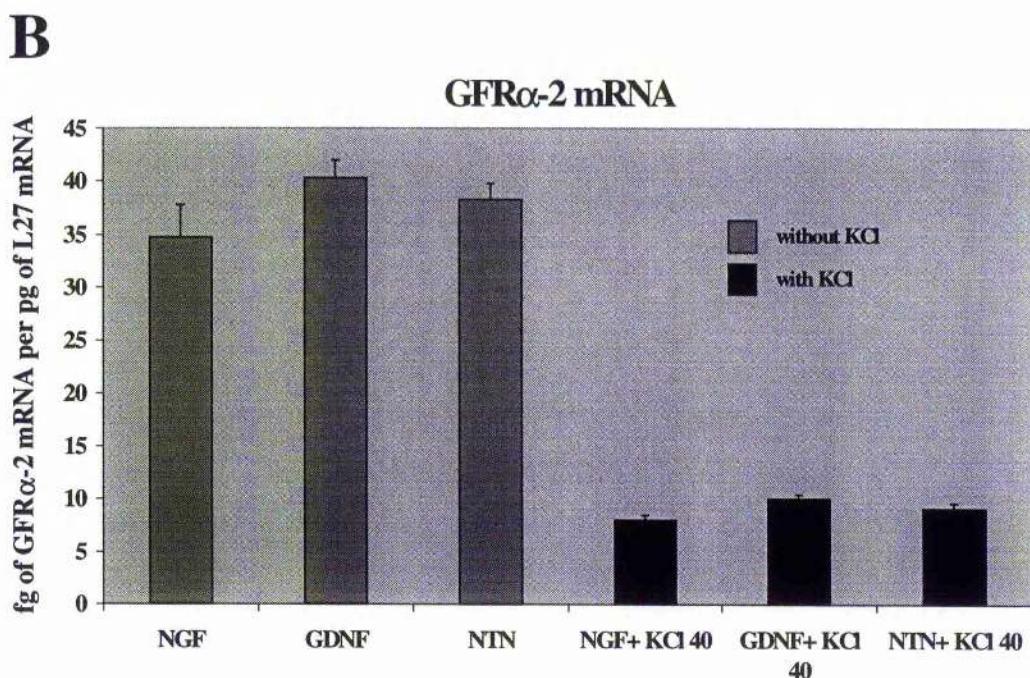
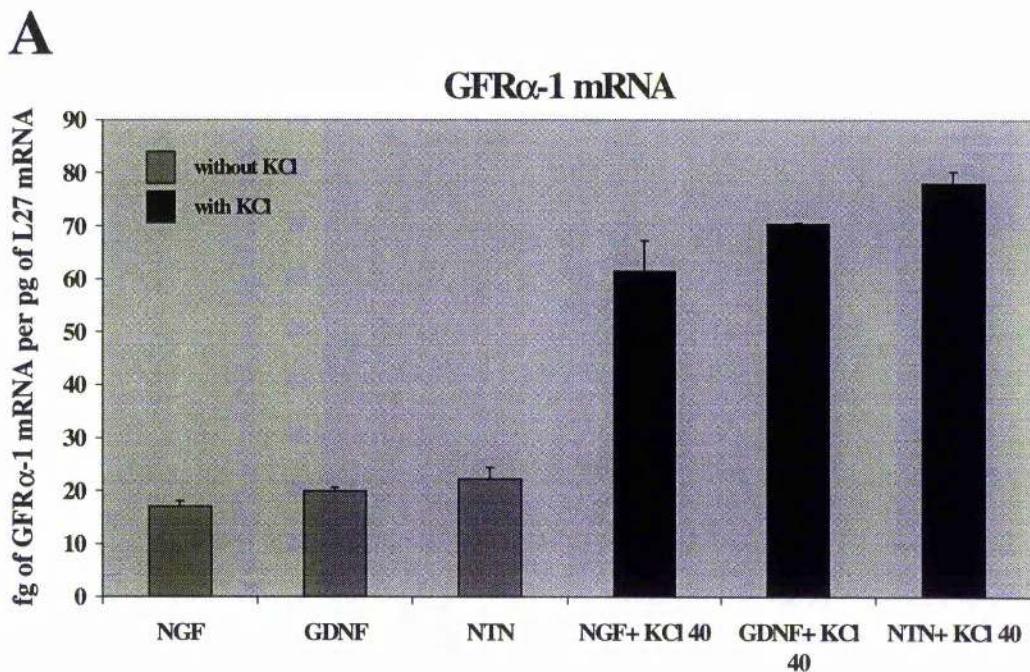
### I. GFR $\alpha$ -1, GFR $\alpha$ -2 and ret mRNA expression is not regulated by GDNF and NTN in PNS neurons

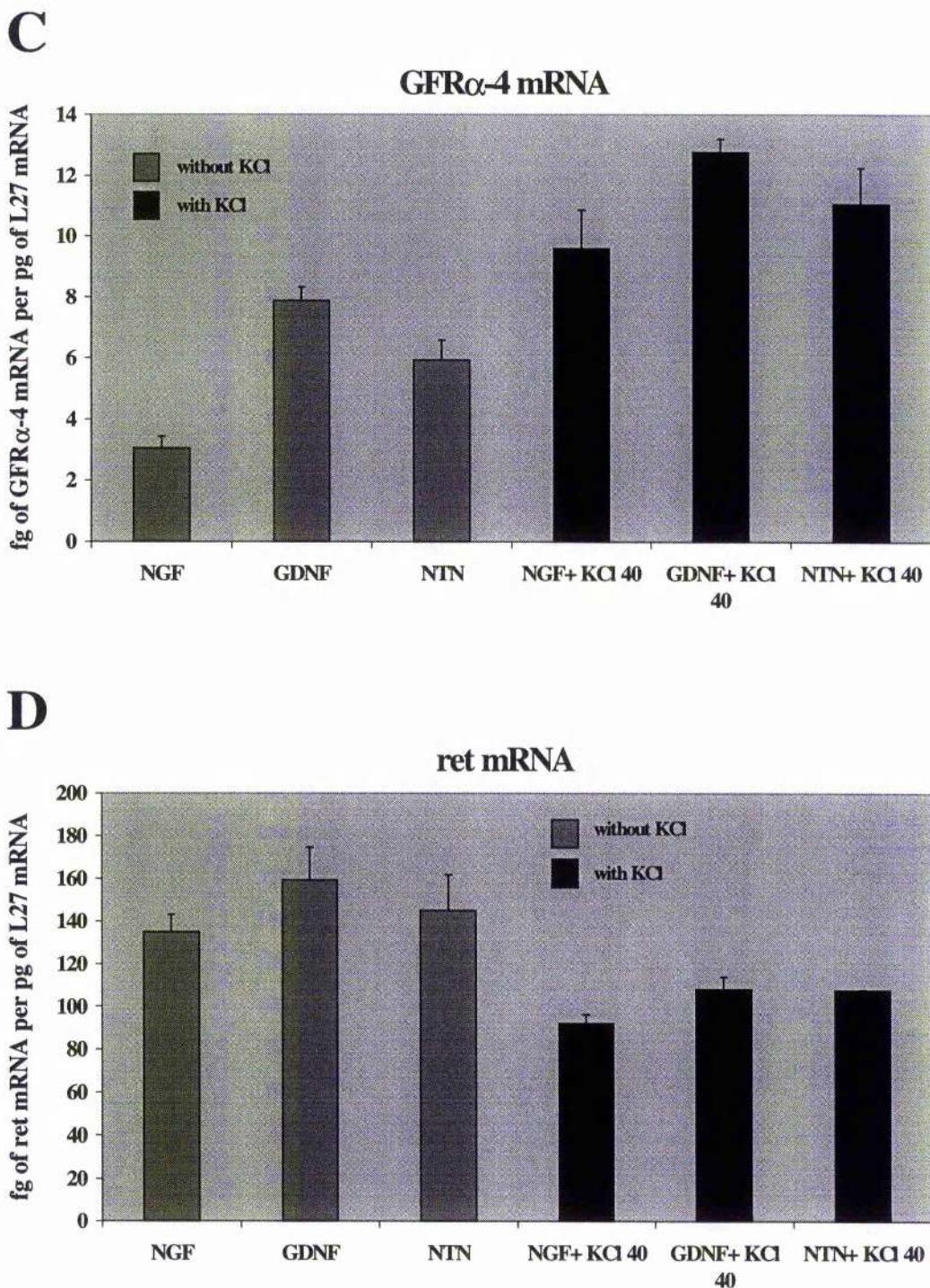
Because numerous previous studies have shown that neurotrophins increase the expression of mRNAs encoding their receptors in neurons, I studied the effects of GDNF and NTN on GFR $\alpha$ -1, GFR $\alpha$ -2 and ret mRNA expression in E12 sympathetic and E8 nodose, ciliary, SCG, and DMTG neurons.

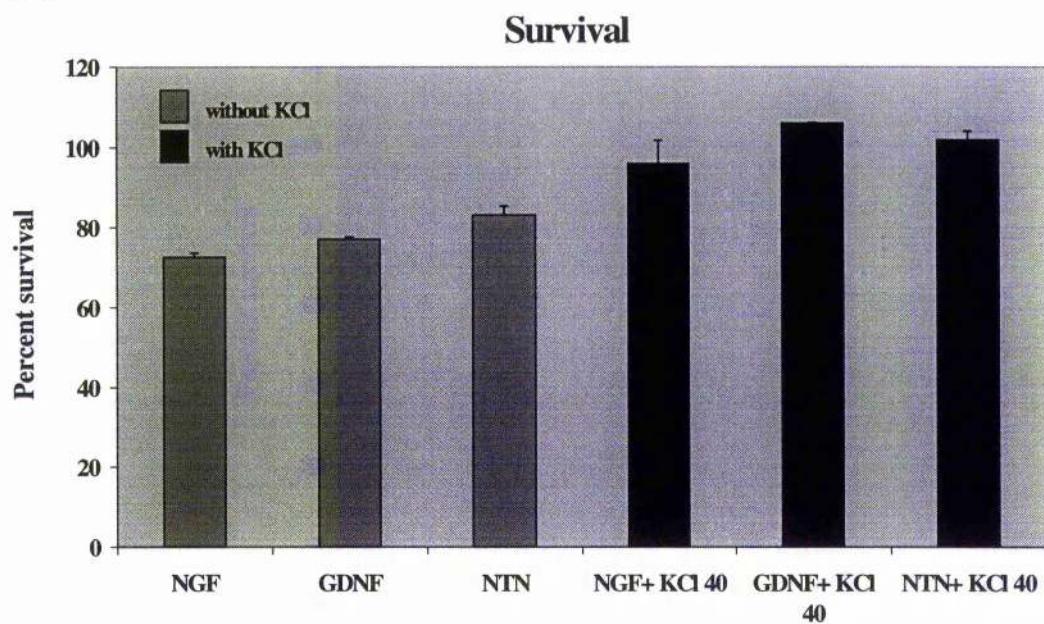
Like NGF, GDNF and NTN promote the *in vitro* survival of the great majority of E12 sympathetic neurons (Figure 3.4E). The levels of GFR $\alpha$ -1, GFR $\alpha$ -2 and ret mRNAs expressed by sympathetic neurons cultured for 24 hours with GDNF or NTN were very similar to those cultures supplemented with NGF (Figure 3.4). Similar results were obtained in cultures supplemented with GDNF and NTN at concentrations ranging from 5 to 500ng/ml (data not shown).

The parasympathetic neurons of the ciliary ganglion are supported by CNTF, GDNF and NTN in culture. These each factors promote the survival of the majority of neurons. Although the neurons cultured with CNTF had distinct expression patterns from those cultured with GDNF and NTN, GDNF and/or NTN at concentrations ranging from 10 to 300ng/ml, either with or without CNTF, yielded very similar data for the expression of all three receptors, indicating the lack of regulation under these conditions (data not shown).

E8 DMTG neurons are well supported by NGF but hardly at all (around 10%) by GDNF or NTN. In NGF containing cultures supplemented with GDNF and/or NTN at concentrations of 30 and 300 ng/ml, the levels of GFR $\alpha$ -1,





**E**

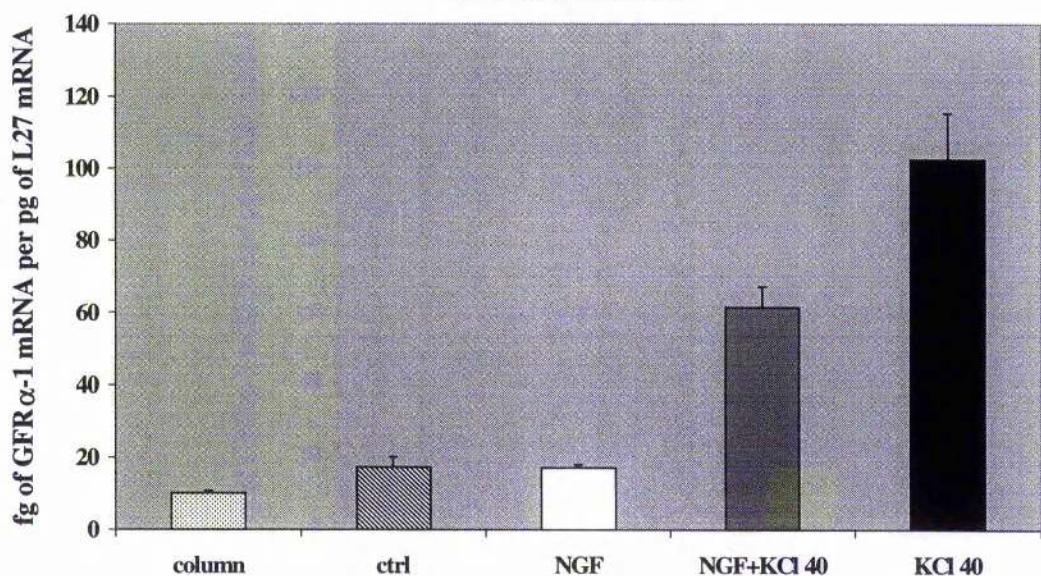
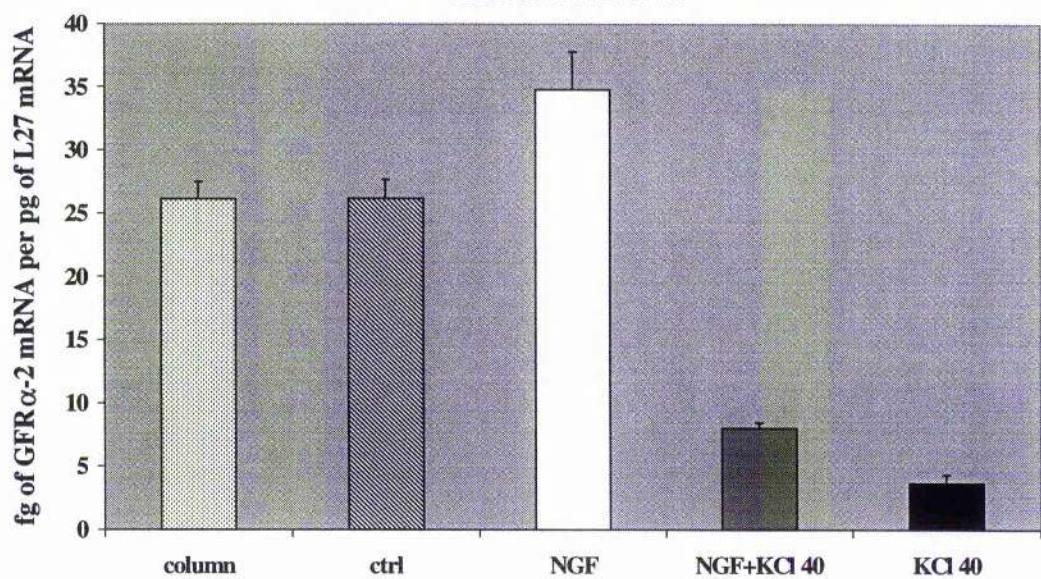
**Figure 3.4.** Bar charts of the percent survival and the levels of GFR $\alpha$  and ret mRNAs relative to L27 mRNA in cultures of purified E12 sympathetic neurons incubated for 24 hours in basal medium (control) and medium containing 10 ng/ml NGF (NGF), GDNF (GDNF) and NTN (NTN) or 40 mM KCl plus 10 ng/ml NGF (NGF + KCl), GDNF (GDNF+KCl) and NTN (NTN+KCl). The mean  $\pm$  standard error of 2 separate assays are represented by each bar.

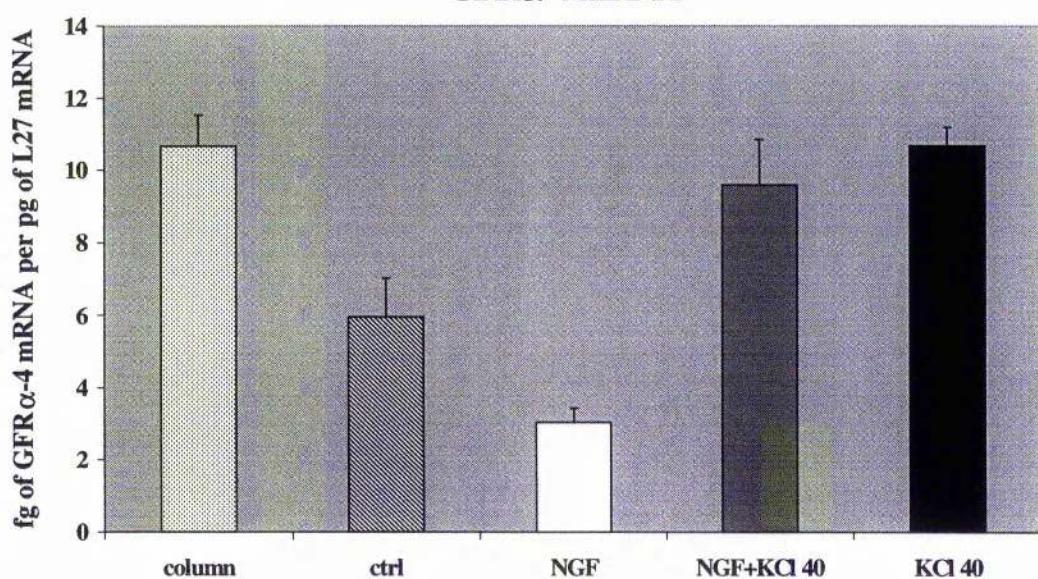
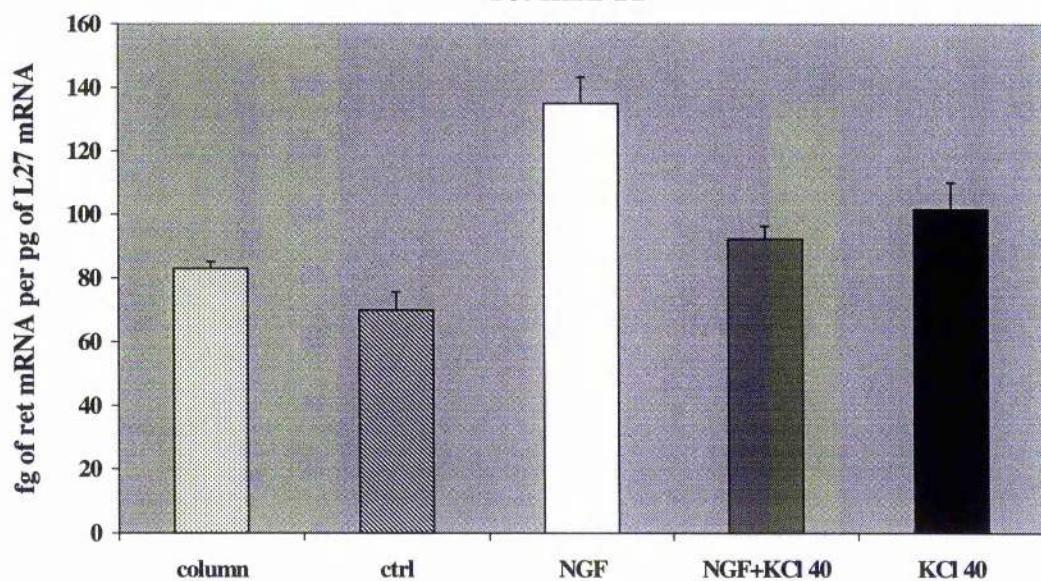
GFR $\alpha$ -2 and ret mRNAs are similar to those cultures supplemented with NGF alone (data not shown).

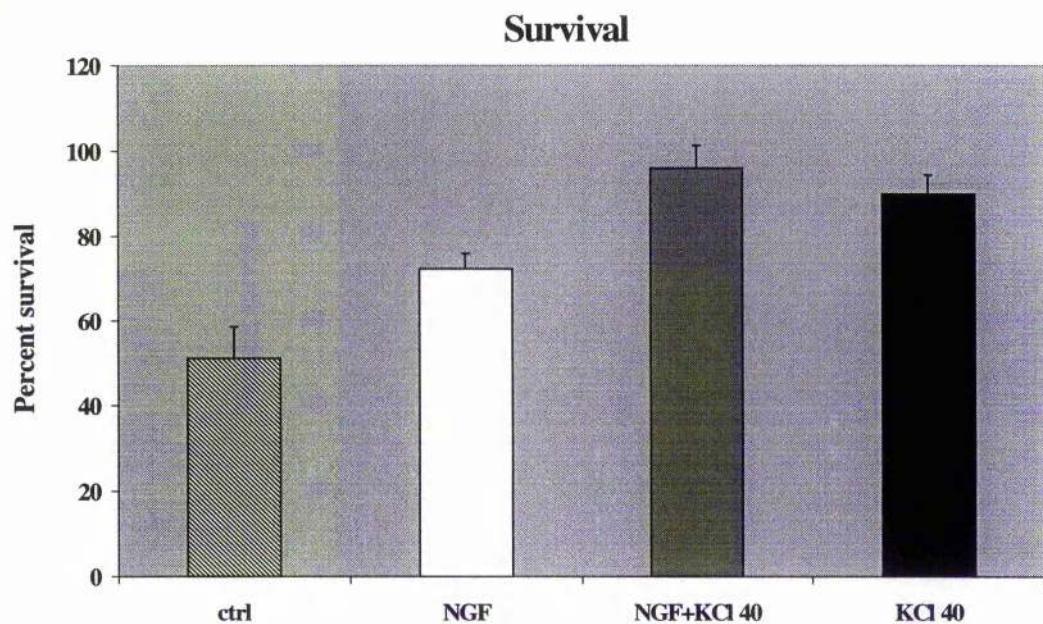
Similar results were obtained from the study of E8 SCG and E8 nodose neurons. These data suggest that neither GDNF nor NTN significantly affect the expression of mRNAs encoding their ligands in developing sympathetic neurons. Preliminary experiments also indicate that PSP and ART do not regulate the expression of GFR $\alpha$  and ret mRNAs in E12 sympathetic neurons (data not shown).

## **II. Regulation of GFR $\alpha$ and ret mRNA expression by depolarization**

To determine if depolarization affects GFR $\alpha$  and ret mRNA expression, I exposed cultures of E12 sympathetic neurons to depolarizing levels of potassium ions (40mM KCl) for a period of 24 hours. Figure 3.5 shows that a depolarizing level of KCl (40mM) caused a marked increase in the level of GFR $\alpha$ -1 and GFR $\alpha$ -4 mRNAs, a marked decrease in the level of GFR $\alpha$ -2 mRNA and a slight decrease in ret mRNA. Neurons grown for the same length of time in medium containing basal KCl (5mM) and supplemented with NGF expressed similar levels of GFR $\alpha$ -1, GFR $\alpha$ -2 and ret mRNAs to those measured in freshly isolated neurons prior to plating, whereas GFR $\alpha$ -4 mRNA expression decreased with time in culture (Figures 3.5 and 3.6). The marked effects of 40mM KCl on GFR $\alpha$ -1, GFR $\alpha$ -2 and GFR $\alpha$ -4 mRNAs were observed in both the presence and absence of NGF but were more pronounced in its absence. Compared with the levels in control cultures, cultures supplemented with 40mM KCl alone exhibited a 6-fold increase in the level of GFR $\alpha$ -1 mRNA, a 6-fold decrease in the level of GFR $\alpha$ -2 mRNA, a 3-fold increase in the level of GFR $\alpha$ -4 mRNA and a slight decrease in the level of ret mRNA. Although the levels of GFR $\alpha$  and ret mRNAs in Figure 3.5 are shown relative to L27 mRNA to facilitate direct comparison of the relative levels of GFR $\alpha$ -1 and GFR $\alpha$ -2 mRNAs in purified neurons freshly isolated from the sedimenting column and neurons that had been maintained in culture, very similar results were observed when the levels of GFR $\alpha$  and ret mRNAs were expressed relative to neuron number (data not shown). The levels of GFR $\alpha$  and ret mRNAs in neurons grown with NGF plus 40mM KCl were also very similar to the levels

**A****GFR $\alpha$ -1 mRNA****B****GFR $\alpha$ -2 mRNA**

**C****GFR $\alpha$ -4 mRNA****D****ret mRNA**

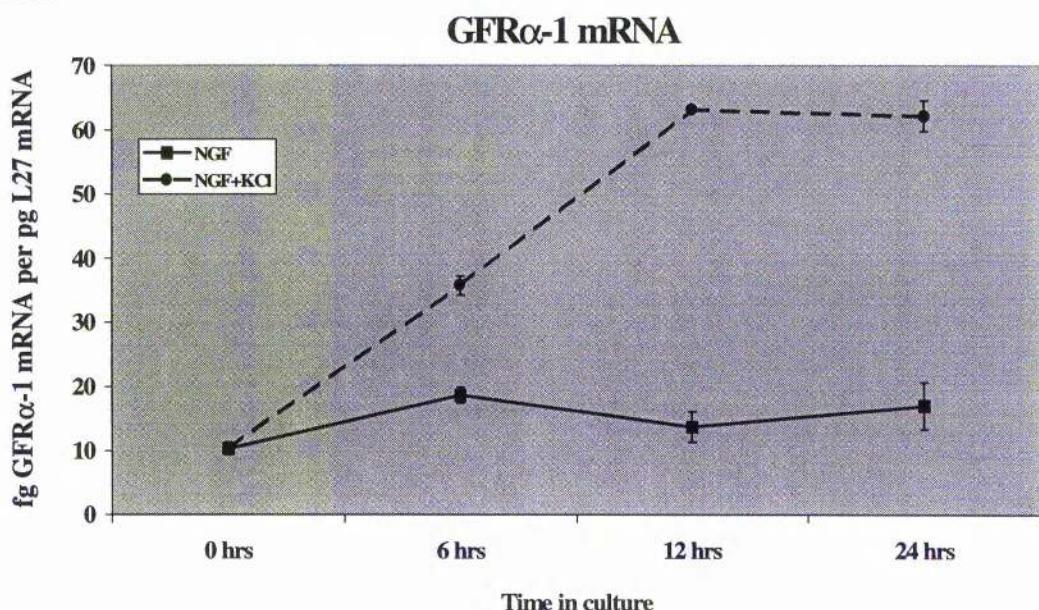
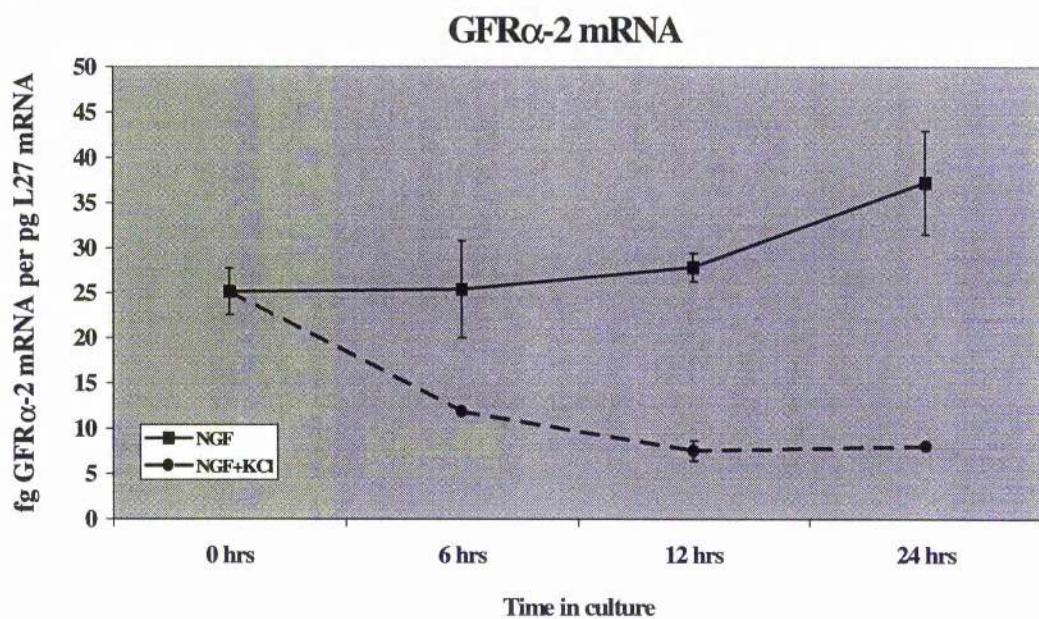
**E**

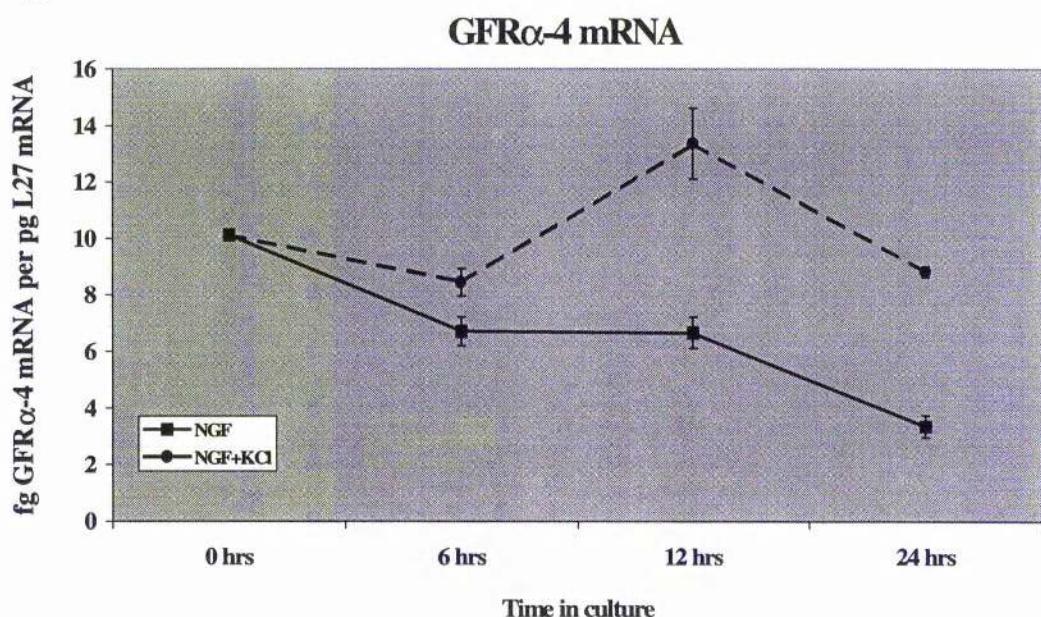
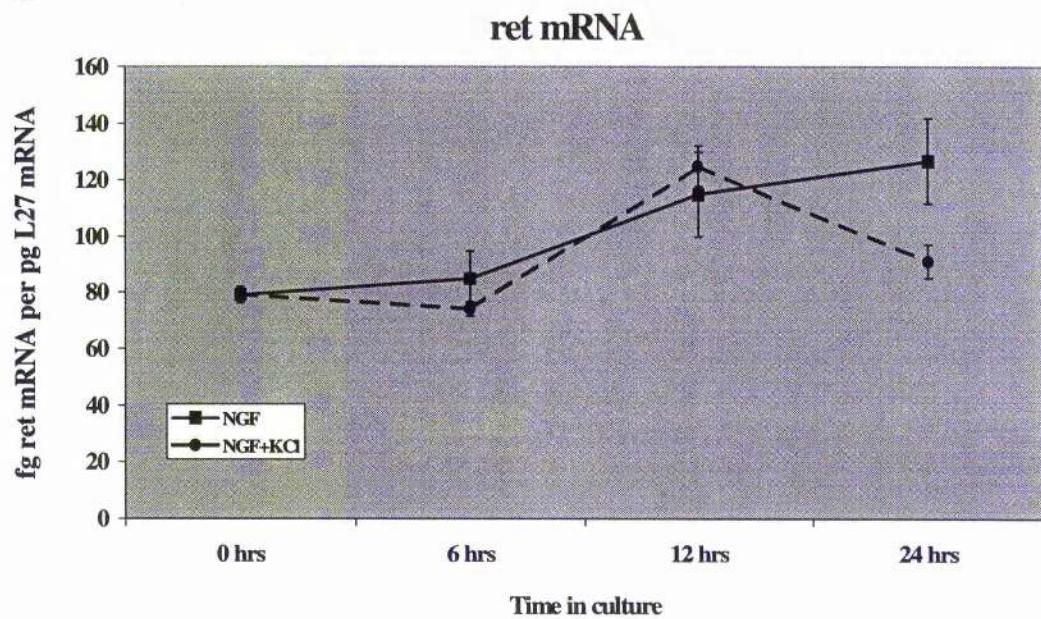
**Figure 3.5.** Bar charts of the percent survival and the levels of GFR $\alpha$  and ret mRNAs relative to L27 mRNA in cultures of purified E12 sympathetic neurons incubated for 24 hours in basal medium (control) and medium containing 10 ng/ml NGF (NGF), 40 mM KCl (KCl) or 40 mM KCl plus 10 ng/ml NGF (NGF + KCl). For comparison, the levels of GFR $\alpha$ -1 and ret mRNAs relative to L27 mRNA in purified E12 sympathetic neurons freshly isolated from the sedimenting column (column) are also shown. The mean  $\pm$  standard error of 6 separate assays are represented by each bar.

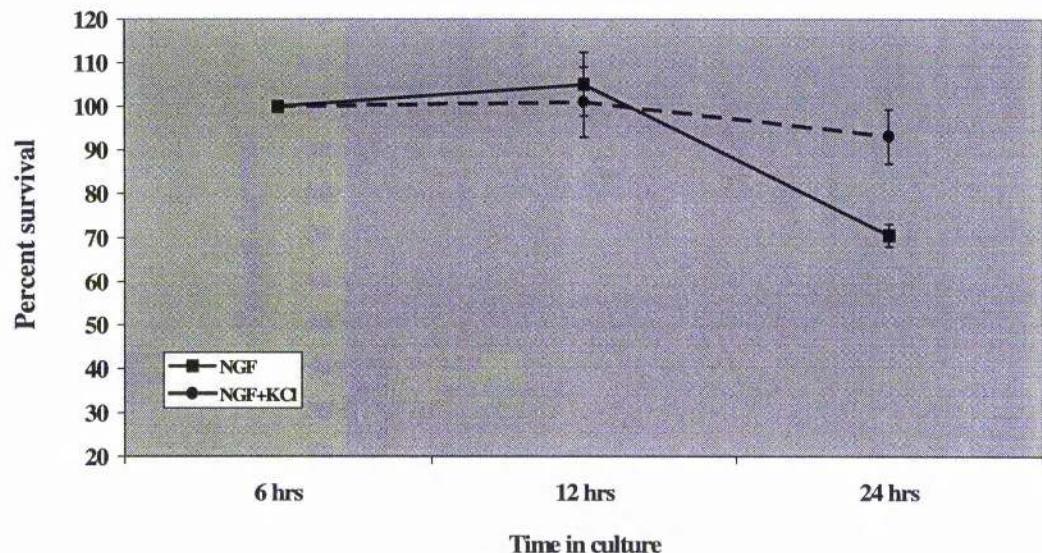
of these mRNAs in neurons grown with GDNF or NTN plus 40mM KCl (Figure 3.4).

To determine how rapidly depolarization affects the expression of GFR $\alpha$  mRNAs, I measured the levels of these mRNAs at time intervals in E12 sympathetic neuron cultures grown in NGF-supplemented medium containing KCl at either 5 mM or 40 mM. Figure 3.6 shows that within 6 hours of exposure to 40 mM KCl there were marked changes in the level of GFR $\alpha$ -1 and GFR $\alpha$ -2 mRNAs, with less pronounced changes in the levels of GFR $\alpha$ -4 and ret mRNAs. These changes in the expression of GFR $\alpha$ -1 and GFR $\alpha$ -2 mRNAs became more dramatic after 12 hours exposure to 40 mM KCl, and there were no further changes after 24 hours exposure. The changes in the expression of GFR $\alpha$ -4 and ret mRNAs were most pronounced after 48hrs exposure, the last time-point examined. The finding that all neurons were surviving 12 hours after plating in cultures containing NGF alone and NGF plus 40 mM KCl (Figure 3.6), demonstrates that 40 mM KCl has a direct effect on GFR $\alpha$ -1, GFR $\alpha$ -2 and GFR $\alpha$ -4 mRNA expression rather than selectively supporting the survival of a subset of neurons that has high levels of GFR $\alpha$ -1 and GFR $\alpha$ -4 mRNAs, and low levels of GFR $\alpha$ -2 mRNA.

To determine if depolarization exerts a similar effect on GFR $\alpha$  and ret mRNA expression throughout sympathetic neuron development, I grew sympathetic neurons at intervals from E8 to E16 in medium containing 40 mM KCl, with or without NGF, or in medium containing 5 mM KCl plus NGF to sustain neuronal survival for a 24 hour period. Figure 3.7 shows that at all ages studied the level of GFR $\alpha$ -1 mRNA was much higher and the level of GFR $\alpha$ -2 mRNA much lower in cultures containing 40 mM KCl compared with cultures containing 5 mM KCl.

**A****B**

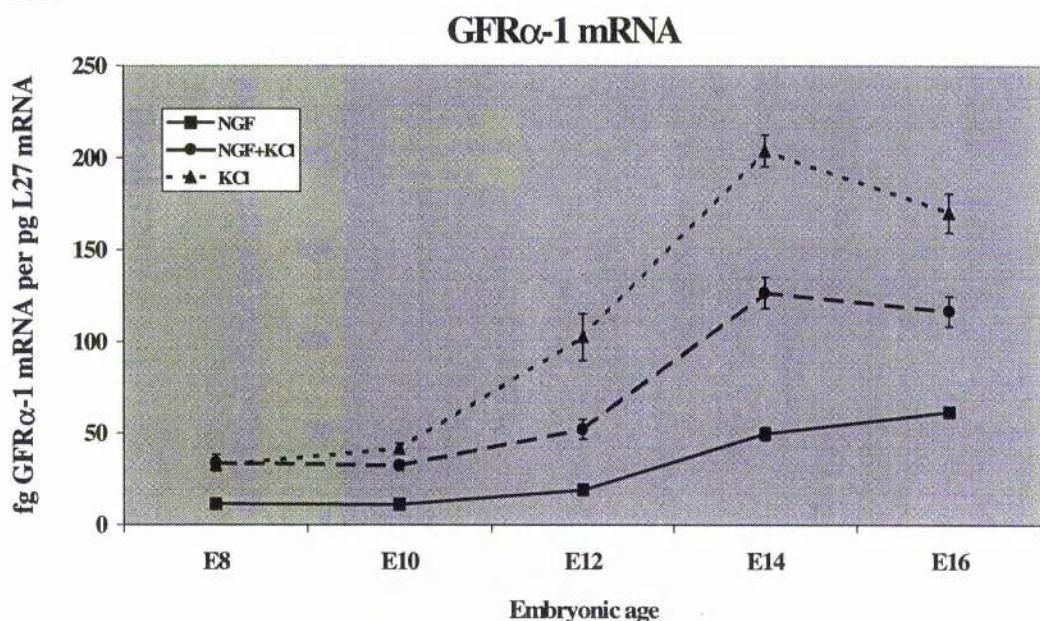
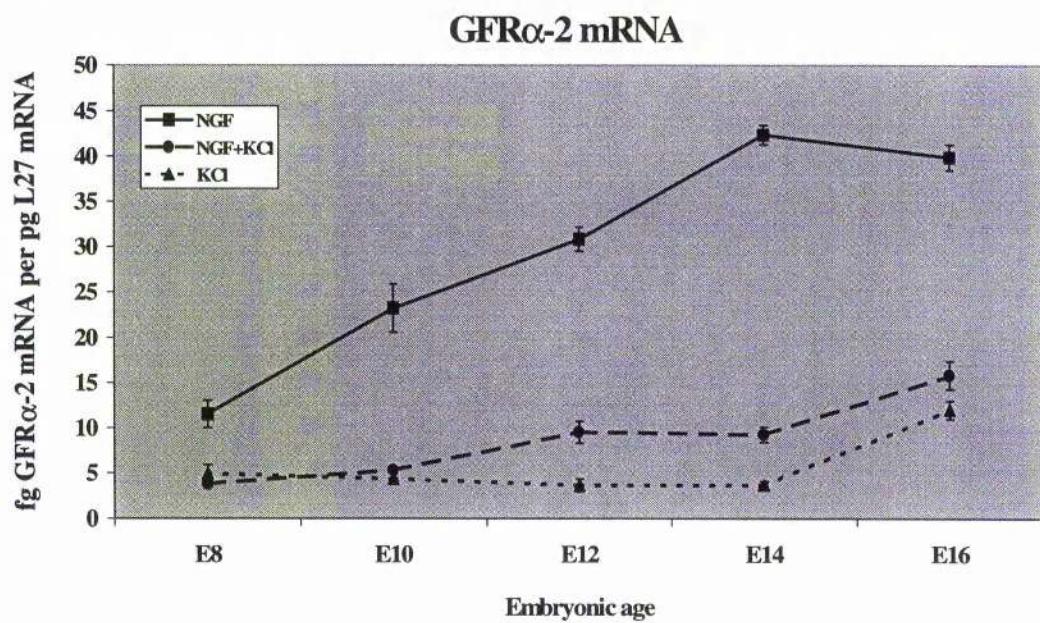
**C****D**

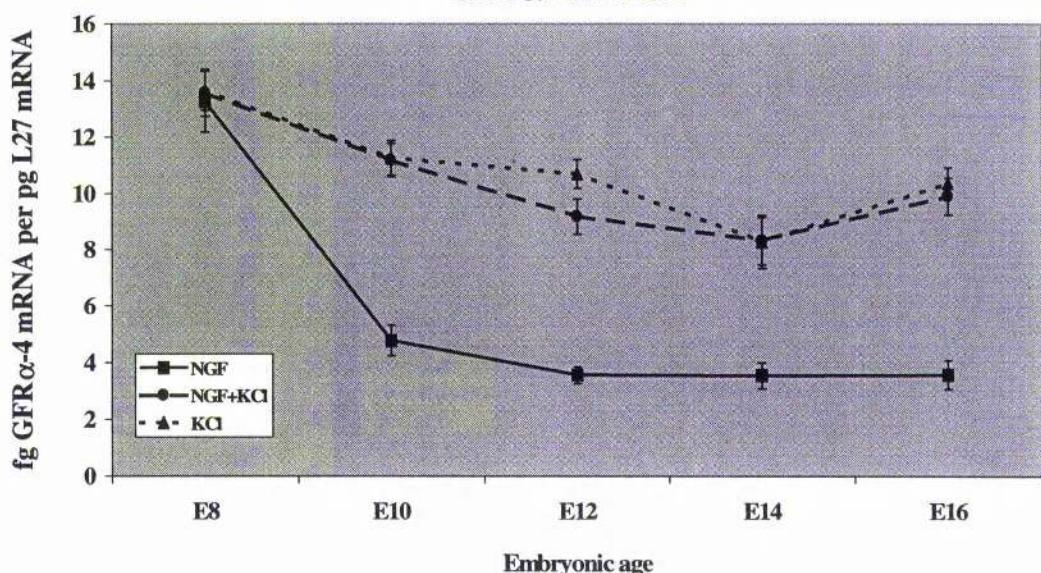
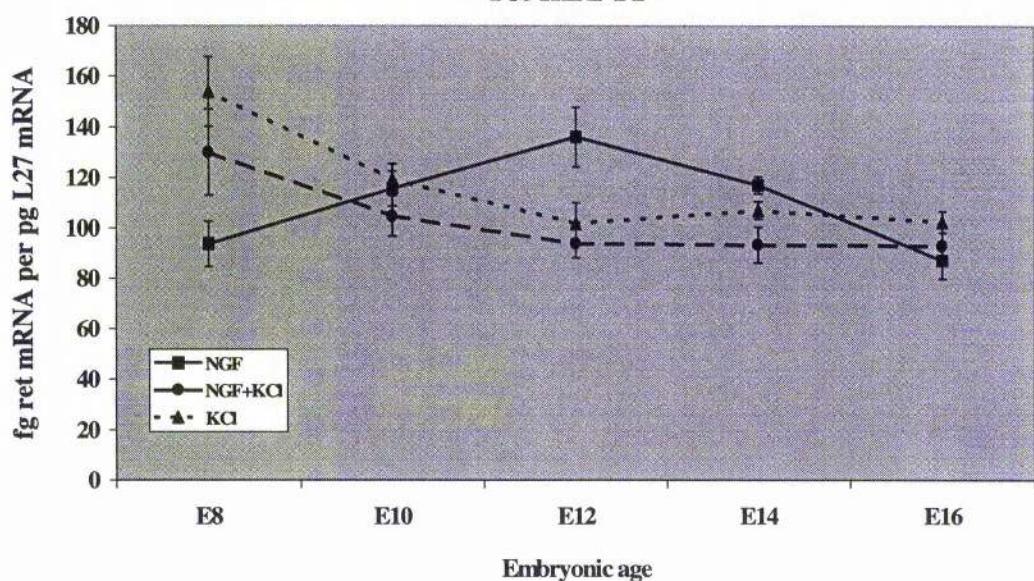
**E****Survival**

**Figure 3.6.** Bar charts of the percent survival and graphs of the levels of GFR $\alpha$  and ret mRNAs relative to L27 mRNA in cultures of purified E12 sympathetic neurons incubated for different times in medium containing 10 ng/ml NGF (NGF) or 40 mM KCl plus 10 ng/ml NGF (KCl + NGF). The zero time point shows the levels of GFR $\alpha$  and ret mRNAs relative to L27 mRNA in purified E12 sympathetic neurons freshly isolated from the sedimenting column. The mean  $\pm$  standard error of 2 separate assays are represented by each data point.

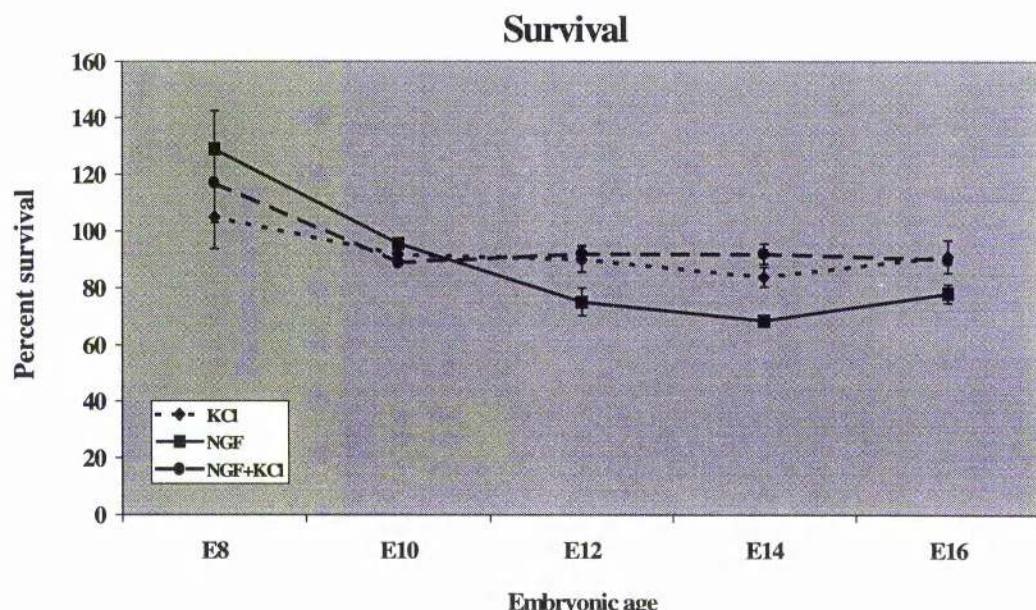
With the exception of E8 cultures, the level of GFR $\alpha$ -4 mRNA expression was higher in cultures supplemented with 40mM KCl. In contrast to the marked effects of 40 mM KCl on GFR $\alpha$  mRNA expression in sympathetic neurons cultured at stages throughout their embryonic development, 40 mM KCl did not have a consistent effect on ret mRNA expression in these cultures (Figure 3.7). The levels of ret mRNA in cultures containing 40 mM KCl were very similar to those containing 5 mM KCl at E10, E14 and E16, and were slightly higher at E8 and slightly lower at E12. These results suggest that depolarization does not have a marked effect on ret mRNA expression in developing sympathetic neurons.

At all ages studied, the majority of neurons survived under each experimental condition (Figure 3.7). The increase in the total number of neurons in E8 cultures between 6 and 24 hours incubation was due to the proliferation of sympathetic neuroblasts present in these early cultures (Ernsberger et al., 1989b).

**A****B**

**C****GFR $\alpha$ -4 mRNA****D****ret mRNA**

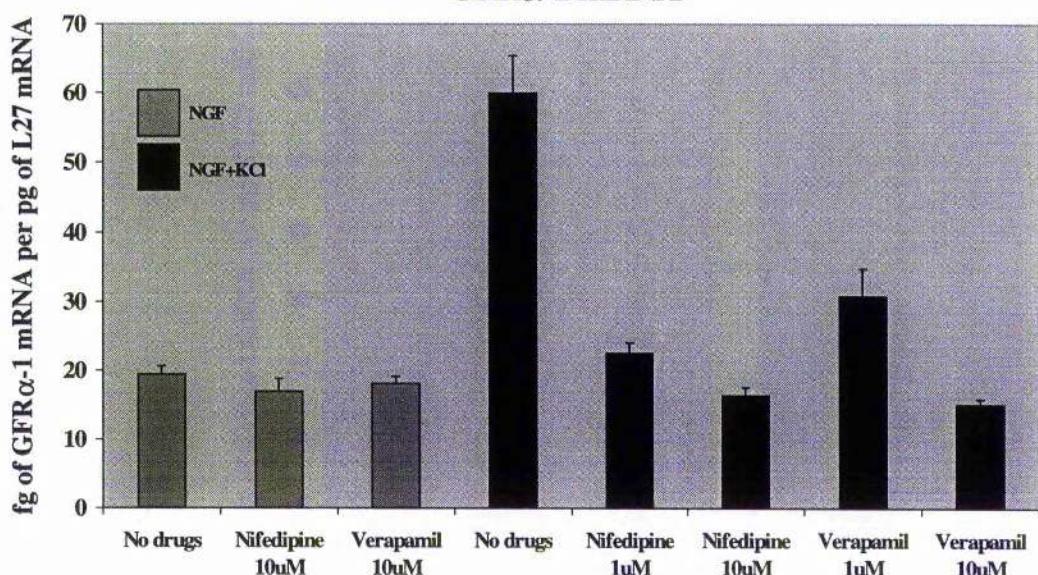
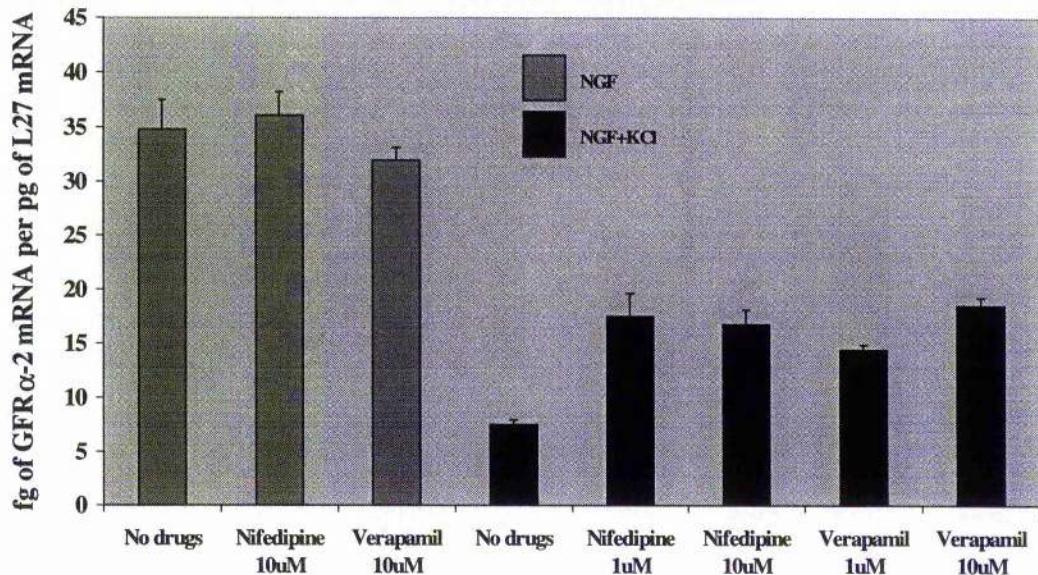
**E**

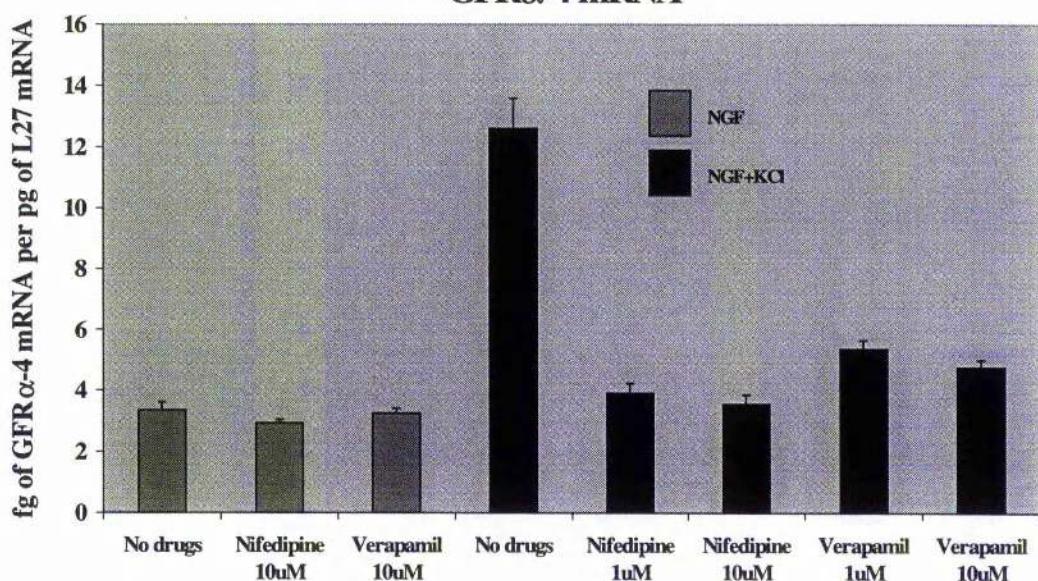
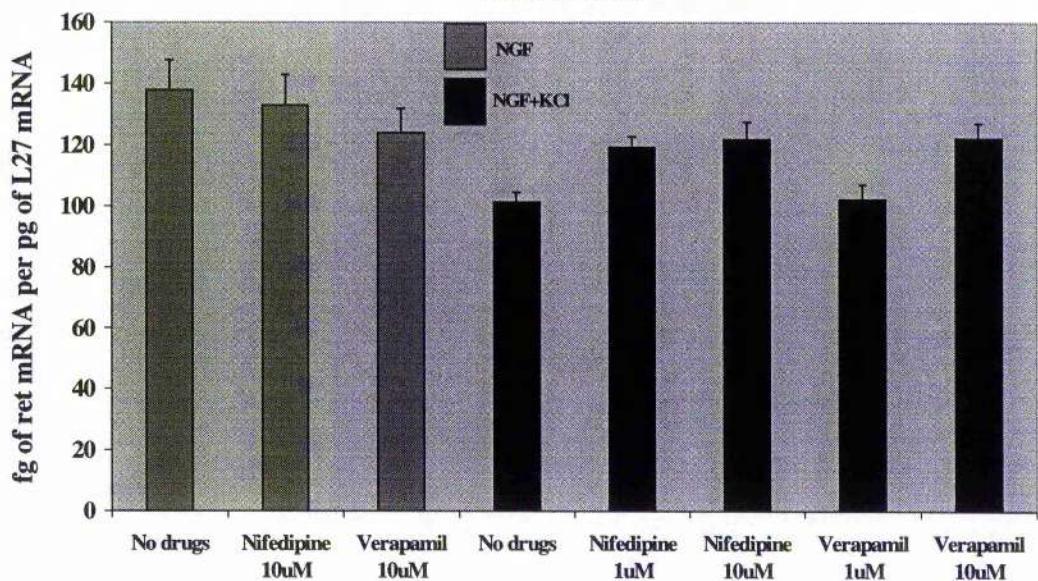


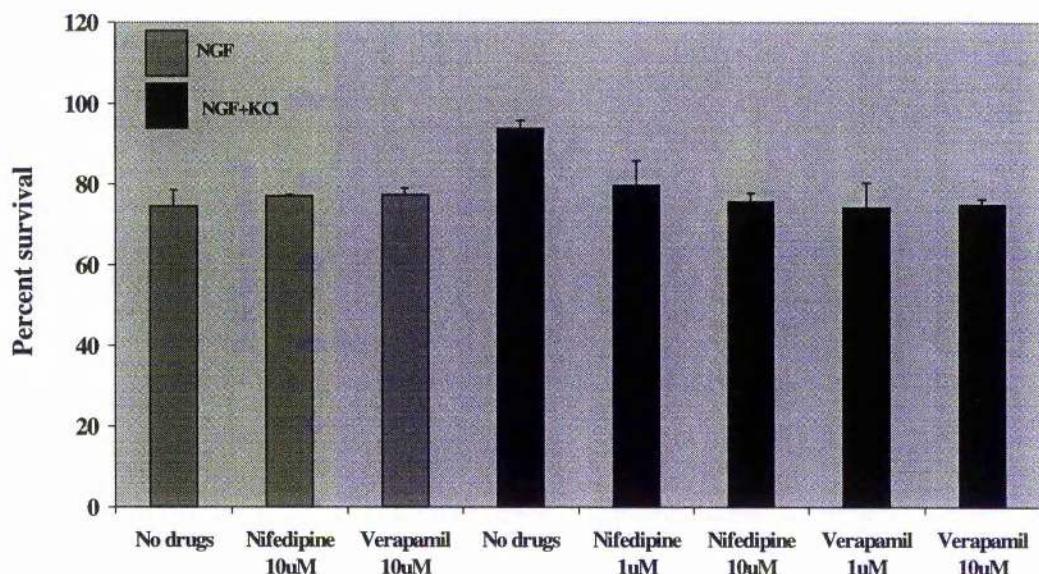
**Figure 3.7.** Graphs of the percent survival and the levels of GFR $\alpha$  and ret mRNAs relative to L27 mRNA in cultures of purified E8, E10, E12, E14 and E16 sympathetic neurons incubated for 24 hours in medium containing 10 ng/ml NGF (NGF), 40 mM KCl (KCl) or 40 mM KCl plus 10 ng/ml NGF (NGF + KCl). The mean  $\pm$  standard error of 2 separate assays are represented by each data point.

### **III. The effects of elevated KCl on GFR $\alpha$ mRNA expression depends on Ca $^{2+}$ influx through voltage-sensitive L-type Ca $^{2+}$ channels**

To determine if the marked changes in GFR $\alpha$  mRNA expression caused by depolarizing levels of KCl were due to Ca $^{2+}$  influx through voltage-sensitive Ca $^{2+}$  channels, I investigated if these changes could be prevented by the L-type Ca $^{2+}$  channel antagonists nifedipine and verapamil. E12 sympathetic neurons were grown for 24 hours in NGF-supplemented medium containing either basal KCl (5mM) or a depolarizing level of KCl (40mM) with and without these drugs. Figure 3.8 shows that nifedipine and verapamil completely inhibited the 40mM KCl induced increase in GFR $\alpha$ -1 and GFR $\alpha$ -4 mRNAs, the slight decrease in ret mRNA and partially inhibited the 40mM KCl induced decrease in GFR $\alpha$ -2 mRNA. Neither of these drugs affected the levels of GFR $\alpha$  and ret mRNAs in medium containing basal levels of KCl. These results suggest that the effects of depolarizing levels of KCl on GFR $\alpha$  and ret mRNA expression are mediated by Ca $^{2+}$  influx through voltage-sensitive L-type Ca $^{2+}$  channels. Neither nifedipine nor verapamil affected the survival of neurons incubated with NGF, but did inhibit the slightly enhanced survival of neurons grown with NGF plus 40mM KCl. This shows that these drugs are not neurotoxic at the concentrations used and that the survival-promoting effects of 40mM KCl are also due to Ca $^{2+}$  influx through voltage-sensitive Ca $^{2+}$  channels, as shown previously (Collins and Lile, 1989; Koike et al., 1989; Koike and Tanaka, 1991; Larmet et al., 1992).

**A****GFR $\alpha$ -1 mRNA****B****GFR $\alpha$ -2 mRNA**

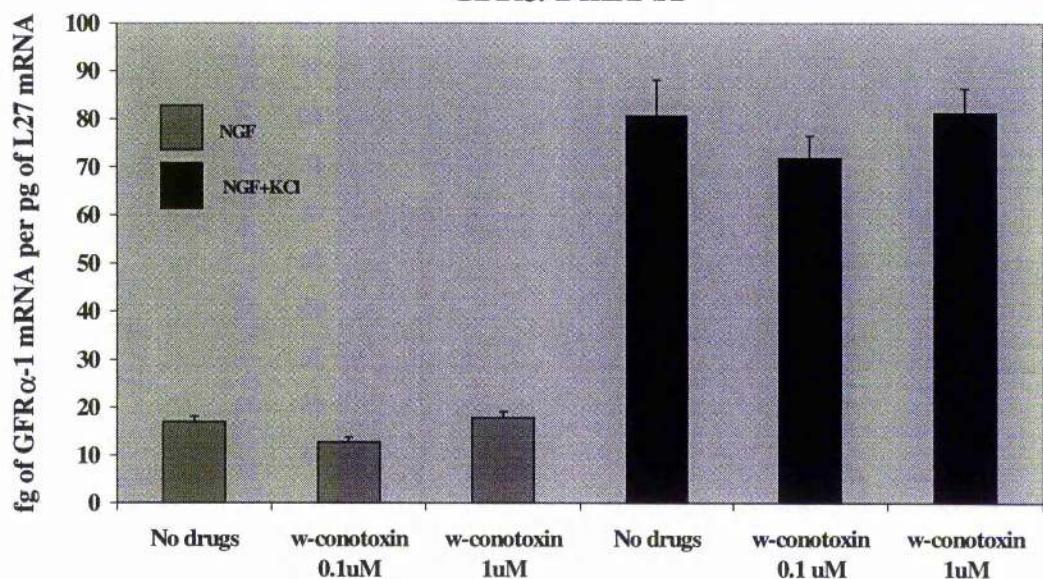
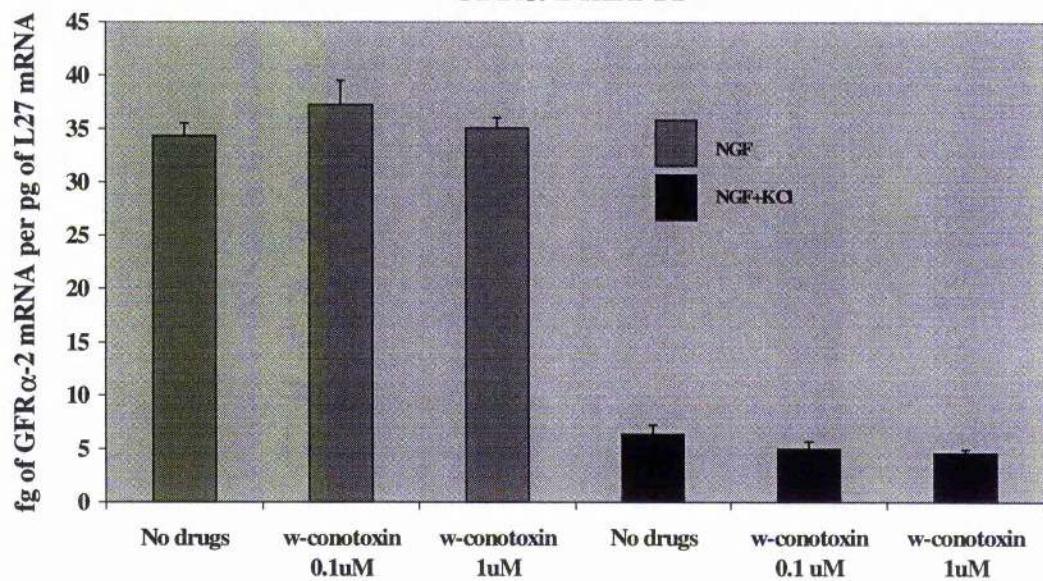
**C****GFR $\alpha$ -4 mRNA****D****ret mRNA**

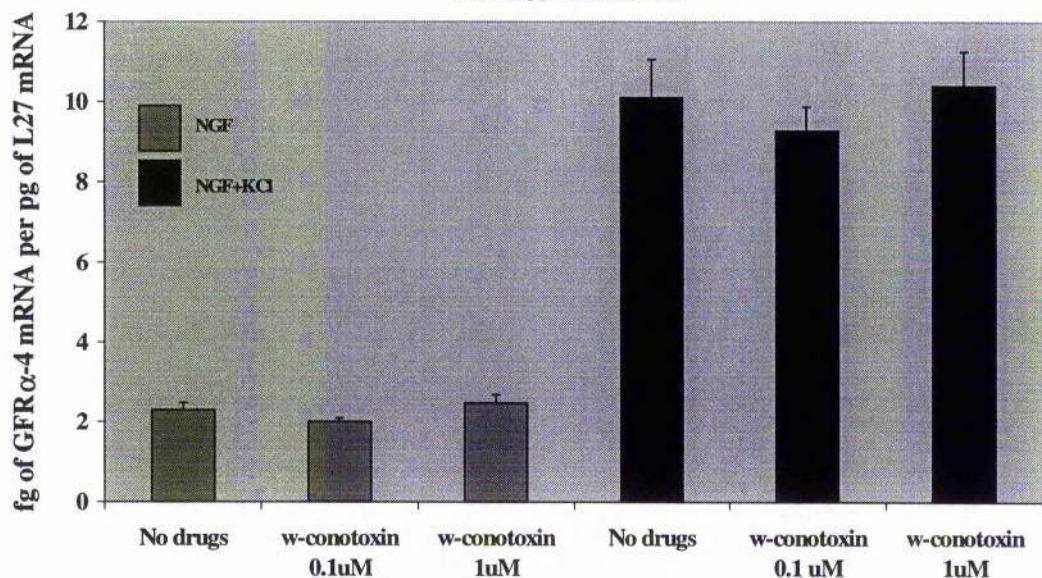
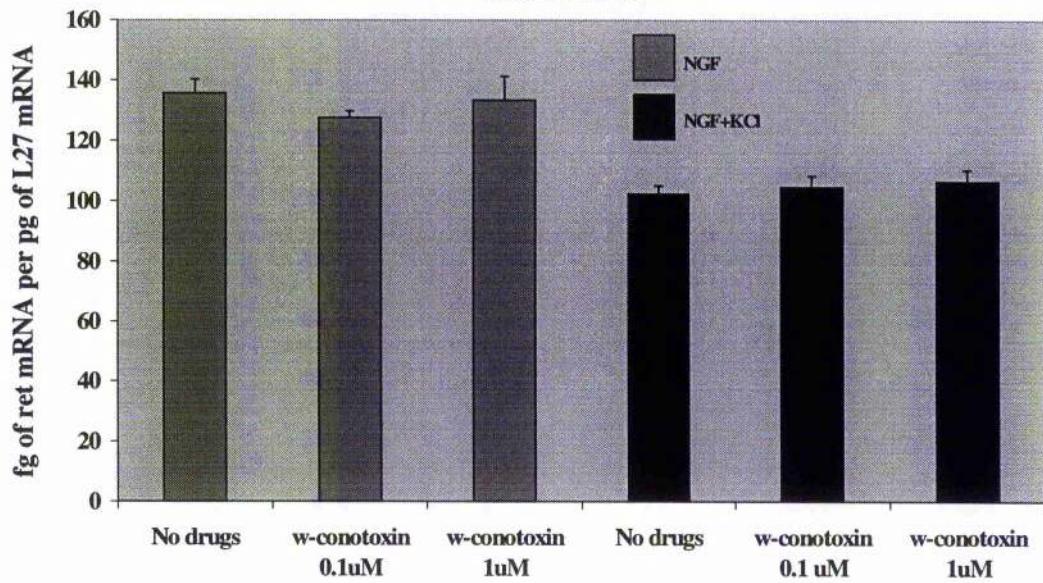
**E****Survival**

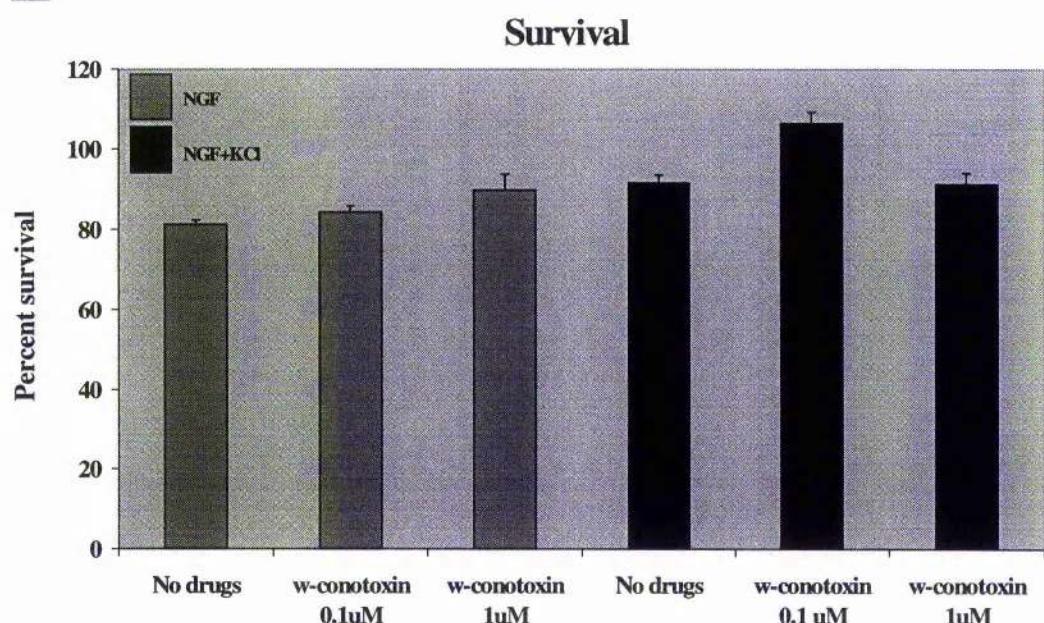
**Figure 3.8.** Bar charts of the percent survival and the levels of GFR $\alpha$  and ret mRNAs relative to L27 mRNA in cultures of purified E12 sympathetic neurons incubated for 24 hours in medium containing 10 ng/ml NGF (NGF) or 40 mM KCl plus 10 ng/ml NGF (NGF + KCl) with and without nifedipine or verapamil (at 1 or 10  $\mu$ M). The mean  $\pm$  standard error of 2 separate assays are represented by each bar.

We also investigated if  $\text{Ca}^{2+}$  influx through N-type  $\text{Ca}^{2+}$  channels plays a role in mediating the effects of depolarization on GFR $\alpha$  and ret mRNA expression. The N-type  $\text{Ca}^{2+}$  channel antagonist  $\omega$ -conotoxin GVIA at concentrations known to effectively block N-type  $\text{Ca}^{2+}$  channels (0.1 to 1 mM) did not influence the effect of 40mM KCl on the levels of GFR $\alpha$ -1 and GFR $\alpha$ -2 mRNAs and did not affect neuronal survival with or without KCl (Figure 3.9). These results indicate that the changes in GFR $\alpha$  and ret mRNA expression induced by elevated extracellular  $\text{K}^+$  do not depend on  $\text{Ca}^{2+}$  influx through N-type  $\text{Ca}^{2+}$  channels.

Tetrodotoxin, a  $\text{Na}^+$  channel antagonist, had also no effect on GFR $\alpha$  and ret mRNA expression in sympathetic neurons grown with either 5 mM or 40 mM KCl in the culture medium (data not shown). This indicates that the changes in GFR $\alpha$ -1 and GFR $\alpha$ -2 mRNA expression induced by elevated extracellular  $\text{K}^+$  do not depend on  $\text{Na}^+$  influx through voltage-gated  $\text{Na}^+$  channels.

**A****GFR $\alpha$ -1 mRNA****B****GFR $\alpha$ -2 mRNA**

**C****GFR $\alpha$ -4 mRNA****D****ret mRNA**

**E**

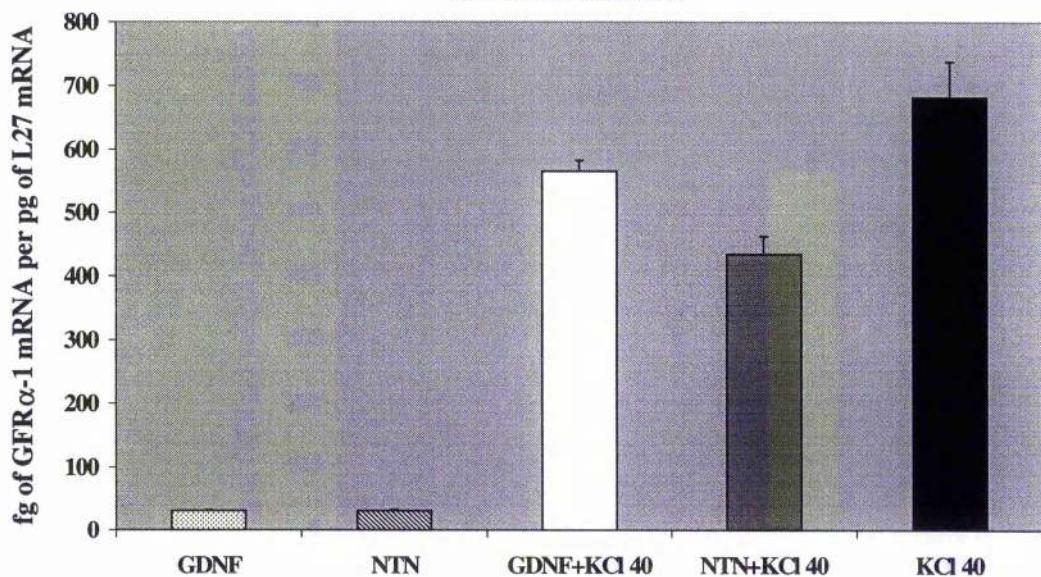
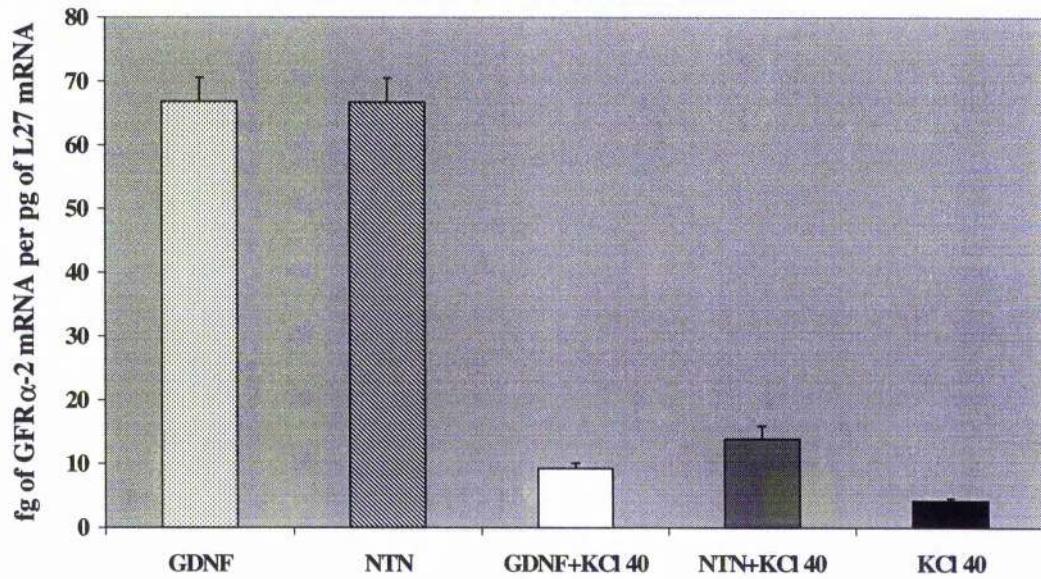
**Figure 3.9.** Bar charts of the percent survival and the levels of GFR $\alpha$  and ret mRNAs relative to L27 mRNA in cultures of purified E12 sympathetic neurons incubated for 24 hours in medium containing 10 ng/ml NGF (NGF) or 40 mM KCl plus 10 ng/ml NGF (NGF + KCl) with and without w-conotoxin GVIA at concentrations of 0.1 or 1 mM. The mean  $\pm$  standard error of 2 separate assays are represented by each bar.

#### **IV. The effects of depolarization on regulation of GFR $\alpha$ and ret mRNA expression in parasympathetic and sensory neurons**

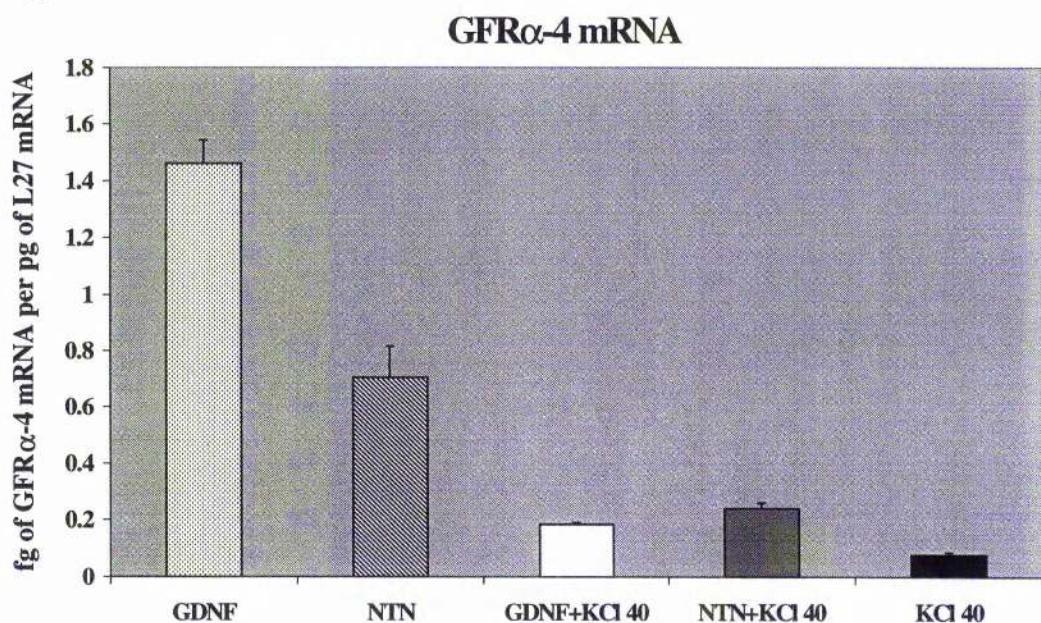
To determine if depolarization has similar effects on the expression of GFR $\alpha$  and ret mRNAs in other kinds of neurons, I studied the effect of 40mM KCl on the levels of these mRNAs in cultures of parasympathetic neurons from the ciliary ganglion and sensory neurons from the nodose ganglion. These experiments were carried out by incubating purified neurons for 24 hours in medium containing basal (5mM) or depolarizing (40mM) levels of KCl together with a neurotrophic factor that very effectively promoted their survival (GDNF or NTN in the case of ciliary neurons and BDNF in the case of nodose neurons). The neurons were also grown in medium containing 40mM KCl alone, which promoted the survival of the majority of ciliary neurons and promoted the survival of a subset of nodose neurons (Figures 3.10 and 3.11).

In cultures of E8 ciliary ganglion neurons, 40mM KCl caused a substantial increase in the levels of GFR $\alpha$ -1 and ret mRNAs and a substantial decrease in the level of GFR $\alpha$ -2 and GFR $\alpha$ -4 mRNAs (Figure 3.10). Compared with the levels in cultures containing GDNF or NTN alone, cultures supplemented with 40mM KCl alone exhibited a 23-fold increase in the level of GFR $\alpha$ -1 mRNA, a 17-fold decrease in the level of GFR $\alpha$ -2 mRNA, an average of 13-fold decrease in GFR $\alpha$ -4 mRNA and a 1.7-fold increase in ret mRNA. These results indicate that depolarization has a similar effect on the expression of GFR $\alpha$ -1 and GFR $\alpha$ -2, but not GFR $\alpha$ -4 and ret mRNAs in sympathetic and parasympathetic neurons. They also show that the changes in GFR $\alpha$  and ret mRNA expression are more pronounced in parasympathetic neurons.

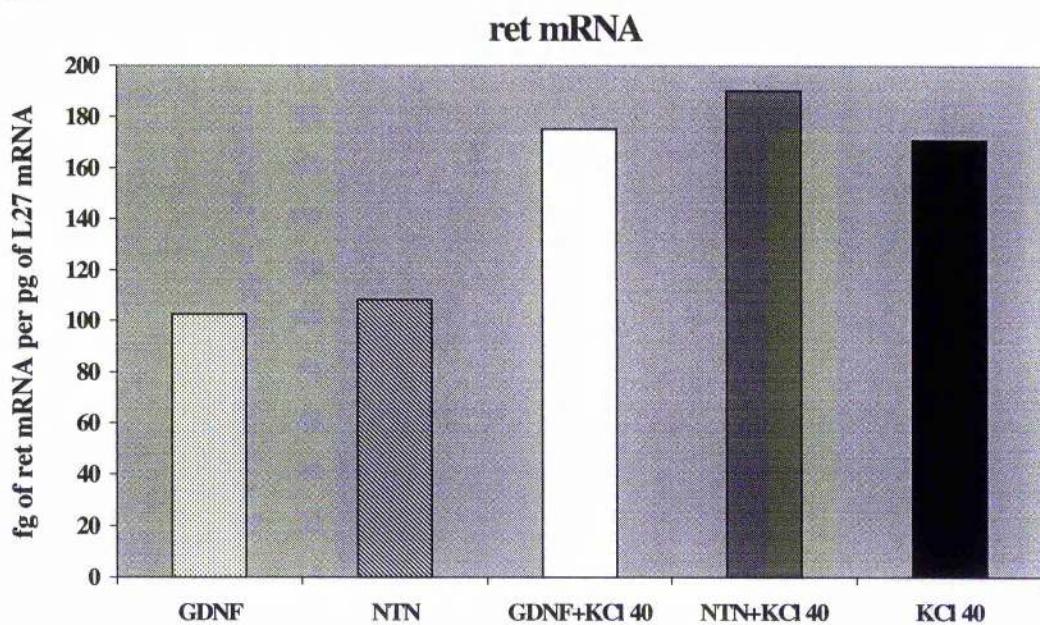
In cultures of E8 nodose ganglion neurons, 40mM KCl increased the level of GFR $\alpha$ -1 and ret mRNA, decreased the level of GFR $\alpha$ -2 mRNA and had no

**A****GFR $\alpha$ -1 mRNA****B****GFR $\alpha$ -2 mRNA**

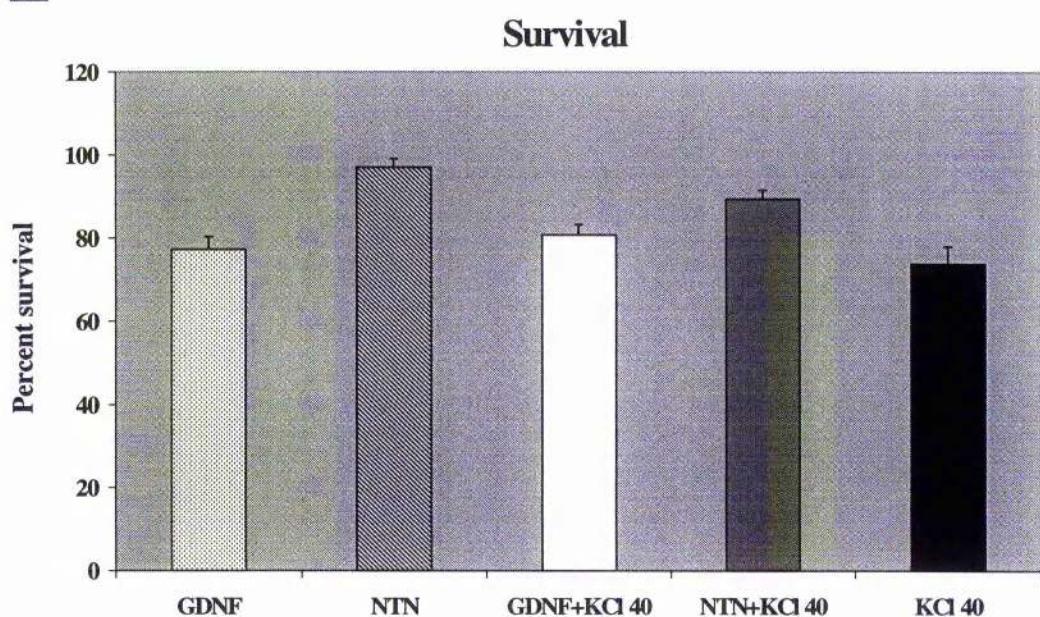
C



D

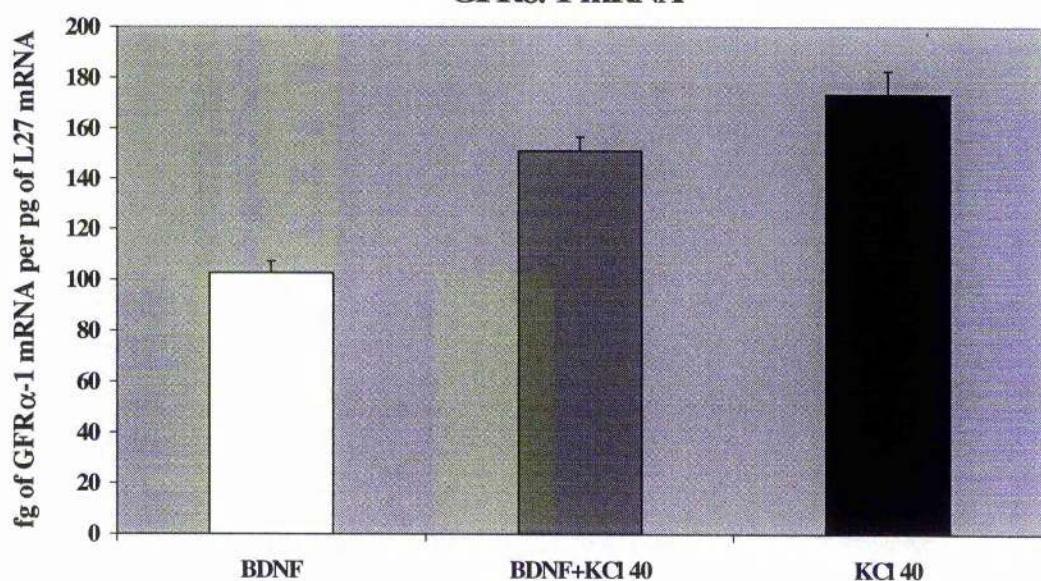
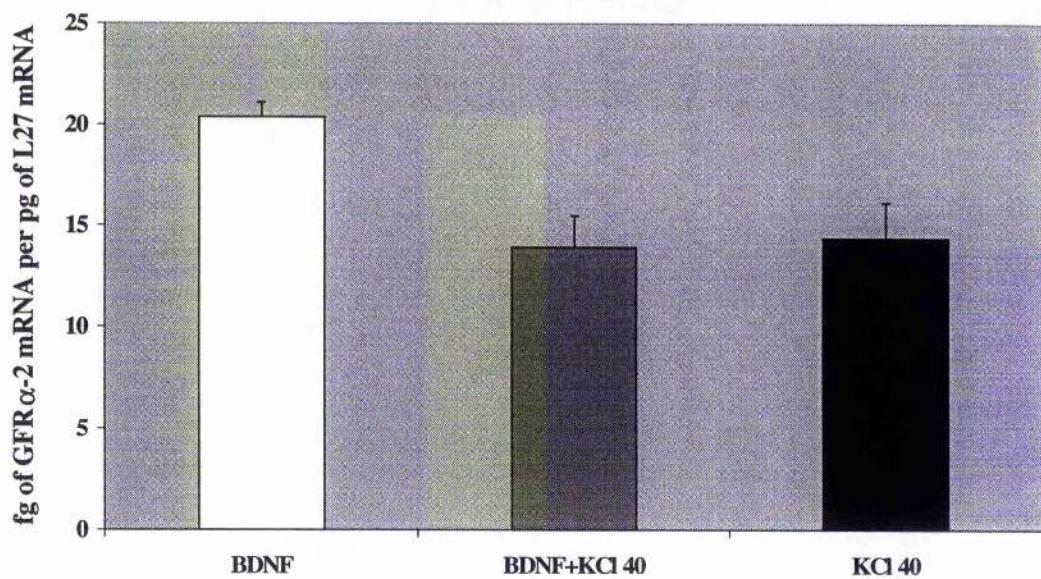


**E**



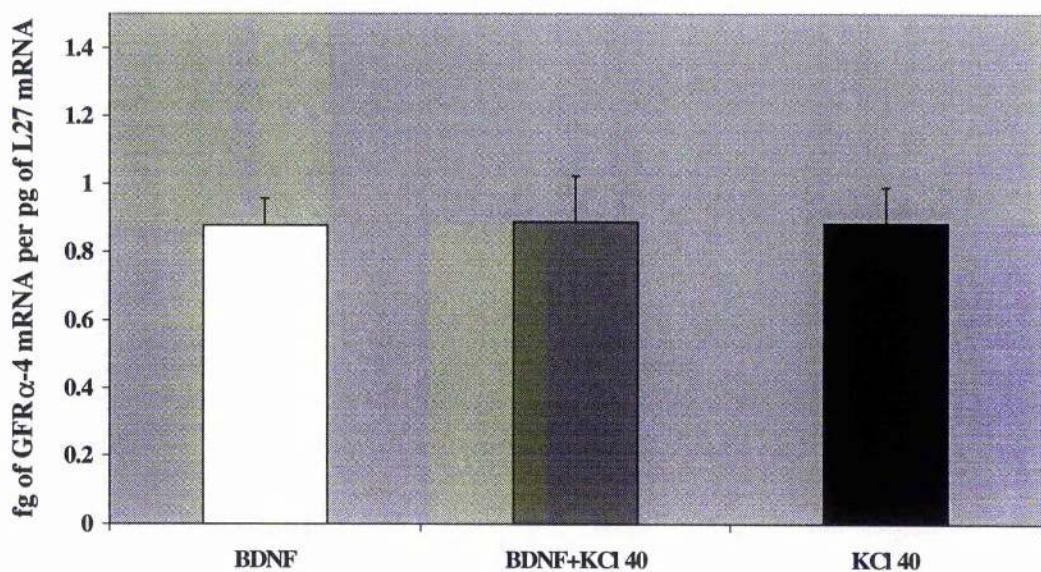
**Figure 3.10.** Bar charts of the percent survival and the levels of GFR $\alpha$  and ret mRNAs relative to L27 mRNA in cultures of purified E8 ciliary neurons incubated for 24 hours in medium containing 10 ng/ml GDNF (GDNF), 10 ng/ml NTN (NTN), GDNF plus 40 mM KCl (GDNF+KCl), NTN plus 40 mM KCl (NTN+KCl) or 40 mM KCl (KCl). The mean  $\pm$  standard error of 2 separate assays are represented by each bar.

effect on GFR $\alpha$ -4 mRNA expression (Figure 3.11). Compared with the levels in cultures containing BDNF, cultures supplemented with 40mM KCl alone exhibited only a 1.7-fold increase in the level of GFR $\alpha$ -1 mRNA, a 1.4-fold decrease in the level of GFR $\alpha$ -2 mRNA and had no effect on GFR $\alpha$ -4 and ret mRNAs. These results suggest that depolarization does influence GFR $\alpha$  (with the exception of GFR $\alpha$ -4) and ret mRNA expression in sensory neurons, although not as markedly as in autonomic neurons.

**A****GFR $\alpha$ -1 mRNA****B****GFR $\alpha$ -2 mRNA**

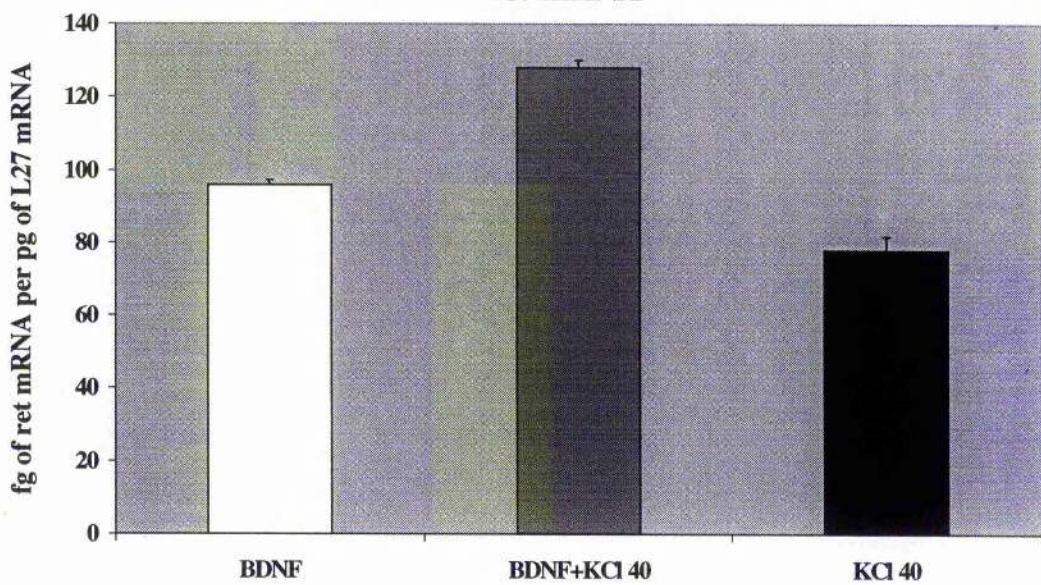
C

GFR $\alpha$ -4 mRNA



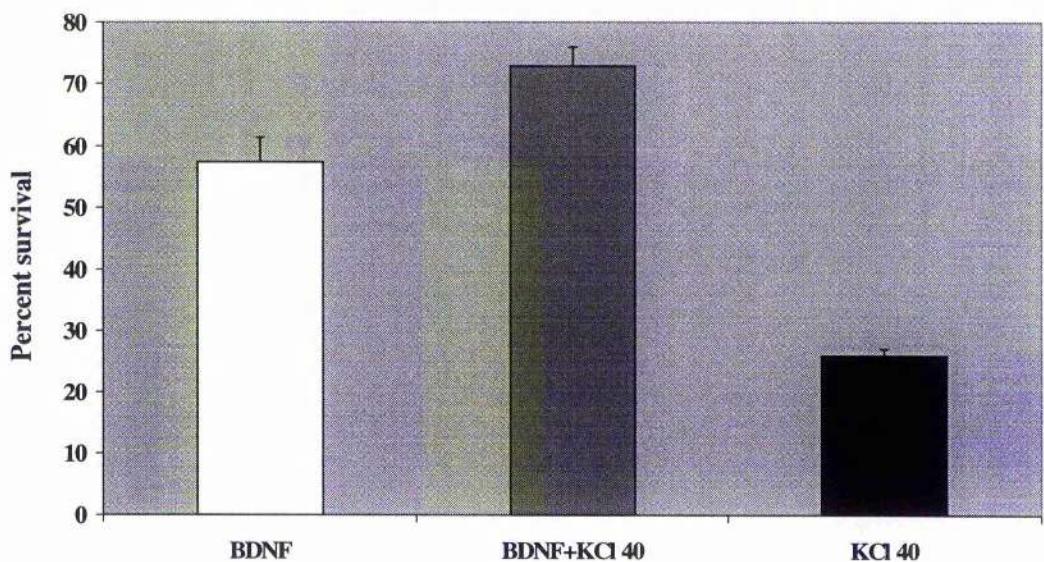
D

ret mRNA



**E**

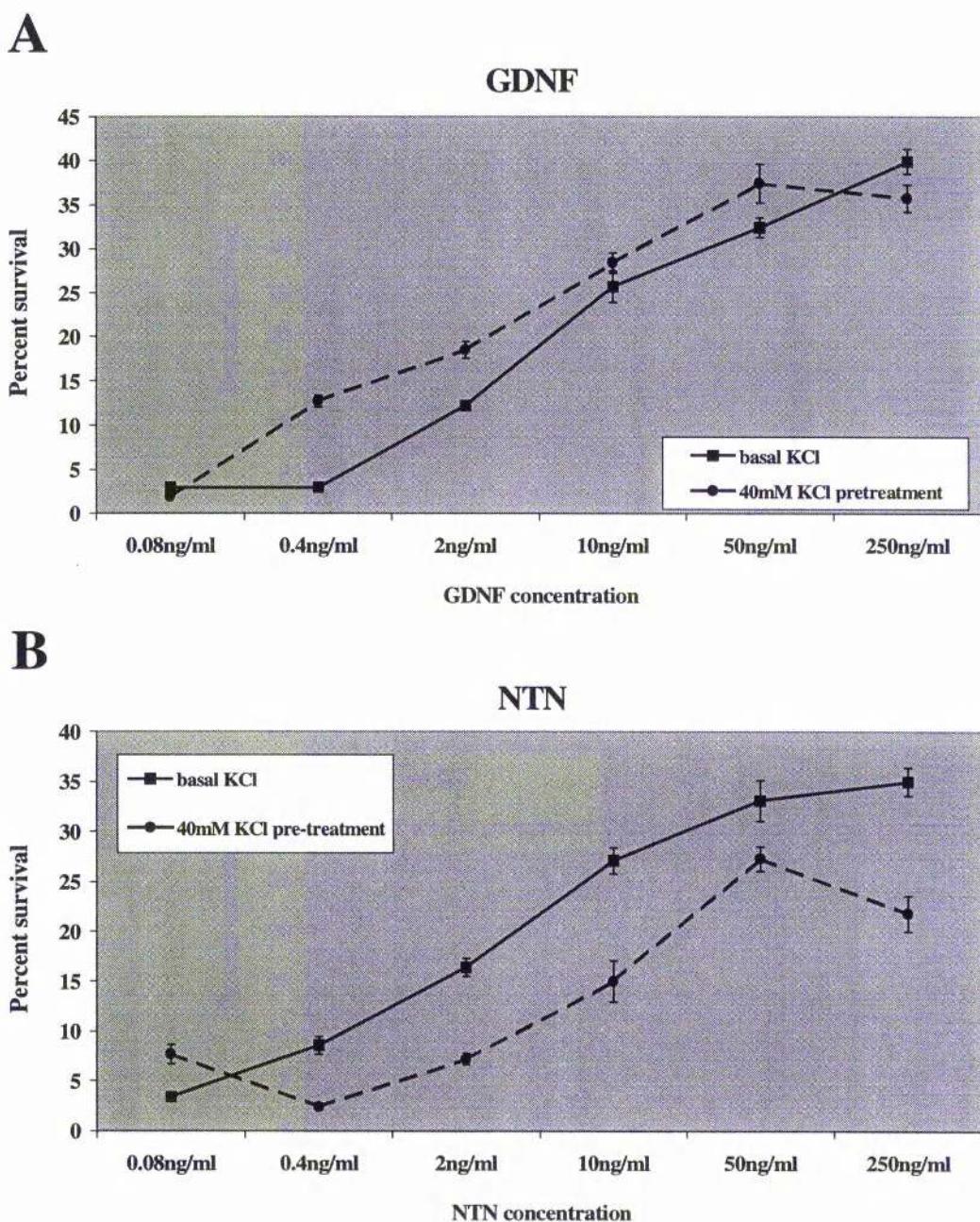
**Survival**



**Figure 3.11.** Bar charts of the percent survival and the levels of GFR $\alpha$  and ret mRNAs relative to L27 mRNA in cultures of purified E8 nodose neurons incubated for 24 hours in medium containing 10 ng/ml BDNF (BDNF), BDNF plus 40 mM KCl (BDNF+KCl) or 40 mM KCl (KCl). The mean  $\pm$  standard error of 2 separate assays are represented by each bar.

## **V. Depolarization shifts the response of neurons to GDNF and NTN**

To investigate if the changes in the levels of GFR $\alpha$ -1 and GFR $\alpha$ -2 mRNAs caused by depolarizing levels of KCl are translated into changes in the response of the neurons to GDNF and NTN, I incubated E12 sympathetic neurons with and without depolarizing levels of KCl for 12 hours and then switched to medium containing a range of concentrations of either GDNF or NTN for 24 hours to see if an initial period of depolarization would shift the dose responses of these neurons to these neurotrophic factors. To ensure maximal survival during the initial incubation period, both 5 mM and 40 mM KCl cultures were supplemented with saturating levels of either GDNF or NTN. The dose response of neurons to GDNF that were initially incubated with depolarizing levels of KCl was shifted to lower concentrations than neurons that were not exposed to depolarizing levels of KCl (Figure 3.12). In contrast, the dose response of neurons to NTN that were initially incubated with depolarizing levels of KCl was shifted to high concentrations than neurons that were not exposed to depolarizing levels of KCl (Figure 3.12). Thus, whereas depolarization increases expression of GFR $\alpha$ -1 mRNA and makes neurons more sensitive to its preferred ligand GDNF, it decreases expression of GFR $\alpha$ -2 mRNA and makes neurons less sensitive to its preferred ligand NTN.



**Figure 3.12.** Graphs of the percent survival after 24 hours incubation of E12 sympathetic neurons that were initially incubated for 12 hours in medium containing 20 ng/ml GDNF (A) or 20 ng/ml NTN (B) with either 5 mM KCl (basal KCl) or 40 mM KCl and then washed and switched to medium containing 5 mM KCl with different concentrations GDNF (A) or NTN (B). The percent survival in control cultures was subtracted. The mean  $\pm$  standard error of 2 separate assays are represented by each bar.

## **2.3. Discussion**

I have used an *in vitro* approach to study the regulation of GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNA expression in the neurons of the embryonic chicken peripheral nervous system. Because preliminary experiments revealed that the non-neuronal cells of peripheral ganglia express low levels of these mRNAs, I used differential sedimentation (Davies, 1988c) to remove most of the non-neuronal cells before plating the neurons at low density in laminin-coated petri dishes (>95% neurons). The most extensive analysis was carried out on sympathetic neurons because the majority of these neurons can be sustained in culture with several different neurotrophic factors (NGF, CNTF, GDNF and NTN) and with depolarizing levels of KCl, thereby enabling me to study receptor expression under different experimental conditions. Also, at early developmental stages, many of these neurons are able to survive for up to 24 hours *in vitro* without neurotrophic factors in the culture medium, thereby providing a valuable control for these studies.

I have shown that depolarizing levels of KCl induce rapid, substantial and opposite changes in the expression of GFR $\alpha$  in developing PNS neurons. In sympathetic neurons, elevated KCl markedly increases the levels of GFR $\alpha$ -1 and GFR $\alpha$ -4 mRNAs, substantially decreases the level of GFR $\alpha$ -2 mRNA and slightly decreases the levels of ret mRNA (Figure 3.5). Similar results were observed with

intact ganglia cultured as explants. However, the effects of depolarization were less pronounced (data not shown).

In parasympathetic neurons, KCl causes a substantial increase in the levels of GFR $\alpha$ -1 and ret mRNAs and substantial decrease in the level of GFR $\alpha$ -2 and GFR $\alpha$ -4 mRNAs (Figure 3.10). In sensory neurons, KCl increases the level of GFR $\alpha$ -1 and ret mRNAs, decreases the level of GFR $\alpha$ -2 mRNA, and has no effect on GFR $\alpha$ -4 mRNA expression (Figure 3.11). However the marginal effect of KCl on GFR $\alpha$  and ret mRNA expression in nodose neurons together with the low survival in KCl and the additive survival of BDNF with KCl make it possible that the data could reflect the different expression of GFR $\alpha$  and ret by different neuronal subtypes.

I have shown that changes in the levels of GFR $\alpha$ -1 and GFR $\alpha$ -2 mRNAs are translated into corresponding changes in the survival responses of the neurons to GDNF and NTN. Whereas sympathetic neurons exposed to depolarizing levels of KCl become more responsive to GDNF, the preferred GFR $\alpha$ -1 ligand, they become less responsive to NTN, the preferred GFR $\alpha$ -2 ligand (Figure 3.12).

The demonstration that these effects of elevated KCl are blocked by L-type Ca $^{2+}$  channel antagonists suggests that they are due to Ca $^{2+}$  influx and elevation of the intracellular free Ca $^{2+}$  concentration (Figure 3.8). These results imply that neural activity plays a role in selectively regulating the expression of the GPI-linked receptors for GDNF and NTN and in modulating the response of neurons to these factors.

The observation that elevated levels of KCl induce expression of transcripts encoding the NGF receptor tyrosine kinase trkA in a retrovirally immortalized sympathoadrenal precursor cell line led to the proposal that depolarization induces trkA expression and NGF responsiveness in sympathetic neuroblasts (Birren et al., 1992). However, in primary cultures of sympathetic neuroblasts and post-mitotic sympathetic neurons, depolarizing levels of KCl do not increase trkA mRNA expression before, during or after the onset of NGF dependence, suggesting that depolarization does not regulate trkA expression in normal sympathetic neuroblasts and neurons during development (Wyatt and Davies, 1995). Elevated levels of the BDNF/NT-4 receptor tyrosine kinase trkB are found in the hippocampus and striatum after generalized seizures produced by electrical stimulation or pharmacological methods (Bengzon et al., 1993; Dugich-Djordjevic et al., 1995b; Salin et al., 1995). Although these observations have raised the possibility that neural activity increases trkB expression in the central nervous system, the functional significance of these changes is unclear, especially as they are observed in both neurons and glia and involve transcripts encoding both catalytic and non-catalytic trkB isoforms. Although my studies have clearly shown that depolarization promotes marked changes in the mRNA expression of the GPI-linked receptors for the GDNF family, depolarization does not have a dramatic effect on the expression of ret mRNA, the common receptor tyrosine kinase receptor for members of the GDNF family of neurotrophic factors.

A large number of *in vitro* and *in vivo* studies have shown that neurotrophins up-regulate the expression of transcripts encoding their receptors. The level of the common neurotrophin receptor p75 mRNA is increased by NGF

in sympathetic (Miller et al., 1991, 1994; Verdi and Anderson, 1994; Wyatt et al., 1995), sensory (Lindsay et al., 1990; Verge et al., 1992; Wyatt and Davies, 1993) and basal forebrain cholinergic neurons (Cavicchioli et al., 1989; Fusco et al., 1991; Higgins et al., 1989; Kojima et al., 1992), and by BDNF in sensory neurons (Wyatt et al., 1993). Exogenous NGF increases the level of trkA mRNA in forebrain cholinergic neurons (Holtzman et al., 1992; Kojima et al., 1994; Venero et al., 1994) and in combination with oestrogen increases trkA mRNA expression in PC12 cells (Sohrabji et al., 1994). BDNF increases the expression of trkB transcripts in cultured sensory neurons (Ninkina et al., 1996) and NT-3 increases expression of its receptor tyrosine kinase trkC in sensory neurons both *in vitro* and *in vivo* (Wyatt et al., 1999). In contrast to these studies, I find that expression of GFR $\alpha$ -1, GFR $\alpha$ -2, GFR $\alpha$ -4 and ret mRNAs are not significantly affected by either GDNF or NTN.

Although I have focused on the consequences that depolarization-induced changes in GFR $\alpha$ -1 and GFR $\alpha$ -2 expression have on the survival response of developing neurons to GDNF and NTN, it is possible that the effects of depolarization on the expression of GFR $\alpha$ -1 and GFR $\alpha$ -2 may have a wider significance in modulating the actions of GDNF and NTN. In addition to promoting neuronal survival, GDNF has been shown to have a variety of other effects on neuronal morphology and physiology. Overexpression of GDNF in muscle increases the number of motor axons innervating neuromuscular junctions in neonatal mice (Nguyen et al., 1998) and GDNF treatment of cultured Purkinje increases dendritic thickening and the development of spines and filopodial

extensions (Mount et al., 1995). GDNF increases dopamine release from midbrain dopaminergic neurons (Feng et al., 1999; Hebert et al., 1996; Pothos et al., 1998) and increases acetylcholine release at neuromuscular junctions (Liou et al., 1997; Ribchester et al., 1998). Furthermore, administration of NTN into the striatum of intact adult induces behavioral and biochemical changes associated with functional upregulation of nigral dopaminergic neurons (Horger et al., 1998). Thus, depolarization induced changes in GFR $\alpha$ -1 and GFR $\alpha$ -2 expression may not only play a role in regulating neuronal survival by GDNF and NTN during development, but may play an important role in modulating the effects of GDNF and NTN on the structural and functional plasticity of neurons in the developing and mature nervous system.

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## **Appendix I**

The avian nervous system arises from the neural plate, which closes to form the neural tube, first in the rostral region and then progressively in more caudal regions of the embryo. In the head, the neural tube expands to form the brain, which partitions into the forebrain, midbrain and hindbrain. The hindbrain (rhombencephalon) is subsequently subdivided into seven or eight distinct neuromeres, the rhombomeres. The borders between adjacent rhombomeres restrict cell movement (Fraser et al., 1990; Birgbauer and Fraser, 1994), rendering each rhombomere as a somewhat separate developmental unit. These developmental subdivisions are paralleled by molecular specialization. After neural tube closure, neural crest cells emerge from the neuroepithelium and begin their migration. In the hindbrain, neural crest cell migration is patterned into three broad streams of cells, emanating laterally adjacent to r1/2, r4 and r6. Cells within the first stream populate the trigeminal ganglion and maxillomandibular arch; those within the second populate the hyoid arch, as well as the geniculate and vestibulocochlear ganglia; those following the third stream populate the third and fourth branchial arches and the remaining peripheral cranial ganglia (the superior, petrosal and nodose; Noden, 1975; D'Amico-Martel and Noden, 1983; Lumsden et al., 1991). Neural crest cells from rhombomeres 3 and 5 do not migrate through the mesoderm surrounding them, but either die or enter into the streams of crest cells on either side of them (Graham et al., 1993; Sechrist et al., 1993).

## **Appendix II**

### **Solutions and media for tissue culture**

#### **Solutions**

##### **HBSS**

Hanks' balanced salts without calcium or magnesium (GibcoBRL).

This medium (serum-free and carbonate-free) was used to trypsinize and triturate the ganglia, and to load the cell suspension onto the dropping funnel during differential sedimentation.

##### **1% Trypsin**

500mg of trypsin (Worthington) was added to 5ml of Ca/Mg-free PBS (GibcoBRL) and sterilized with a 0.22 $\mu$ m filter (Nalgene).

Aliquots of 100 $\mu$ l in 1ml microfuge tubes were stored in a -30°C freezer.

##### **Laminin**

Stored in 20 $\mu$ l aliquots (-30°C) at 1mg/ml, then diluted to 20 $\mu$ g/ml in fresh F14.

##### **Poly-DL-ornithine**

0.5mg/ml poly-DL-ornithine (SIGMA) in 0.15M boric buffer [4.6g boric acid (BDH) in 500ml ddH<sub>2</sub>O, pH8.4] was sterilized with a 0.22 $\mu$ m filter (PallGelman Sc).

### **HIHS**

Heat inactivated horse serum (GibcoBRL).

### **Media**

#### **L15**

A 1L unit of L15 powder (GibcoBRL) was added to 1L of ddH<sub>2</sub>O plus 100mg streptomycin (SIGMA) and 60mg penicillin (SIGMA). After adjusting pH to 7.3 the L15 was sterilized with an 0.22μm filter (PallGelman Sc).

#### **HAM's F12**

A 1L unit of F-12 powder (GibcoBRL) was added to 1L of ddH<sub>2</sub>O plus 100mg streptomycin (SIGMA) and 60mg penicillin (SIGMA). The pH was adjusted to 7.3 and the F12 was sterilized with a 0.22μm filter (PallGelman Sc).

#### **HAM's F14**

10x concentrated stock solution:

A 5L unit of F14 powder (Imperial) was added to 500ml of ddH<sub>2</sub>O containing 500mg streptomycin (SIGMA) and 300mg penicillin (SIGMA). The 10x stock was stored in 50ml aliquots at -30°C.

1x solution:

A 50ml of 10x aliquot was diluted into 450ml ddH<sub>2</sub>O. Next, 1g of NaHCO<sub>3</sub> (BDH) was added and the solution was adjusted to pH7.0, with dry ice or 5M HCl before being sterilized with a 0.22μm filter (PallGelman Sc).

### **Neurotrophic factors**

Neurotrophic factors were kept in F12 plus 10% HIHS, pH5.5, at -80°C until required.