CLONING, CHARACTERISATION AND EXPRESSION OF NOVEL GPI-LINKED RECEPTORS OF THE GDNF FAMILY.

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for the degree of

Doctor of Philosophy (Ph.D.)

By

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I dedicate this thesis to my parents for their love and patience

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DECLARATIONS.

I, Jane Victoria Thompson, hereby certify that this thesis, which is approximately 50,766 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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ABSTRACT.

The main aim of this project was to clone and characterise novel members the GFRα family of glycosyl-phosphatidylinositol (GPI)-linked receptors for the GDNF family of neurotrophic factors and to examine their expression in the nervous system and other tissues throughout development.

The cloning and characterisation of chicken GFRα-1, GFRα-2 and GFRα-4 is described. Determination of the primary structure revealed that these members of the GFRα family possess an N-terminal, hydrophobic signal peptide and features that are characteristic of GPI linked proteins, namely, a C-terminal hydrophobic domain separated by a consensus sequence for GPI-linkage. GFRα-4 mRNA was shown to have a distinctive pattern of expression in the CNS and several other organs and tissues of the chicken embryo.

In addition, a novel splice isoform of GFRα-1 (GFRα-1sv) was cloned in mouse that is expressed predominantly in peripheral tissues during development and in maturity. Ectopic co-expression of GFRα-1sv together with the Ret receptor tyrosine kinase (the signalling component for the GDNF family of neurotrophic factors) in cultured neurons conferred an increased survival response to neurturin but not to GDNF (in contrast to GFRα1 plus Ret which conferred a survival response to GDNF). GFRα-1sv and GFRα-1 were also observed to exhibit different patterns of expression. These findings suggest that GFRα-1sv and GFRα-1 have distinctive roles in development.

The identification of a putative GFRα-4 homologue in mouse is also described. Expression of GFRα-4 mRNA is restricted to the CNS of the developing and adult mouse, which is in contrast to the more widespread distribution of chicken GFRα-4.

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ABBREVIATIONS.

aa amino acid

Aids acquired immuno-deficiency syndrome

ARTN Artemin

BDNF Brain-derived neurotrophic factor

BH bcl-2 homology region

BLAST basic local aligment search tool

BMP bone morphogenetic protein

bp base pair

BSA Bovine serum albumin

CNS Central nervous system

CNTF Ciliary neurotrophic factor

CT-1 Cardiotropin-1

ddATP dideoxyadenosine triphosphate

ddCTP dideoxycytidine triphosphate

ddGTP dideoxyguanosine triphosphate

ddNTP dideoxynucleotide triphosphate

ddTTP dideoxythimidine triphosphate

DIG digoxigenin

DNA deoxyribonucleic acid

dNTP deoxynucleotide triphosphate

DNase deoxyribonuclease

DPP decapentaplegic

DRG Dorsal root ganglia

DTT dithiothreitol

DVR decapentaplegic-Vg1-related

E Embryonic day

E.coli Escherichia coli

EDTA Ethylenediaminetetraacetic acid

ENS enteric nervous system

F14 Ham nutrient mixture F-14

FGF Fibroblast growth factor

FMTC familiar medullary thyroid carcinoma

GC granule cell

GDNF Glial cell line-derived neurotrophic factor

GFRα GDNF family receptor alpha

gp130 glycoprotein 130

GPI glycosyl-phosphatidylinositol

HIHS Heat-inactivated horse serum

IL-6 Interleukin-6

IL-6-Rα IL-6 receptor alpha

JAK jak kinase

JNK jun kinse

Kb Kilobase

kDa Kilodalton

LB luria broth

LIF Leukaemia inhibitory factor

LIFRβ LIF receptor beta

L litre

MAD mothers against DPP

MAPK mitogen-activated protein kinase

mCi millicurie

MEN mutiple endocrine neoplasia

μg microgram

μl microlitre

μm micromolar

mg milligram

ml milli litre

M molar

mM milli molar

mRNA messenger Ribonucleic Acid

MOPS 3-Morpholinepropanesulfonic acid

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

ng nanogram

NGF Nerve growth factor

NT-3 Neurotrophin-3

NT-4 Neurotrophin-4

NT-5 Neurotrophin-5

NT-6 Neurotrophin-6

NT-7 Neurotrophin-7

NTN Neurturin

OD optical density

6-OHDA 6-hydroxydopamine

ORF open reading frame

OSM Oncostatin-M

OSMR-β OSM receptor beta

P postnatal

pBS Bluescript plasmid

PBS phosphate buffered saline

PC purkinje cell

PCR Polymerase chain reaction

PEG polyethylene glycol

pfu plaque forming unit

pmol picomolar

PD parkinsons disease

P-ORN Poly-DL-ornitine

PNS Peripheral nervous system

PSP Persephin

PTC papillary throid carcinoma

Ret rearranged in transformation

RNA ribonucleic acid

RNase ribonuclease

RT-PCR reverse transcription-polymerase chain reaction

RTK receptor tyrosine kinase

SCG Superior cervical ganglia

SDS sodium duodecylsuphate

SH2 src homology 2

SM sodium magnesium

STAT signal transducer and activator of transcription

STE sodium-tris-EDTA

STET sucrose-Triton-EDTA-tris

TAE tris-acetate EDTA

TBE tris-boric acid-EDTA

TβR TGF-β receptor

TGF -β transforming growth factor beta

TNF tunor necrosis factor

TNFR TNF receptor

TMN Trigeminal mesencephalic nucleus

tRNA transfer ribonucleic acid

Trk tropomyosin-related kinase

UV ultraviolet

W Watts

CHAPTER 1.

INTRODUCTION.

1. GENERAL INTRODUCTION.

The vertebrate nervous system is the most diverse and complex organ to have evolved. It is divided into two components, the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS consists of the brain and spinal cord. The PNS, consists of ganglia and peripheral nerves which arise from the brain and spinal cord. Functionally, the central and peripheral nervous systems are interconnected and interactive with information being transferred between the two. This occurs via a diverse pattern of connections formed between neurons, which are the principle functional units of the nervous system. In humans the nervous system is estimated to contain about 10^{12} neurons. It is these highly specialised neuronal cells along with support cells such as glia that establish specific connections and are required to process information for the higher functions of the nervous system such as cognition, learning, memory, motor co-ordination, and intellect.

1.1. Early Development of the Vertebrate Nervous system.

Development of the vertebrate nervous system involves a series of co-ordinated events which begin during gastrulation. At this stage of development the three principle layers of cells of the vertebrate embryo are formed. The endoderm, is the innermost layer and gives rise to the gut, lungs and liver. The mesoderm, the middle layer, gives rise to the connective tissues, muscle and the vascular system. The ectoderm, the outermost layer gives rise to all the major tissues of the central and the peripheral nervous system. During early

development the dorsal midline of the ectoderm undergoes thickening to form the neural plate from which the CNS is derived. The lateral edges of the neural plate then begin to fold, forming the neural groove. The folds of the neural groove then become opposed and fuse in a rostral caudal direction, sealing the neural groove and forming a hollow structure known as the neural tube (Schoenwolf and Smith 1990). The cavity of the neural tube gives rise to the ventricular system of the CNS, while the neuroepithelial walls of the tube generate all the neurons and glial cells of the CNS. In addition, the neuroepithelium also gives rise to a specialised group of migratory cells known as the neural crest. These cells emerge from the dorsal region of the neural tube and migrate away to form most of the PNS, including sensory and autonomic neurons, satellite cells and schwann cells. Neural crest cells also give rise to melanocytes in the skin and to some connective tissues of the face. The only group of neural crest cells which remain in the CNS are those that give rise to sensory neurons of the trigeminal mesencephalic nucleus (Weston, 1962). Although neural crest cells generate the majority of neurons in the PNS, certain groups of cranial sensory neurons are derived from another group of progenitor cells known as neurogenic placodes which are epithelial thickenings on the rostral ectoderm of the developing embryo (Le Douarin, 1982).

1.2. Development of the Peripheral Nervous System.

Once PNS progenitor cells have migrated to the appropriate locations these cells differentiate into sensory, sympathetic, parasympathetic and enteric ganglia neurons and associated satellite and Schwann cells.

The sensory ganglia of the PNS consist of the cranial sensory ganglia and the dorsal root ganglia. There are seven pairs of the cranial sensory ganglia which reside on five of the cranial nerves. They are the: trigeminal (cranial nerve

V), geniculate (cranial nerveVII), vestibular (cranial nerve VIII), acoustic (cranial nerve VIII), jugular (cranial nerveI X/X), petrosal (cranial nerve IX) and nodose (cranial nerve X) ganglia (Weston, 1962; Le Douarin, 1982; 1986).

The sympathetic and parasympathetic ganglia of the autonomic nervous system are involved in an autonomic motor system. The sympathetic ganglia are found in two groups known as the paravertebral and prevertebral ganglia. The paravertebral ganglia comprise a chain of ganglia lying on the ventral aspect of the vertebral column, and the prevertebral ganglia are found in defined groups in relation to major blood vessels in the abdomen. Postganglionic fibres originating from the paravertebral ganglia (superior cervical, middle cervical, stellate, and sympathetic chain ganglia) are known to innervate the head, neck, heart, lungs, bronchi, and other thoracic viscera, respectively. Postganglionic fibres originating from the prevertebral ganglia (coeliac, aorticorenal, superior mesenteric and inferior mesenteric ganglia) innervate the stomach spleen, liver, kidney, intestine, ureter, and genital organs, respectively. The parasympathetic ganglia (ciliary, pterygopalatine, submandibular, otic, and terminal ganglia) are found close to visceral targets or are actually embedded within them. Postganglionic fibres originating from these ganglia innervate the ciliary muscle, lacrimal gland, pharayngeal gland, submandibular gland, parotid gland, and heart, lungs, intestine, liver, and kidneys respectively.

Although the enteric nervous system functions autonomously, it is also regulated by extrinsic innervation supplied by the parasympathetic and sympathetic systems. Enteric, sympathetic prevertebral and paravertebral ganglia innervate the interconnected myenteric and submucous plexuses present in the gastrointestinal walls of the intestine to provide control of motility and secretion within the gastrointestinal tract.

2. NEUROTROPHIC THEORY.

In the developing nervous system neurons initially generated in excess die, shortly after innervating their target fields. This loss is thought to match the number of neurons to the size and requirements of their target fields because altering target field size prior to innervation affects the number of neurons that survive. The neurotrophic theory provides an explanation for how target fields regulate the size of the neuronal populations that innervate them. Originating from work on nerve growth factor (NGF), the founder member of the neurotrophin family of secreted proteins. It states that the survival of developing neurons depends on the supply of a neurotrophic factor that is synthesized in limiting amounts in their target fields.

The neurotrophic theory (Levi-Montalcini and Angeletti 1968; Thoenen and Barde 1980; Purves 1988) has gained support from work on NGF and has been substantiated by studies of more recently identified neurotrophic factors. The most direct evidence for the neurotrophic theory is that populations of developing neurons that are supported by NGF *in vitro*, namely sympathetic neurons and certain kinds of sensory neurons, also depend on NGF *in vivo*. Anti-NGF antibodies administered during the phase of target field innervation eliminate these neurons whereas exogenous NGF rescues neurons that would otherwise die (Levi-Montalcini and Angeletti 1968; Hamburger and Yip 1984; Johnson *et al.*, 1980). Likewise, these same neurons are lost in mice that have targeted null mutations in the NGF gene (Crowley *et al.*, 1994) or NGF receptor tyrosine kinase (*trk*A) gene (Smeyne *et al.*, 1994).

In the peripheral target fields of sensory and sympathetic neurons NGF synthesis commences with the arrival of the earliest axons (Davies *et al.*, 1987; Korsching and Thoenen 1988). At the onset of neuronal death in sensory ganglia, NGF synthesis in cutaneous territories is proportional to their final innervation density; high levels in future densely innervated territories and low

levels in future sparsely innervated territories (Harper and Davies 1990). NGF is conveyed by fast axonal transport to the cell bodies of the innervating neurons following uptake by sensory and sympathetic fibres in their target fields, where it exerts its survival-promoting effects (Hendry *et al.*, 1974; Korsching and Thoenen 1983).

The purification of a second neurotrophin, brain-derived neurotrophic factor (BDNF) a protein with extensive homology to NGF and studies investigating the physiological significance of this factor established the generality of the neurotrophic theory (Barde *et al.*, 1982; Leibrock *et al.*, 1989). BDNF promotes the survival of certain populations of embryonic sensory neurons *in vitro* and prevents loss of these neurons *in vivo* following administration to embryos during the phase of naturally occuring neuronal death (Hofer and Barde 1988). Accordingly, mice with targeted disruption of the BDNF gene (Ernfors *et al.*, 1994a; Jones *et al.*, 1994) or BDNF receptor tyrosine kinase (*trk*B) gene (Klein *et al.*, 1993) have deficiencies in these BDNF-dependent neurons.

3. PROGRAMMED CELL DEATH.

Programmed cell death occurs during development and is a mechanism responsible for the widespread removal of excess cells and cells that have served their purpose (Oppenheim *et al.*, 1991; Williams, 1991). Neurons die as normal part of development by an active process known as apoptosis. This occurs when a cell activates an internally encoded suicide program following either extrinsic or intrinsic signals. Apoptotic cell death is characterized by plasma membrane blebbing, cytoplasmic condensation, pyknosis and degradation of nuclear DNA into oligosomal lengths (Wyllie *et al.*, 1980). Several extracellular signals regulating apoptosis in many different cell types have been identified, but

less is known about the intracellular mechanisms that activate apoptosis within the cell. Initial studies demonstrating the inhibition of transcription or translation in various cell types was able to inhibit apoptosis suggested that it was dependent upon the activation of a program of gene expression within the dying cell (Cohen and Duke, 1984; Scott and Davies, 1990). Genetic analysis in nematode, *Caenorhabditis elegans* has led to the identification of two pro-apoptotic genes ced-3 and ced-4. Mutation of these genes results in increased cell survival suggesting their activation is necessary for apoptosis (Ellis and Horvitz, 1986). In contrast, it appears that ced-9 is necessary for cell survival, since a loss-of-function mutation in the gene results in extensive cell death via the ced-3 and ced-4 pathways (Hengartner *et al.,* 1992). Ced-3 encodes an aspartate-specific cysteine protease (Yaun *et al.,* 1993; Xue *et al.*, 1996). Studies have shown the presence of several mammalian homologues of the nematode gene ced-3 which have been termed 'caspases' (for cysteine aspase). The first mammalian homology identified was interleukin-1β (IL-1β) coverting enzyme (ICE), a cysteine protease responsible for the processing of pro-IL-1β to the active cytokine (Yuan *et al.,* 1993). It is thought that ICE is functionally and structurally related to ced-3, since overexpression of either gene in Rat-1 fibroblasts induces apoptosis. (Miura *et al.,* 1993). In contrast the cowpox virus CrmA gene product and the baculovirus p35 protein both interfere with the protease function of ICE-related proteases and inhibit apoptosis (Tewari *et al.*, 1995; Xue *et al.*, 1995; Miura *et al.*, 1995; Bump *et al.*, 1995) suggesting that ICE-related proteases are involved in apoptotic pathways. Eight mammalian homologues of ced-3 and ICE have been characterised: ICH-1 (Wang *et al.*, 1994), CPP-32 (Fernandes-Alnemri et al., 1994; Nicholson *et al.,* 1995; Tewari *et al.*, 1995), ICH-2 (Faucheu *et al.*, 1995; Kamens *et al.*, 1995; Munday *et al.*, 1995), ICErel-111 (Munday *et al.,* 1995) Mch-2 (Fernandes-Alnemri *et al.,* 1995a), Mch-3 (Duan *et al.*, 1996; Fernandes-Alnemri et al., 1995b; Lipke *et*

al., 1996), FLICE (Muzio *et al.,* 1996) and ICE-LAP6 (Duan *et al.*, 1996). Ectopic expression of these ICE/Ced-3 homologues in a variety of cells causes apoptosis. All caspases cleave their substrates after specific aspartic acids and are themselves activated by cleavage at specific aspartic acids. Apoptosis is mediated by the caspases cleaving selected intracellular proteins, including proteins of the nucleus, nuclear lamina, cytoskeleton, endoplasmic reticulum, and cytosol. Some of the cleaved proteins subsequently activate other destructive processes within the cell thereby enhancing apoptosis (Chinnaiyan and Dixit, 1996). Ced-9 has since been identifed as a homologue of the mammalian proto-oncogene bcl-2 (Hengartner and Horvitz, 1994). Overexpression of Bcl-2 is able to prevent the ectopic cell death that occurs in Ced-9 loss-of-functions mutants (Hengartner and Horvitz 1994), demonstrating that the molecular mechanism of apoptosis is conserved throughout evolution.

The Bcl-2 gene was first isolated from the breakpoint of the t (14;18) chromosomal translocation found in many human B cell lymphomas (Tsujimoto *et al.*, 1985; Bakhshi *et al.*, 1985; Cleary and Sklar 1985). This translocation results in overexpression of Bcl-2 in B cells causing the development of neoplasia due to the suppression of cell death (Seto *et al.,* 1988). The Bcl-2 protein is an intracellular integral membrane protein and has been shown to play an important role in regulating the survival of B and T lymphocytes within the immune system (linnette and Korsmeyer 1994). Several genes that are homologous with Bcl-2 have been identified recently in mice and humans and are known to comprise the Bcl-2 gene family. These include A1, Mcl-1, Bcl-x, Bax and Bad. While some members of this family are anti-apoptotic other members appear to be pro-apoptotic. Among those that inhibit death are Bcl-2, McI-1, A1, Bcl-x (Hockenbery *et al.,* 1990; Boise *et al.,* 1993; Lin *et al.,* 1993; Reynolds *et al.,* 1994) and those that promote death are Bax and Bad (Oltvai *et al.,* 1993; Yang *et al.,* 1995). Homology among the Bcl-2 related proteins is

concentrated in two regions termed BH1 and BH2 domains. Recent work has identified another two homologous regions BH3 and BH4. BH3 is found only in pro-apoptotic family members suggesting it is a potent death domain (Kelekar and Thompson 1998). In addition there is a less well conserved hydrophobic C-terminus domain that is thought to be important for the the attachment of the proteins to the cytosolic side of cell membranes (Davies *et al.,* 1995).

4. NEUROTROPHIC FACTORS.

Since the discovery of nerve growth factor half a century ago, an increasing number of proteins have been shown to promote neuronal survival. These include the NGF family of neurotrophic factors, the neurotrophins (NGF, BDNF, NT-3, NT-4/5, NT-6 and NT-7), the neurotrophic cytokines (CNTF, LIF, IL-6,OSM, and CT-1), and the GDNF family (GDNF, neurturin, artemin and persephin). The neurotrophins signal via the Trk family of receptor tyrosine kinases, and also bind to a common receptor, p75, which, among other functions, modulates Trk signalling; the neurotrophic cytokines signal via oligomeric receptors consisting of two related proteins, gp130 and LIFR-β, and several acessory proteins, members the GDNF family signal via the Ret receptor tyrosine kinase, and ligand specificity is confered by a family of GPI-linked receptors. Each of these neurotrophic factors and their receptors will be discussed in turn.

4.1. The Neurotrophins.

Several families of neurotrophic factors have been identified. The most intensively studied of these is the nerve growth factor family of neurotrophins. This comprises seven structurally related proteins: NGF, BDNF, NT-3, NT4/5, NT-6 and NT-7.

4.1.1. Nerve Growth Factor.

NGF was the first member of the neurotrophin family to be described. Initially identified in snake venom for its nerve growth-promoting activity on populations of sensory and sympathetic ganglia, NGF was purified from the mammalian counterpart of the snake venom gland, the male mouse submandibular gland, where the protein was found to be present in extremely large amounts (Cohen, 1960; Angeletti and Bradshaw 1971). Purification and amino acid sequencing of mouse NGF led to subsequent cloning of the NGF cDNA in mouse and several other species namely human, cow, and chicken (Angeletti and Bradshaw 1971; Scott *et al.*, 1983; Ullrich *et al.*, 1983; Meier *et al.*, 1986; Ebendal *et al.*, 1986).

The NGF protein isolated from the salivary gland consists of three subunits α, ß, and γ. The β subunit is solely responsible for the trophic activity of NGF. This is synthesised as a precursor protein of 307 amino acids that is enzymatically processed to give a mature β-NGF glycosylated protein which exists as a homodimer of two identical non covalently bound 118 amino acid chains (Angeletti and Bradshaw 1971; Berger and Shooter 1977; Maness *et al.*, 1994).

Structural analysis of mature NGF by X-ray crystallography has revealed a novel tertiary fold dominated by two central pairs of anti-parallel β-strands that define the elongated shape of the molecule. The β strands are connected by highly flexible hairpin loops in which most of the sequence variability among the different neurotrophins is located. The molecule contains three disulphide bridges, two of these form a ring structure through which the third passes to form what is known as a 'cystine knot' motif (McDonald *et al.*, 1991; Ibanez, 1998).

In vitro and *in vivo* studies have shown that NGF promotes the survival of sympathetic neurons, populations of sensory neurons and basal forebrain cholinergic neurons. NGF also increases the synthesis of rate-limiting enzymes involved in the production of catecholamines in sympathetic neurons and increases neuropeptide expression in sensory neurons (Thoenen and Barde 1980; Hamburger *et al*, 1981; Gnahn *et al.*, 1983; Davies and Lindsey, 1985; Levi- Montalcini, 1987; Barde, 1989; Davies, 1994). The timing of NGF synthesis has been shown to occur at the onset of target field innervation by NGF responsive neurons.

In situ hybridisation and Northern blot analysis have revealed high expression of NGF mRNA in several sympathetic and sensory innervated tissues, including the heart, iris, skin and submandibular gland,with lower levels of expression in the adrenal gland, liver and skeletal muscle (Heumann *et al.*, 1984; Shelton and Riechardt, 1984, Maness *et al.*, 1994). NGF synthesis in these tissues is proportional to the density of innervation by sympathetic neurons (Korsching and Thoenen, 1983; Heumann *et al.*, 1984; Shelton and Riechardt, 1984; Korsching *et al.*, 1985; Unsicker, 1993). In the mouse whisker pad, a tissue rich in sensory innervation from the trigeminal ganglion, the highest levels of NGF mRNA are expressed in the epithelium (Davies *et al.*, 1987). Moreover, the concentration of NGF mRNA in the epithelia of the opthalmic, maxilliary and mandibular territories of the trigeminal ganglion is proportional to the innervation density of these target territories (Harper and Davies 1990). The most direct evidence that NGF is synthesised in cells innervated by NGF-responsive neurons, was provided by *in situ* hybridisation studies carried out on adult rat brains which localised NGF mRNA to cell bodies of pyramidal neurons in the hippocampus which are innervated by the NGF-responsive cholinergic neurons of the basal forebrain (Ayer-LeLievre *et al.*, 1988; Whittemore *et al.*, 1988).

Analysis of mice that are homozygous for a null mutation in the NGF gene have confirmed earlier studies of the effects of anti-NGF antibodies on nociceptive peptidergic and thermoceptive neurons that depend on NGF for survival (Crowley *et al.*, 1994). These mice fail to respond to noxious and thermal stimuli and have lost the small diameter neurons in dorsal root ganglia thought to convey nociceptive and thermoceptive information (Crowley *et al.*, 1994). In contrast, transgenic mice overexpressing NGF within the epidermis exhibit hypertrophy of peripheral sensory neurons and sympathetic neurons. In addition, both trigeminal and superior cervical ganglia contain more neurons in NGF over expressing mice, indicating a reduction of programmed or naturally occuring cell death (Albers *et al.*, 1994)

4.1.2. Brain-Derived Growth Factor.

The second member of the neurotrophin family to be identified was brain -derived neurotrophic factor (BDNF). Initially purified from pig brain, BDNF is a 12KDa basic protein which exists as a homodimer of two non-covalently linked subunits (Barde *et al.*, 1982; Radziejewski *et al.*, 1992). The protein is synthesised as a 252 amino acid polypeptide precursor, processed to release a 119 amino acid mature BDNF that has 50% sequence identity to NGF. Molecular cloning of the pig full length BDNF cDNA, led to subsequent isolation of the gene in several other species including human, mouse and rat (Leibrock *et al.*, 1989; Jones and Reichardt *et al.*, 1990; Maisonpierre *et al.*, 1991; Hofer *et al.*, 1990).

In vitro and *in vivo* studies have demonstrated that BDNF promotes the survival of several neuronal populations in the PNS, including both neural crest and placode derived peripheral sensory neurons that do not show a survival response to NGF (Barde *et al.*, 1982; Lindsey *et al.*, 1985; Davies *et al.*, 1986). In the CNS, a survival response has also been observed in specific neuronal

populations including embryonic motoneurons, basal forebrain cholinergic neurons, mesencephalic dopaminergic neurons, serotonergic neurons, GABAergic neurons, cerebellar granule neurons, corticospinal neurons and retinal ganglion cells have also been shown to survive in response to BDNF *in vitro* (Knüsel *et al.*, 1991; Hyman *et al.*, 1991; Oppenheim *et al.*, 1992; Lindholm *et al.*, 1993; Mamounas *et al.*, 1995; Ventimiglia *et al.*, 1995; Giehl *et al.*, 1996; Loudes *et al.*, 1999). In addition, to its survival promoting effects, BDNF is known to influence maturation of developing sensory neurons, and to influence synaptic strength and neuronal plasticity (Wright *et al.*, 1992; Thoenen, 1995; Lo, 1995).

Widespread BDNF mRNA expression has been detected in a variety of neurons in the CNS and PNS, as well as in other tissues. In the adult CNS, BDNF mRNA and protein have been detected at high concentrations in the hippocampus, amygdala, and cerebral cortex, and lower levels of expression have been found in the hypothalamus, septum, substantia nigra, brainstem and spinal cord (Ernfors *et al.*, 1990; Friedman *et al.*, 1991; Katoh-Semba *et al.*, 1997; Yan *et al.*, 1997). BDNF mRNA has been detected in subsets of neurons in dorsal root, trigeminal and geniculate ganglia. Low levels of BDNF expression have also been demonstrated in the heart, lung, muscle and skin (Ernfors *et al.*, 1990; Schecterson and Bothwell 1992; Kobayashi *et al.*, 1996; Conner and Dragunow 1998).

Mice lacking the BDNF gene display severe deficiencies in coordination and balance as a result of the reduced numbers of sensory neurons (Ernfors *et al.*, 1994; Jones *et al.*, 1994). The sensory neuronal populations particularly affected are those of the dorsal root, trigeminal, geniculate, vestibular, and nodose ganglia. These *in vivo* results are consistent with previous findings demonstrating the survival promoting effects of BDNF on these populations of neurons. In contrast, neuronal populations of the CNS previously shown to

respond to BDNF, such as motoneurons, dopaminergic neurons and cholinergic neurons, appear largely unaffected in BDNF$^{-/-}$ mice. This could be due to compensation by other neurotrophic factors. Interestingly, the expression of calcium binding proteins and neuropeptide Y is altered in many of the CNS neurons in BDNF$^{-/-}$ mice, which may indicate a functional deficit (Ernfors *et al.*, 1994; Jones *et al.*, 1994). BDNF deficient mice also exhibit learning difficulties due to impaired formation of the connections within the cerebral cortex and hippocampus that are thought to mediate certain forms of learning and memory (Linnarsson *et al.*, 1997).

4.1.3. Neurotrophin-3.

Identification of neurotrophin-3, was based upon the sequence homology between NGF and BDNF. Degenerate oligonucleotides based on highly conserved regions between BDNF and NGF were designed and used to screen a rat cDNA library resulting in the cloning of the full length cDNA (Ernfors *et al.*, 1990). NT-3 is secreted as a 282 amino acid polypeptide precursor that is enzymatically cleaved to give a 119 amino acid mature glycosylated protein. Mature NT-3 exists as a biologically active homodimer, that shares 50-60% structural homology with NGF and BDNF (Hohn *et al.*, 1990; Radziejewski *et al.*, 1992).

In the PNS, NT-3 has been shown to promote the survival of spinal sensory and sympathetic neurons (Maisonpierre *et al.*, 1990; Rosenthal *et al.*,1990; Wyatt *et al.*,1997), effectively supporting lumbar and cervical neurons of the DRG which contain many proprioceptive neurons (Hory-Lee *et al.*,1993) In addition, NT-3 promotes the differentiation of sensory neurons from their progenitors *in vitro*, and enhances early maturational changes in dorsal root ganglion neurons before they become dependent on neurotrophins for survival (Wright *et al.*,1992; Buchman and Davies 1993; Elshamy and Ernfors, 1996;

Elshamy *et al.*,1996). Several *in vitro* and *in vivo* studies have also shown that developing muscle sensory neurons also require NT-3 for survival (Hohn *et al.*, 1990; Ericksson *et al.*, 1994; Oakley *et al.*, 1995). Additional, sensory neuron populations supported by NT-3 include those of the trigeminal and nodose ganglia (Buchman and Davies 1993; Wilkinson *et al.*, 1996) and neurons from the spiral ganglia (Ernfors *et al.*, 1995). More recently NT-3 has also been shown to be required for a subset sympathetic neurons that respond to NGF in vitro (Francis *et al.*, 1999).

In the CNS, NT-3 promotes the survival of noradrenergic neurons and motoneurons *in vitro* (Henderson *et al.*, 1993; Friedman *et al.*, 1993). NT-3 has also been shown to support specific neuronal populations and glia. Interestingly NT-3 stimulates the expression of c-fos in hippocampal neurons and promotes their survival. Furthermore, NT-3 also regulates the survival of non-neuronal oligodendrocyte precursor cells in the embryonic rat hippocampus (Bertollini *et al* , 1997). NT-3 has been shown to promote neurite outgrowth from embryonic sensory neurons, and stimulate dendritic growth of pyramidal neurons within the developing visual cortex (McAllister *et al.*, 1995; 1997; Dijkhuizen *et al.*, 1997).

Accordingly, northern blot hybridisation has revealed NT-3 mRNA is expressed in several peripheral tissues including the kidney, spleen, heart, adrenal gland, ovary, intestine, lung, muscle and liver (Hohn *et al.*, 1990; Jones and Reichardt *et al.*, 1990; Maisonpierre *et al.*, 1990). The widespread distribution of NT-3 mRNA in peripheral tissues suggests that NT-3 could serve as a target derived factor for sympathetic and sensory neurons. The spatio-temporal expression pattern of NT-3 mRNA has also been determined by *in situ* hybridisation. In embryonic and postnatal rats NT-3 transcripts have been found in tissues of the inner ear, iris and the epithelium of whisker follicles, the target of trigeminal neurons (Pirvola *et al.*, 1992; Ernfors *et al.*, 1992; Hallbook *et al.*, 1993). Northern blot hybridisation has also demonstrated that NT-3 mRNA is

expressed in the peripheral trigeminal target field with expression peaking during the early stages of target field innervation and declining following the loss of neuronal responsiveness to NT-3 (Buchman and Davies, 1993). The distribution of NT-3 in the adult rat brain demonstrates remarkable regional specificity, with the highest levels being observed in the hippocampus and cerebellum, nucleus basalis, cerebral cortex and pons (Ernfors *et al.*, 1990; Hohn *et al.*, 1990;Jones *et al.*,1990; Rosenthal *et al* , 1990).

Further evidence for the physiological role of NT-3 during development has been provided by mice carrying a targeted disruption of the NT-3 gene. Homozygous NT-3 deficient mice are viable at birth but exhibit abnormal limb movements indicating deficits in proprioception (Ernfors *et al.*, 1994; Farinas *et al.*, 1994). Significantly the main components of the proprioceptive system are missing, including golgi tendons, muscle spindles and large diameter myelinated dorsal root sensory neurons (Ernfors *et al.*, 1994). However, selective expression of NT-3 in skeletal muscle of mice lacking endogenous NT-3 rescues proprioceptive neurons (Wright *et al.*, 1997). Severe deficits are also observed in trigeminal, superior cervical, nodose and spiral ganglia (Ernfors *et al.*, 1994; Fritzsch *et al.*, 1997; Wyatt *et al.*, 1997). In contrast, motoneurons, the enteric nervous system, and the major anatomical regions of the CNS appear to develop normally (Ernfors *et al* , 1994; Farinas *et al* , 1994).

4.1.4. Neurotrophin-4/5.

Recognition of the amino acid sequence homology between NGF, BDNF and NT-3, facilitated the identification of Neurotrophin 4/5. Using degenerate PCR primers, the NT4 gene was isolated from both *Xenopus laevis* and Viper (Hallbook *et al* ., 1991, Berkemeier *et al* ., 1991, Ip *et al* ., 1992). The NT-4 protein is secreted as a 236 amino acid precursor which is enzymatically processed giving rise to a 123 amino acid polypeptide. Mature

NT-4 shares 51%-60% homology with the other neurotrophins and retains all six cysteines thought to be involved in the formation of intrachain disulphide bridges. Following the isolation of NT-4 in *Xenopus* and Viper, mammalian homologues were isolated in human and rat. Originally designated mammalian NT-4 and NT-5, these mammalian homologues had identical amino acid sequences and were therefore renamed NT4/5 (Hallbook *et al.*, 1991, Berkemeier *et al.*, 1991, Ip *et al* ., 1992). More recently, genomic NT-4 has also been identified in rat enabling the production of a transgene to aid in functional analysis of the gene (Salin *et al.*, 1997).

Although NT-4 exerts distinct biological effects on several neuronal populations in the PNS and CNS, overlapping activities of NT-4 and BDNF *in vitro* and *in vivo* have been demonstrated in several studies. In the developing PNS, NT-4/5 promotes the survival of BDNF-dependent mouse nodose neurons during the phase of naturally occuring cell death, and mouse trigeminal and jugular neurons in the early stages of target field innervation (Davies *et al* , 1993; Ibanez *et al.*, 1993).

NT4/5 exerts neuroprotective effects in the CNS promoting the survival of both developing and adult spinal and facial motoneurons (Henderson *et al.*, 1993; Koliatsos *et al.*, 1994; Freidman *et al.*, 1995; Junger *et al.*, 1997). Hippocampal neurons and certain neuronal populations of the developing and adult visual system also respond to NT-4/5 (Ip *et al.*, 1993; Mcallister *et al.*, 1995). NT4/5 also supports the *in vitro* survival and, or differentiation of cerebellar granule cells and dopaminergic neurons of the substantia nigra (Studer *et al* ., 1995; Gao *et al* ., 1995). More recently, NT-4/5 has also been shown to promote the development and maintenance of sympathetic preganglionic neurons innervating the adrenal medulla (Schober *et al.*, 1998).

Analysis of NT-4/5 mRNA expression by northern blot hybridisation and RNase protection assays has revealed low levels throughout adult and

embryonic rat tissues. In the adult rat, several peripheral tissues express NT-4/5 mRNA including, the thymus, muscle, lung, heart, stomach and kidney (Berkemeier *et al.*, 1991). The highest level of NT-4/5 mRNA expression in peripheral tissues appears to coincide with the period of naturally occuring neuronal death in peripheral ganglia suggesting that NT-4/5 may act as target derived factor. In the CNS, NT-4/5 mRNA has been detected in the thalamus, hypothalamus, hippocampus, cerebral cortex, brain stem and cerebellum, demonstrating a more widespread distribution compared to the other neurotrophins (Timmusk *et al.*, 1993; Persson *et al.*, 1993).

Analysis of mice carrying a null mutation in the NT-4/5 gene, has revealed severe deficiencies of the nodose-petrosal and geniculate ganglia. However, motoneurons of the facial nucleus, sympathetic neurons of the SCG, and dopaminergic neurons of the substantia nigra which are responsive to NT-4/5 *in vitro* appeared largely unaffected. Furthermore, analysis of mice deficient for both NT-4/5 and BDNF revealed a substantial loss of peripheral sensory neurons which was additive compared to that of the single mutants alone. (Lui *et al.*, 1995; Conover *et al.*, 1995;).

4.1.5. Neurotrophin-6.

More recently, neurotrophin-6 has been identified in the teleost fish *Xiphophorus* by screening a genomic library with a mouse NGF probe. As with the other neurotrophins, NT-6 is synthesised as a precursor polypeptide that is proteolytically cleaved, giving rise to a mature secreted protein of 143 amino acids. Structural analysis has revealed NT-6 shares 40-60% amino acid identity with the other members of neurotrophin family, and conservation of all six cysteine residues. However, a unique feature of NT-6 is the presence of a 22 amino acid basic domain between cysteine residues 2 and 3 which is thought to

be responsible for proteoglycan binding that affects the release of NT-6 from the extracellular matrix and surface of producing cells (Götz *et al* ., 1994).

In vitro NT-6 has been shown to exert survival promoting effects on peripheral neurons including, NGF-dependent chick sympathetic and DRG sensory neurons (Götz *et al* ., 1994). Furthermore, neurite outgrowth of DRG explants is stimulated by heparin beads coated with NT-6 (Xiaoling *et al* ., 1997).

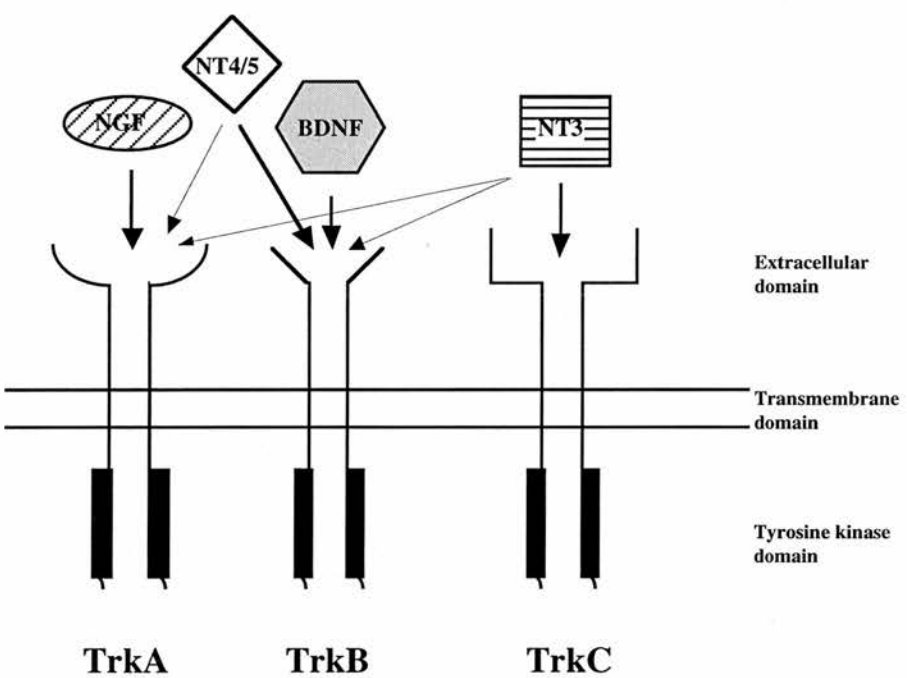
Analysis of NT-6 mRNA in *Xiphophorus* by northern blotting, revealed moderate expression in the adult gill, liver, and eye, with weaker expression in skin, spleen, heart, and skeletal muscle. Furthermore, *in situ* hybridisation in the developing embryo revealed a detailed expression pattern in the cerebellum, and midbrain (Götz *et al* ., 1994).

4.1.6. Neurotrophin-7.

The most recent member of the NGF family to be identified is neurotrophin-7 (NT-7). Cloned in the carp *Cyprinus carpio*, and zebrafish *Danio rerio,* NT-7 was isolated by degenerate PCR using oligonucleotides designed to highly conserved regions of *Xiphophorus* NT-6 (Nilsson *et al.*, 1998; Kwok-On *et al.*, 1998). NT-7 possesses the structural characteristics common to all known neurotrophins, including conservation of the six cysteine residues. Amino acid alignment of NT-7 with NT-6 reveals 66% amino acid identity between the two proteins. Furthermore, NT-7 contains a 15 amino acid insertion in the same position to that found in NT-6 (Kwok-On *et al.*, 1998).
NT-7 promotes the survival of DRG neurons and is predominantly expressed in peripheral tissues, including skin, heart and intestine (Kwok-On *et al.*, 1998). NT-7 has also been shown to bind the human p75 receptor and induce tyrosine phosphorylation of rat TrkA (Nilsson *et al.*, 1998; Kwok-On *et al.*, 1998).

4.2. The Neurotrophin Receptors.

The NGF family of neurotrophic factors is known to interact with two types of transmembrane glycoproteins, the lower affinity p75 receptor and the high affinity Trk receptors. Whereas the p75 receptor interacts with all neurotrophins with equal affinity, the Trk family of receptor tyrosine kinases show a greater degree of specificity. NGF binds exclusively to Trk A (Klein *et al.*, 1991; Kaplan *et al.*, 1991), BDNF binds exclusively to Trk B (Berkemeier*et al.*, 1991), NT-3 binds predominantly to Trk C (Lambelle *et al.*, 1991), but also to a lesser extent Trk A and TrkB (Cordon-Cardo *et al.*, 1991; Berkemeier *et al.*, 1991; Klein *et al.*, 1991; Kaplan *et al.*, 1991; Squinto *et al* ., 1991) and NT-4/5 binds predominantly to trkB but also binds to a lesser extent to trkA (Figure 1.1).



Adapted From Davies, 1994

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

4.2.1. Trk Receptors.

The trk family of receptor tyrosine kinases consists of three related receptors TrkA, TrkB, and TrkC to which the neurotrophins bind differentially. Each receptor is composed of an extracellular ligand binding domain and an intrinsic cytoplasmic tyrosine kinase domain (Barbacid *et al.*, 1994; 1995). The first member of this family to be identified, was isolated from a human colon carcinoma as a partial gene fragment (Martin-Zanca *et al.,* 1986). The full-length receptor tyrosine kinase cDNA was subsequently isolated and called Trk (Trk A) for tropomyosin receptor kinase. TrkA encodes a 140KDa transmembrane receptor ($p140^{trkA}$) which consists of a 32 amino acid putative signal peptide, a 374 amino acid extracellular domain containing N-linked glycosylation sites, a transmembrane domain, and a cytoplasmic region similar to previously identified tyrosine kinase domains. TrkB and TrkC have been identified based on nucleotide sequence similarity with TrkA. TrkB cDNA was isolated from a mouse brain library (Klein *et al.,* 1989). It encodes a 145KDa transmembrane protein ($p145^{trkB}$) that is structurally similar to that of TrkA, sharing 37% amino acid identity in the extracelluar domain and 75% amino acid identity in the cytoplasmic tyrosine kinase domain (Klein *et al.,* 1989; Middlemas *et al.*, 1991). TrkC, originally isolated from a porcine brain cDNA library, also encodes a 145KDa transmembrane protein ($p145^{trkC}$) and displays similar homology to TrkA and TrkB, with ~ 55% amino acid identity in the extracellular domain, and ~80% in the tyrosine kinase domain (Lamballe *et al.*, 1991).

Interestingly, the trk family members appear to encode several transcripts. TrkA encodes two isoforms which differ in the presence of 6 amino acid residues located in the extracellular domain (Martin-Zanca *et al.*, 1989; Meakin *et al.*, 1992). Expression of full length TrkA is found primarily in neuronal cells and although the isoform that lacks the 6 amino acids in the

juxtamembrane region has been found in non-neuronal cells both isoforms are thought to have similar functional characteristics (Barker *et al* ., 1993). TrkB has a more complex transcriptional pattern, encoding at least eight different isoforms. These include isoforms that possess or lack the receptor tyrosine kinase, domain (Klein *et al.*, 1990; Middlemas *et al.*, 1991). Splice variants that lack either two or all three of the leucine rich motifs in the extracellular domain have been identified. These variants do not bind any of the TrkB ligands, suggesting that the leucine rich motifs in TrkB are essential for ligand binding and signalling (Ninkina *et al.*, 1997). The TrkC gene encodes nine isoforms, the full length receptor, four isoforms with small insertions in the kinase domain, and four truncated receptor isoforms lacking the intracellular tyrosine kinase domain (Valenzuela *et al.,* 1993; Tsoulfas *et al.,* 1993).

Expression of murine TrkA, TrkB, and TrkC mRNA in embryonic, early postnatal, and adult brain, suggest that these receptors and their ligands play roles in the developing and mature nervous system. In the CNS, TrkA mRNA is expressed in the brainstem, striatum, and septum as well as cholinergic neurons of the striatum and basal forebrain (Ringstedt *et al.*, 1993). TrkA mRNA is also expressed in several neuronal populations of the PNS, including those of the dorsal root, sympathetic and cranial sensory ganglia (Martin-Zanca *et al.*, 1990; Vazquez and Ebendal 1991; Holtzman *et al.*, 1992). $TrkA^{-/-}$ homozygous null mice are insensitive to noxious and thermal stimuli, they display extensive neuronal deficits in dorsal root, sympathetic and trigeminal ganglia. However, cholinergic neurons of the basal forebrain appear largely unaffected (Smeyne *et al.,* 1994).

Northern blotting and *in situ* hybridisation have revealed TrkB mRNA expression in the brain and spinal cord, and in spinal and cranial sensory ganglia (Klein *et al.*, 1989; Ringstedt *et al.*, 1993). $TrkB^{-/-}$ mice die within the first postnatal week due to cardiovascular and respiratory impairment, and they

display severe neuronal losses in trigeminal, dorsal root and nodose-petrosal ganglia (Klein *et al.*, 1993).

Northern blot hybridisation has revealed TrkC mRNA expression in the cerebral cortex, hippocampus and granular cell layer of the cerebellum, in the dorsal root, trigeminal and cranial ganglia and in the myenteric plexi (Tessarollo *et al.*, 1993; Lambelle *et al.*, 1994). TrkC-deficient mice survive only a few days after birth, displaying abnormal atheotic movements that are due to a defect in proprioception (Klein *et al.*, 1994). There is a complete loss of Ia muscle afferents, accompanied by a 20% loss of DRG neurons, presumably those responsible for the missing afferents (Klein *et al.*, 1994).

4.2.2. p75.

p75 is the founding member of the TNF receptor superfamily (Smith *et al.*, 1994), which includes type I membrane proteins such as TNFR-I, TNFR-II and the FAS-CD95 receptor (Nagata and Golstein 1995). It is a transmembrane glycoprotein of approximately 75KDa, also referred to as $p75^{NTR}$ (Radeke *et al.*, 1987; Johnson *et al.*, 1986). The distinguishing features common to all members of this superfamily are an extracellular ligand binding region containing a cysteine-rich domain and an intracellular region that contains a death domain. In p75, the death domain is a 90 amino acid region that is homologous to the intracellular death domain of FAS and TNFR-I. It is thought this region may mediate interactions between signalling molecules necessary for the activation of apoptosis (Chapman *et al.*, 1995; Carter and Lewin 1997). The extracellular, ligand binding domain consists of four 25-28 amino acid cysteine rich repeats, two putative N-linked glycosylation sites, and a cytoplasmic domain of 151 amino acids (Welcher *et al.*, 1991).

The biological roles of the p75 receptor are complex. p75 can collaborate with trk receptors to either enhance or reduce neurotrophin-mediated trk receptor activation and can activate signalling cascades involved in apoptosis independently of the signalling (Hantzopoulos *et al.,* 1994; Barker and Shooter 1994; MacPhee and Barker 1997).

When p75 and trkA receptors are co-expressed, the affinity of NGF binding is increased and there is enhanced trkA activation compared with trkA expression alone (Barker and Shooter 1994; Verdi *et al.*, 1994). Consistent with this, the NGF responsiveness of embryonic sensory and postnatal sympathetic neurons derived from $p75^{-/-}$ mice is reduced compared to neurons from their wildtype counterparts (Davies *et al.*, 1993; Lee 1994). Therefore, in the presence of both receptors, p75 mediates increased NGF responsiveness by increasing the association of NGF with trkA. Two models have been proposed to account for how p75 can enhance trk receptor function. The first is a ligand passing mechanism which predicts the high affinity binding state is a result of NGF ligand presentation by p75 to the trkA receptor. The second is a conformational model, in which there is a ligand independent interaction between p75 and trkA, producing a high affinity binding site for NGF (Barker and Shooter 1994; Ross *et al.*, 1996). More recently, it has been shown, BDNF can block the binding of NGF to p75, thereby causing a reduction in binding of NGF to trkA (Lachance *et al.,* 1997). Mutant forms of NGF that bind trkA but not p75, are less effective than wildtype NGF in activating trkA on cells where p75 and trkA are coexpressed, indicating p75 is necessary for the increased responsiveness of trkA to NGF (Ryden *et al.,* 1997; Horton *et al.*, 1997).

In addition to increasing activation of trkA by its prefered ligand NGF, p75 also reduces trkA activation by its non-prefered ligands. Whereas NT-3 and NT4/5 can activate trkA in mutant PC12 cells lacking p75 neither ligand can

activate trkA in PC12 cells where both trkA and p75 are co-expressed (Ip *et al.*, 1993).

p75 also has the potential to induce cell death; expression of its intracellular domain as a transgene in mouse neurons causes apoptosis in populations of central and peripheral neurons (Majdan *et al.*, 1997). Evidence for this new function of p75 has also been obtained *in vitro* in the avian trigeminal mesencephalic nucleus (TMN) (Davey and Davies, 1998) and *in vivo* in the avian retina, and mouse sympathetic ganglia (Frade and Barde 1999; Bamji *et al.*, 1998). In all cases, trk signalling inhibits the p75 mediated cytotoxic affect. Consistent with this trk independent function of p75, has been shown that p75 is capable of autonomous signalling. Neurotrophin binding to p75 results in sphingomyelinase activation and ceramide production in neural cell lines and glial cells (Dobrowsky *et al.*, 1994; 1995). NGF binding to p75 produces activation of the nuclear factor NF-kB and translocation of this protein to the nucleus in fibroblasts and Schwann cells and enhancement of jun kinase (JNK) activity in oligodendrocytes (Carter *et al.*, 1996; Casaccia-Bonnefil *et al.*, 1996). Such signalling has been implicated in the NGF mediated apoptosis of developing retinal cells and neonatal rat oligodendrocytes which express p75 but lack TrkA. (Frade *et al.*, 1996; Casaccia-Bonnefil *et al.*, 1996). In contrast, adult human oligodendrocytes expressing p75 but not TrkA, do not demonstrate significant apoptosis or JNK activation (Ladiwala *et al.*, 1998).

4.3. The Neurotrophic Cytokines.

The family of cytokines known as the 'neuropoietic cytokines' are a group of distantly related growth factors which include ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), oncostatin M (OSM), and cardiotrophin-1 (CT-1). The cytokines CNTF, LIF, and IL-6,

have similar secondary structures and their receptors share a common signaling subunit gp130. Additional specific receptor subunits, determine the specific activity of each cytokine.

4.3.1. Ciliary Neurotrophic Factor.

CNTF was the first member of this family to be identified based on its ability to support the survival of parasympathetic neurons of the embryonic chick ciliary ganglia (Ardler *et al.*, 1979). Purification of the protein from adult rat sciatic nerve and determination of a partial amino acid sequence led to cloning of the CNTF cDNA by degenerate PCR. CNTF is an acidic protein of 200 amino acids with a molecular mass of 22.8kDa. It appears to be predominantly a cytosolic protein, that lacks an N-glycosylation site and a secretory signal peptide (Lin *et al.*, 1989; Stockli *et al* ., 1989).

CNTF affects the *in vitro* and *in vivo* survival and or phenotypic differentiation of several neuronal populations from both the peripheral and central nervous system. In the PNS, CNTF supports the *in vitro* survival of ciliary, sympathetic and sensory neurons at various stages during development (Manthorpe *et al.*, 1982; Manthorpe *et al.*, 1986). Interestingly, whereas nodose neurons survive in response to CNTF throughout development it has been observed trigeminal neurons do not respond to CNTF until the late fetal period (Horton *et al.,* 1998). Overexpression of recombinant chicken CNTF in the target tissues of ciliary ganglia during the period of naturally occuring cell death increases neuronal survival. This demonstrates CNTF may be necessary in regulating ciliary ganglion neuron survival during development and presumably can be secreted to some extent (Finn *et al* ., 1998).

In addition to promoting neuron survival, CNTF also influences the expression of the neurotransmitter choline acetyltransferase in sympathetic and retinal neurons as well as vasoactive intestinal peptide (VIP) in embryonic

sympathetic neurons (Saadat *et al.*, 1989; Ernsberger *et al.,* 1989). More recently CNTF and LIF have been shown to influence dendritic retraction in cultured rat sympathetic neurons (Guo *et al* ., 1999).

CNTF promotes the long term survival of several neuronal populations in the CNS including, embryonic chick and rat motoneurons *in vitro* (Arakawa *et al.,* 1990; Magal *et al.*, 1991). Similarly, during the period of naturally occuring cell death, administration of CNTF *in ovo* increases the number of spinal cord motoneurons in the embryonic chick (Oppenheim *et al.*, 1991). CNTF also prevents axotomy-induced degeneration of facial and spinal cord motoneurons, dopaminergic neurons of the substantia nigra and cholinergic neurons of the medial septum (Sendtner *et al.*, 1990; Sendtner *et al.*, 1992; Forger *et al* ., 1993; Li *et al* ., 1994; Clatterbuck *et al* ., 1993; Hagg *et al.*, 1992; Hagg and Varon *et al.,* 1993). Following peripheral nerve injury in adult rats, increased retrograde transport of CNTF has been observed in DRG and spinal cord motoneurons, suggesting that CNTF may have an endogenous role in injury and repair (Thoenen *et al.*, 1991; Curtis *et al.*, 1993). Additional neuronal populations of the CNS shown to be CNTF responsive include, cerebellar purkinje neurons and hippocampal neurons (Ip *et al.,* 1991; Larkfors *et al.*, 1994). More recently it has been demonstrated that CNTF enhances BDNF mediated cell survival of cholinergic neurons, and increases choline acetyltransferase activity in these neurons eight fold (Hashimoto *et al.*, 1999). In addition, CNTF regulates the *in vitro* differentiation and survival of astroglia and oligodendrocytes, respectively (Louis *et al* ., 1993; Lillien *et al.*, 1990).

Analysis of CNTF expression by northern blotting and immunocytochemistry has revealed that CNTF is expressed in the sciatic nerve in the adult rat. Expression of CNTF mRNA in the sciatic nerve increases with age, only becoming apparent by postnatal day 4 (Stockli *et al.*, 1989). High CNTF expression has also been detected in the optic nerve, spinal cord and

olfactory bulb, with lower levels in the hippocampus, cerebellum, striatum and brain stem. No CNTF mRNA expression has been detected in skin or skeletal muscle (Stockli *et al.*, 1989; Stockli *et al.*, 1991; Ip *et al.*, 1993). Immunocytochemistry has localised CNTF protein to dorsal root ganglia, spinal motor roots, oligodendrocytes in the spinal cord, and astrocyte-like cells of the optic nerve, which is consistent with the role of CNTF as a growth and survival factor for these cell types (Dobrea *et al.*, 1992). The temporal and spatial pattern of CNTF expression indicates that it is not involved in the regulation of neuronal survival in mammals, since the period of naturally occuring cell death is complete by the time CNTF synthesis begins. It is possible however that CNTF has a physiological role in injury repair (Stockli *et al.*, 1991; Weisenhorn *et al* 1998).

CNTF $^{-/-}$mice grow normally, but develop mild motoneuron deficits in late adulthood, resulting in a significant reduction in muscle strength (Masu *et al.*, 1993). In accordance with previous findings, this suggests that CNTF is not a target derived neurotrophic factor involved in the establishment of the nervous system, but is important for the postnatal maintenance of some neuronal populations, particularly motoneuons.

4.3.2. Leukemia Inhibitory Factor.

LIF is a pleiotropic member of the cytokine family, initially purified and cloned on the basis of its ability to induce differentiation and supress the clonogenicity of the monocytic leukemia cell line M1 (Metcalf *et al.*, 1988). In the nervous system, LIF can act both as a trophic factor enhancing neuronal survival, and a differentiation factor, altering neuropeptide expression in neuronal and glial cells. LIF also has been shown to regulate neurotransmitter

phenotype inducing a switch from adrenergic to cholinergic in sympathetic neurons (Bamber *et al.*, 1994; Horton *et al.*, 1996).

In the PNS, LIF promotes the survival and differentiation of sensory neurons from mouse neural crest and dorsal root ganglia in culture (Murphy *et al* .,1991; Murphy *et al* ., 1993). It also has the potential to regulate the development of placode derived nodose sensory neurons in coordination with BDNF, NT-3 and NT-4/5 (Thaler *et al.,* 1994). Furthermore, LIF is retrogradely transported by sympathetic and sensory neurons following axotomy, which suggests a possible role in peripheral nerve regeneration (Hendry *et al.*, 1992; Curtis *et al.*, 1994).

In the CNS, LIF promotes the survival of cultured fetal motoneurons, and induces differentiation of spinal cord precursor cells into neurons (Martinou *et al.,* 1992; Richards *et al.*, 1996). Up-regulation of LIF mRNA has been demonstrated in several brain regions *in vivo* following injury to the adult rat CNS (Banner *et al.,* 1997; Blesch *et al.*, 1999). LIF also exerts neurotrophic effects on non-neuronal cells, promoting the growth and differentiation of astrocyte and oligodendrocyte precursors *in vitro* (Mayer *et al .,* 1994; Richards *et al.*, 1996). LIF displays a wide pattern of expression in the nervous system, consistent with that of a target derived factor (Lemke *et al.,* 1996). Immunocytochemistry has revealed that the neuronal populations found to express LIF are those which respond to this factor suggesting LIF may have a possible autorine/paracrine mode of action (Cheng *et al.*, 1997).

Analysis of mice carrying a targeted disruption of the LIF gene, display retarded postnatal growth, but no gross developmental abnormalities in the nervous system (Stewart *et al.*, 1992).

4.3.3. Other Neurotrophic Cytokines.

IL-6 was initially described as a pleiotrophic molecule involved in the regulation of inflammatory responses and in hematopoiesis (Hirano *et al.*, 1992). Accumulating evidence has suggested a role for IL-6 during neuronal development. It promotes the survival of sensory and sympathetic neurons, and induces substance P expression in dissociated cultures of sympathetic neurons, and galanin expression in DRG neurons *in vivo*. IL-6 also promotes the survival of basal forebrain cholinergic neurons and catecholaminergic neurons of the mesencephalon (Hama *et al.*, 1989; Kushima *et al.*, 1992). Furthermore, IL-6 can exert protective effects against neuronal insults, attenuating the toxic effects of glutamate on cholinergic neurons of the striatum and hippocampus (Toulmond *et al.*, 1992; Yamada and Hatanaka 1994; Gadient and Otten 1997).

IL-6 mRNA appears to be developmentally regulated in the PNS and CNS, with pronounced accumulation between birth and adulthood. IL-6 is expressed in sympathetic and sensory ganglia, astrocytes and in neuronal subpopulations of the neocortex, hippocampus and cerebellum (Gadient and Otten 1994; 1996). Although IL-6 expression is not apparent in intact nerves, upregulation of IL-6 mRNA following axotomy, suggests a potential role in response to nerve injury (Reichart *et al.*, 1996). Mice carrying a homozygous null mutation for IL-6 display no obvious phenotypic defects within the nervous system (Poli *et al.*, 1994; Cressman *et al.*, 1996)

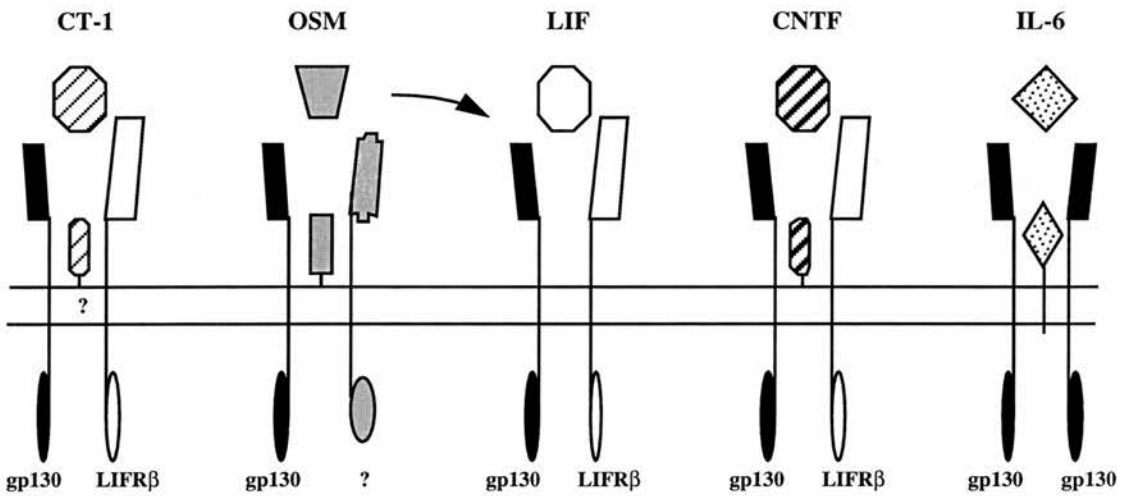
Oncostatin M (OSM) was originally purified on the basis of its ability to inhibit the proliferation of cells derived from solid tumors (Zarling *et al.*, 1986). OSM acts as a mitogen for fibroblasts derived from AIDS-related Kaposi's sarcoma. It induces the differentiation of leukemia cells to macrophage like cells, and causes the synthesis of acute phase proteins in hepatic cells (Bruce *et al.*, 1992). OSM also inhibits the differentiation of totipotent embryonic stem cells, similar to LIF (Rose *et al.*, 1993), and regulates the expression of

lymphokines such as IL-6, granulocyte-colony stimulating factor, and granulocyte-macrophage colony stimulating factor. In the nervous sytem, OSM supports the survival of embryonic nodose and trigeminal ganglion neurons *in vitro* (Horton *et al.,* 1996).

Cardiotrophin-1 (CT-1) was cloned from a mouse embryoid body cDNA library based on its ability to induce the growth of rat heart cells (Pennica *et al.*, 1995). It shares some structural features in common with LIF and CNTF, but has limited amino acid sequence homology with these cytokines. CT-1 inhibits the proliferation of the M1 myeloid leukemic cell line, and the differentiation of embryonic stem cells. It also induces and supresses the expression of the same set of neuropeptide and neurotransmitter mRNAs as CNTF and LIF in sympathetic neurons (Cheng *et al.*, 1997). CT-1 has neurotrophic effects on the central and peripheral nervous systems, promoting the survival of ciliary, sympathetic, midbrain dopaminergic, dorsal root ganglion, and motoneurons (Pennica *et al.*, 1995a; 1996; Their *et al.*, 1999). Furthermore, *in vitro* glial cell line derived factor (GDNF) and CT-1 derived from Schwann and muscle cells, produce synergistic effects on motoneuron survival *in vitro* (Arce *et al.,* 1998). CT-1 mRNA is detected in a wide range of tissues, including postnatal and embryonic, mouse brain, spinal cord, dorsal root ganglia, muscle, heart, and skin, suggesting it may be a target derived factor, and/or act in an autocrine/paracrine manner (Sheng *et al.,* 1996; Pennica *et al.*, 1995).

4.4. The Neurotrophic Cytokine Receptors.

The neurotrophic cytokines are known to signal through a muticomponent receptor complex (Figure 1.2), consisting in most cases of a ligand specific α component, and two signal transducing β subunits. Although not all cytokines have ligand specific α components in their receptors, known α components include the CNTF receptor α, (CNTFRα) and IL-6 receptor α (IL-6Rα). β subunits include the LIF receptor β (LIF-Rβ) and glycoprotein 130 (gp130) which are present in various combinations in receptors for different members of the cytokine family. The LIFRβ/gp130 heterodimer and CNTFRα form the specific receptor for CNTF whereas LIFRβ/gp130 form a receptor for LIF, (Ip *et al.*, 1992), CT-1 (Taga *et al.*, 1997) and OSM (Mosley *et al.*, 1996). IL-6 signals through a gp130/gp130 homodimer and IL-6Rα (Bruce *et al.*, 1992).



Adapted from Stahl and Yancopoulos, 1994

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

The CNTFRα is attached to the plasma membrane surface via a phospholipase-C sensitive glycosyl-phosphatidylinositol link (GPI-link) whereas IL-6Rα is a transmembrane protein. Following ligand binding to the α receptor, the ligand/receptor complex binds to the β component of the cytokine receptors (Bazan *et al.*, 1991; Ip *et al.*, 1992; Davies *et al.*, 1993; Stahl and Yancopoulos, 1994). The β components are transmembrane signalling proteins with no intrinsic enzymatic domains, however ligand induced hetero- or homo-dimerization of β receptor components activates members of the JAK family of non-receptor tyrosine kinases which are bound constitutively to LIFR-β, and gp130 at the plasma membrane (Stahl *et al.,* 1994). Transactivation by the JAK proteins leads to the phosphorylation of specific tyrosine residues on LIFR-β and gp130. Phosphorylated LIFR-β and gp130 then bind SH2 domain containing components of the signal transduction machinary and activate several signal transduction pathways including PLCγ, PI-3 kinase and the Ras/MAP kinase pathway (Frank and Greenberg, 1996; Inoue *et al.*, 1996). Recent evidence also suggest that NFkB plays an important role in mediating cytokine signalling (Middleton *et al.*, 1999). The signal transducer and activator of transcription (STAT) proteins are key substrates for activated JAK kinases. Following phosphorylation, STAT proteins dissociate from LIFR-β and gp130, translocate to the nucleus and activate transcription of specific genes. Specific STAT factors activated by CNTF include STAT1, STAT3 and STAT5B (Shuai *et al.*, 1994; Stahl *et al.*, 1995; Boulton *et al.*, 1995).

4.5. The TGF-ß Family Of Neurotrophic Factors.

The transforming growth factor ß (TGFß) superfamily comprises a large group of around 30 structurally related cytokines functioning throughout development to regulate cell growth, proliferation and differentiation.

The TGFß protein is considered the prototype of this superfamily, and was originally discovered as a secreted factor that induced malignant transformation *in vitro* (Roberts *et al.,* 1981). Since then, many family members have been identified in species ranging from drosophila to human. These have been separated into different sub-families depending on their function, and include the TGF-ß (TGF-ß 1-3), inhibin/activin, decapentaplegic (Dpp), 60A, DVR (Dpp andVgl Related proteins), and a divergent sub-family (Kingsley *et al.*, 1994). Four recently isolated closely related proteins that comprises a subgroup of the TGFß family members, GDNF, neurturin, persephin and artemin (Lin *et al.*, 1993; Kotzbauer *et al.*, 1996; Milnbrandt *et al.,* 1997; Baloh *et al.*, 1998b), have been shown to promote the survival of a number of neuronal populations within the CNS and PNS.

Nearly all TGF-ß family members signal through a receptor complex formed by two distantly related types of serine/threonine kinase receptors, TßR-I and TßR-II (Wrana *et al.*, 1994; Massague *et al.*, 1996; Josso *et al.*, 1997). The exception are members of the GDNF subfamily which signal via a multicomponent receptor consisting of the transmembrane tyrosine kinase receptor Ret and a GPI-linked receptor component (Durbec *et al.*, 1996; Jing *et al.*, 1996; Treanor *et al.,* 1996; Baloh *et al.,* 1998a).

4.5.1. TGF-ß Superfamily Members In The Nervous System.

Increasing evidence suggests that numerous members of the TGF-ß superfamily have pleiotropic roles in the developing nervous system. The functions of the TGF-ßs range from conferring ventral versus dorsal identities on neural precursors to the specification of glial cell fates and the regulation of neuronal survival (Mehler *et al.*, 1997). From previous studies, TGF-ß (1-3) have been implicated in promoting the survival of motoneurons, sensory neurons and midbrain dopamingergic neurons (Martinou *et al.*, 1990; Poulsen *et al.*, 1994). More recently, TGF-ß has been shown to exert neurotrophic effects on peripheral autonomic, sensory and CNS dopaminergic neurons by acting in synergy with GDNF (Krieglstein *et al.*, 1998; Schober *et al.*, 1999). Furthermore, the rapidly expanding bone morphogenetic protein (BMP) subfamily has been implicated in the early formation of the nervous system, where they act as gradient morphogens, specify cell lineage commitment of neural cells and affect cell differentiation (Mehler *et al.*,1997). BMP-4 and BMP-7 are thought to be involved in the regulation of neurulation, and BMP-2 or BMP-4 play an instructive role in programming the elaboration of neuronal cell lineage from neural crest stem cells. Furthermore, BMP-2, -6 and -7 have been shown to promote the survival and differentiation of dopaminergic neurons (Jordan *et al.*, 1997; Mehler *et al.*, 1997).

4.6. Glial Cell Line Derived Neurotrophic Factor Family.

4.6.1. Glial Cell Line Derived Neurotrophic Factor.

Glial cell line derived neurotrophic growth factor (GDNF) is a distantly related member of the transforming growth factor ß family that was originally isolated from the rat glial cell line B49 on the basis of its ability to promote dopamine uptake in midbrain cultures (Lin *et al.*, 1993). GDNF is a 16KDa basic protein which exists as a disulphide bonded homodimer (Lin *et al.*, 1993). Molecular cloning of the full length rat and human GDNF cDNAs using degenerate oligonucleotides to a partial amino acid sequence of purified GDNF revealed 93% amino acid identity between them (Lin *et al.*, 1994).

GDNF is secreted as a mature protein of 134 amino acids that contains two potential N-linked glycosylation sites and seven conserved cysteine residues. The cysteine residues display the same relative spacing to those found in transforming growth factor-ß, indicating that GDNF is a member of the TGF-ß superfamily. However, GDNF shares less than 20% homology with other TGF-ß family members and therefore is a member of a new sub-family (Lin *et al.*, 1993).

GDNF has been shown to promote the survival of several neuronal populations in the central and peripheral nervous system. In the CNS, GDNF promotes the survival, morphological differentiation and high affinity dopamine uptake of dopaminergic neurons in dissociated cultures of rat embryonic midbrain (Lin *et al.*, 1993; Akerud *et al.*, 1999). GDNF also exerts trophic effects on mesencephalic dopaminergic neurons *in vivo*. For example, the addition of GDNF to intraocular brain grafts of the ventral mesencephalon results in a marked increase in the numbers of surviving dopaminergic neurons

and their associated innervating nerve fibres (Stromberg *et al.*, 1993). GDNF also promotes the survival of embryonic motoneurons in the early phases of developmental death and increases choline acetyltransferase activity (Zurn *et al.*, 1994). At low concentrations, GDNF is more effective in supporting the survival of motoneurons than BDNF, CNTF and LIF, making it the most potent survival factor for embryonic motoneurons yet identified (Henderson *et al.*, 1994). GDNF also promotes the survival and development of several other neuronal populations in the CNS. GDNF has been shown to rescue noradrenergic neurons of the locus coeruleus from 6-OHDA lesion induced cell death as well as increasing soma size, neurite outgrowth and tyrosine hydroxylase activity (Arenas *et al.*, 1995). In addition, GDNF also promotes the survival, differentiation and morphologic maturation of Purkinje cells *in vitro*, without affecting the overall number of neurons or glial cells (Mount *et al.*, 1995). Furthermore, GDNF promotes the survival and increased choline acetyl transferase activity of basal forebrain cholinergic neurons in adult rat following axotomy of the fimbria/fornix, the effects of which are comparable to those shown by BDNF (Williams *et al.*, 1995).

GDNF exerts neuroprotective effects in the CNS, rescuing several neuronal populations from axotomy-induced degeneration. Following transection of axons within the rat medial forebrain bundle, administration of GDNF *in vivo* has been shown to prevent the degeneration of dopaminergic neurons, whilst NT-4/5, TGF-α, CNTF and bFGF failed to have any effect (Beck *et al.*, 1995). In addition, GDNF prevents the axotomy induced degeneration and atrophy of facial motoneurons in adult and neonatal rat by retrograde transport of GDNF in a receptor mediated fashion (Yan *et al.*, 1995). Local administration of GDNF to an avulsed ventral root of the spinal cord prevents the loss of half the motoneuron population and results in hypertrophy of the surviving motoneurons (Li *et al.*, 1995). More recently, GDNF has also

been shown to prevent the death and atrophy of a population of corticospinal neurons following axotomy in adult rat (Giehl *et al.*, 1997).

Initial experiments have demonstrated that GDNF can promote the survival of dopaminergic neurons and motoneurons *in vitro* and exert neuroprotective effects on these neurons following lesion induced cell death *in vivo*. Since then much research has been directed towards investigating whether GDNF has therapeutic value in the treatment of neurodegenerative disorders such as Parkinson's disease (PD), amyolateral sclerosis (ALS), Alzheimer's disease and Huntington's chorea. In particular, much research has focused on the ability of GDNF to reverse or prevent lesion induced damage in animal models of PD. Perhaps the best studied experimental models are those in which 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) have been used to cause degeneration of the dopaminergic neurons of the nigrostriatal system (Olson, 1997). GDNF has been shown to restore dopamine levels to normal and partially prevent the reduction in tyrosine hydroxylase levels in ipsilateral substantia nigra when administered intranigrally one month after a 6-OHDA lesion of the medial forebrain bundle (Hoffer *et al.*, 1994; Bowenkamp *et al.*, 1995). Pre-treatment with GDNF has also been shown to have neuroprotective effects against 6-OHDA. Administration of GDNF intranigrally over a one month period starting on the day of a 6-OHDA lesion, completely prevents nigral death and atrophy (Sauer *et al.*, 1995). Injection of a GDNF-adenoviral expression construct into a region adjacent to the dopaminergic cell bodies one week prior to 6-OHDA lesioning also prevents 6-OHDA induced death and atrophy of dopaminergic neurons (Choi-Lundberg *et al.*, 1997). GDNF can also reverse the neurotoxic effect on MPTP whether administered by injection, intranigrally or whether provided by an adenovirus/GDNF expression construct injected into the striatum (Tomac *et al.*, 1995; Kojima *et al.*, 1997; Chou Cheng *et al.*, 1998).

Furthermore, GDNF can induce similar functional recovery in MPTP-treated monkeys, and increase the efficacy of foetal mesencephalic grafts transplanted into the lesioned striatum of rats (Gash *et al.*, 1996; 1998; Granholm *et al.*, 1997; Tang *et al.*, 1998). The neuroprotective actions of GDNF on the nigrostriatal system provides evidence in both rodents and primates that delivery of GDNF to the CNS not only counteracts lesions of dopaminergic neurons but stimulates them to perform better; which makes GDNF a promising candidate for the treatment of PD.

In the PNS, GDNF also has widespread survival effects on embryonic sympathetic, parasympathetc and sensory neurons. It promotes the survival and neurite outgrowth of sympathetic neurons in embryonic chick and induces robust bundle-like fasciculated outgrowth from sympathetic ganglion explants. GDNF has also been shown to promote the survival of newborn rat superior cervical ganglion, which is accompanied by an increase in vasoactive intestinal peptide (VIP) and preprotachykinin-A mRNA (Trupp *et al.*, 1995). A more thorough study has shown that chicken autonomic and sensory neurons, change their responsiveness to GDNF with age (Buj-Bello *et al.*, 1995). In particular, sensory neurons of the nodose ganglia show increased sensitivity to GDNF throughout development compared to neurturin (Forgie *et al.*, 1999). Interestingly, following transection of the sciatic nerve in neonatal and adult rat, GDNF reduces cell death of DRG neurons and prevents other axotomy induced changes, including the down-regulation of isolectin B4 (IB4) binding, thiamine monophosphatse (TMP) activity and somatostatin expression.(Bennett *et al.*, 1998). Consistent with this, GDNF is retrogradely transported by dorsal root sensory neurons in neonatal rats (Matheson *et al.*, 1997). Furthermore GDNF enhances the *in vitro* survival of neonatal dorsal root sensory neurons, and increases the expression of substance P and the capsaicin receptor,VR1, however the efficacy of GDNF decreases with age (Adler *et al.*, 1998; Ogun-

Muyiwa *et al.*, 1999). GDNF has also been shown to promote the survival and proliferation of ENS precursor cells and to induce their differentiation into enteric neurons and glia (Heuckeroth *et al.*, 1998; Hearn *et al.*, 1998). Recently, *in vitro* studies have shown that GDNF and TGFß acts synergistically to promote the survival of embryonic chick ciliary, sensory and sympathetic neurons in serum free cultures at levels comparable to that of CNTF and NGF. It has been proposed that TGFß is required for GDNF to exert its full neurotrophic potential and that TGFß acts by recruiting and/or stabilizing GFRα-1 (Krieglstein *et al.*, 1998).

Analysis of the expression of GDNF mRNA has suggested that it acts as a target derived factor. In the CNS, *in situ* hybridisation has shown that GDNF mRNA is expressed in the targets of striatal and ventral limbic dopaminergic neurons (Lin *et al.*, 1993; Stromberg *et al.*, 1993; Golden *et al.*, 1999). Surprisingly, GDNF mRNA is also expressed by nigral dopaminergic neurons themselves, suggesting a possible autocrine mode of action (Pochon *et al.*, 1997). *In situ* hybridisation, RT/PCR and RNase protection assays have shown that GDNF mRNA has a widespread expression in the developing and adult CNS including: the cerebellar anlage, developing basal ganglia, developing Purkinje cells, trigeminal brainstem nuclei, olfactory tubercle, hippocampus, cortex, cerebellum, thalamic nuclei, and Clarke's column of the spinal cord (Scharr *et al.*, 1993; Stromberg *et al.*, 1993; Springer *et al.*, 1994; Cristina *et al.*, 1995; Arenas *et al.*, 1995; Nosrat *et al.*, 1996; Pochon *et al.*, 1997). In the adult CNS, GDNF mRNA is mainly expressed in neurons and not glial cells (Pochon *et al.*, 1997).

Widespread distribution of GDNF mRNA expression has been demonstrated within both the PNS and in non-neuronal tissues. Low levels of GDNF mRNA have been observed in DRG and SCG by RNase protection assay (Trupp *et al.*, 1995). Following axotomy of the peripheral nerve, GDNF

mRNA expression in DRG is upregulated (Kashiba *et al.*, 1998). In the developing rat, the highest levels of GDNF mRNA are expressed in the developing skin, whisker pad, kidney, stomach, testis, muscle and Schwann cells within peripheral nerves (Henderson *et al.*, 1994; Trupp *et al.*, 1995; Golden *et al.*, 1999). Northern blot hybridisation has shown that in embryonic chick, GDNF mRNA is expressed in skeletal muscle, heart, eye, and skin; all tissues that are innervated by neurons that respond to GDNF. In muscle and eye, GDNF mRNA levels decrease with age, whereas in heart the GDNF mRNA levels increase with age. The changing expression of GDNF in these tissues corresponds to the changing sensitivity to GDNF of neurons that innervate them (Buj-Bello *et al.*, 1995). Developmental changes in GDNF mRNA expression have also been demonstrated in peripheral organs of rat such as kidney, stomach, intestine, liver, lung, testis, ovary, heart, spleen and in mouse limb bud (Trupp *et al.*, 1995; Choi-lundberg and Bohn 1995; Wright and Snider 1996; Golden *et al.*, 1999). The varying patterns of GDNF expression found in these tissues suggest that, as in the CNS, GDNF is a target-derived neurotrophic factor for several neuronal populations and that it may be important for establishing and maintaining neuronal innervation.

The physiological relevance of many of the *in vitro* effects of GDNF have been substantiated by analysis of GDNF knockout mice. Homozygous GDNF null mice show severe neuronal deficits in dorsal root, sympathetic and petrosal-nodose ganglia, and more mild deficits in the trigeminal ganglia. Surprisingly, dopaminergic neurons of the substantia nigra and noradrenergic neurons of the locus coeruleus appear to be completely unaffected (Sanchez *et al.*, 1996; Pichel *et al.*, 1996; Moore *et al.*, 1996). However, this may be because homozygous null mutants die before these neurons normally require GDNF for survival and maintenance. The most striking phenotypic changes observed in homozygous GDNF null mice are agenesis of the kidneys and

complete loss of the enteric nervous system. The disrupted development of the metanephric kidney, from which the entire adult kidney derives, suggests that GDNF is a morphogen for the developing kidney. Consistent with this, GDNF initiates ureteric bud outgrowth from the Wolffian duct in explant cultures of embryonic kidney and promotes branching of the growing buds (Sainio *et al.*, 1997; Pepicelli *et al.*, 1997; Sariola *et al.*, 1997). The absence of the neurons from the myenteric and submucosal plexuses of the enteric nervous system is evident as early as E12.5 (Sariola *et al.*, 1997). Although the precise role of GDNF in the process of enteric nervous system development is unclear, such an early loss in the absence of GDNF suggests that GDNF may be required for the commitment, migration and differentiation of enteric neuron precursors.

4.6.2. Neurturin.

Neurturin was initially purified from a Chinese hamster ovary cell line, on the basis of its ability to promote the survival of sympathetic neurons from the SCG of neonatal rats (Kotzbauer *et al.*, 1996). Mouse and human neurturin cDNA and genomic clones were subsequently isolated by PCR using degenerate primers based on partial N-terminal and internal amino acid sequences obtained from purified neurturin (Kotzbauer *et al.*, 1996). Neurturin is synthesised as a polypeptide precursor that is processed to release a 100 amino acid glycosylated protein. Mature neurturin exists as a disulphide bonded homodimer that shares 42% structural homology with GDNF. Further characterisation of the protein has demonstrated conservation of the seven cysteine residues found in GDNF with the same relative spacing. Therefore, neurturin and GDNF establish the existence of a new sub-family of peptide growth factors distantly related to the TGF-ß superfamily.

Neurturin has been shown to promote the survival of similar central and peripheral neuronal populations to GDNF (Kotzbauer *et al.*, 1996; Horger *et al.*, 1998; Tseng *et al.*, 1998). Purified neurturin added at low concentrations *in vitro* promotes the survival of dorsal root, nodose, and superior cervical ganglion cultures at levels comparable to that of GDNF (Kotzbauer *et al.,* 1996). Furthermore, ciliary neurons show increased responsiveness to neurturin at late embryonic stages of development, whereas sympathetic neurons show increased responsiveness at earlier stages (Forgie *et al.*, 1999). In addition, neurturin is a potent survival factor for enteric neuron precursor cells and can increase the number of enteric neurons and glia that differentiate in culture (Heukeroth *et al.*, 1998). Neurturin has also been shown to support the *in vitro* survival of TH-expressing dopaminergic neurons in the developing rat with the same potency and efficacy to that observed with GDNF (Horger *et al.*, 1998; Tseng *et al.*, 1998). Furthermore *in vivo*, administration of neurturin to the substantia nigra protects mature dopaminergic neurons from transection of the medial forbrain bundle and 6-OHDA-induced cell death (Horger *et al.,* 1998; Tseng *et al.*, 1998; Akerud *et al.*, 1999). More recently, neurturin has been shown to increase choline acetyltransferase activity in postnatal motoneurons, induce neurite outgrowth in the spinal cord and potently protect motoneurons from chronic glutamate mediated degeneration (Bilak *et al.*, 1999).

Analysis of neurturin mRNA expression has revealed a distribution pattern similar to that of GDNF. *In situ* hybridisation has shown neurturin is expressed sequentially in the developing mouse midbrain and striatum, suggesting it may influence the development and maintenance of dopaminergic neurons (Horger *et al.*, 1998). Neurturin mRNA is also expressed in developing and adult rat heart, blood, ovary, salivary gland, testis, adrenal gland, lung, spleen, kidney and intestine (Widenfalk *et al.*, 1997; Xian *et al.*, 1999).

Analysis of mice lacking the neurturin gene has provided evidence for the physiological role of neurturin during development. These mice are viable and fertile but display deficits in the enteric nervous system due to a reduction in innervation density and neuropeptide release which results in abnormal gut motility. In addition, neurturin-/- mice also display minor deficits in neuronal subpopulations of the dorsal root, and trigeminal ganglia. Parasympathetic neurons are lost from the submandibular salivary gland and the ciliary ganglia, and parasympathetic innervation to the lacrimal gland is dramatically reduced. This indicates that neurturin is an important neurotrophic factor for postmitotic enteric and parasympathetic neurons (Heuckeroth *et al.*, 1999)

4.6.3. Persephin.

Persephin, a novel member of the GDNF family, was recently cloned from rat and mouse genomic DNA using degenerate primers based on GDNF and neurturin nucleotide sequences. Initial characterisation led to the identification of a 10-12KDa protein that is synthesised as a precursor of 156 amino acids, and is proteolytically cleaved and secreted as a 96 amino acid mature glycosylated protein. Persephin shares 40% homology with GDNF and neurturin and contains seven cysteine residues with the same relative spacing to those found in other TGF-ß family members (Milbrandt *et al.,* 1998). Persephin exists as a biologically active disulphide bonded homodimer.

Persephin promotes the survival of ventral midbrain dopaminergic neurons in culture and prevents their degeneration after 6-OHDA treatment *in vivo*. In addition, persephin promotes the survival of developing motoneurons *in vitro* and *in vivo* following sciatic nerve axotomy. In contrast to GDNF and neurturin, persephin does not support the *in vitro* survival of SCG, DRG, trigeminal, nodose and enteric neurons (Milbrandt *et al.*, 1998). However, as

with GDNF and neurturin, persephin promotes the branching of the kidney ureteric bud *in vitro,* suggesting it may play a role in metanephric kidney morphogenesis (Milbrandt *et al.*, 1998).

Analysis of persephin mRNA expression in postnatal rat tissue has revealed widespread distribution in both neuronal and non-neuronal tissues. RT/PCR analysis has shown that within the CNS, persephin mRNA is present in the cortex, hippocampus, striatum, diencephalon, mesencephalon, cerebellum, hindbrain and spinal cord. Within the PNS, persephin mRNA expression has been detected in the sciatic nerve, DRG and SCG. Persephin mRNA has also been detected in non-neuronal tissues including skeletal muscle, skin and adrenal gland (Jaszai *et al.*, 1998)

4.6.4. Artemin.

Artemin, the most recent member of the GDNF family to be identified, was isolated on the basis of its sequence homology with mature neurturin in a database. Full length cDNA clones were obtained following rapid amplification of cDNA ends (RACE) from human and mouse cDNA libraries. The artemin protein is ~12-14KDa and is secreted as a mature polypeptide of 113 amino acids. Further characterisation of the protein has revealed that artemin shares ~45% amino acid identity with neurturin and persephin and 36% identity with GDNF (Baloh *et al.*, 1998b).

Artemin demonstrates neurotrophic activity on several neuronal populations in culture. Like GDNF and neurturin, artemin supports the survival of a subset of sensory neurons from both the dorsal root and trigeminal ganglia of P1 rats. Interestingly, it supports a larger number of these neurons than GDNF or neurturin, and a larger number of trigeminal neurons than NGF. Artemin also exerts survival effects on neonatal visceral sensory neurons of the

rat nodose ganglion and sympathetic neurons of the P1 SCG. In the CNS, artemin promotes the survival of dopaminergic neurons of the ventral midbrain.

Consistent with this, northern blot hybridisation has revealed the expression of artemin mRNA in many adult and fetal human tissues. In the adult, the highest levels have been shown in the pituitary gland, placenta and trachea. Fetal kidney and lung also express high levels of artemin mRNA. Low levels of expression have been found in the adult and fetal brain (Baloh *et al.*, 1998b). *In situ* hybridisation has demonstrated a more precise localisation of artemin mRNA in E14 rats. Strikingly high expression has been observed in the nerve roots of DRG and around the superior mesenteric artery, raising the possibility that artemin may act in a paracrine fashion as a survival factor for developing DRG neurons or as a targeted derived factor for autonomic innervation of the superior mesenteric artery (Baloh *et al.*, 1998b).

4.7. Receptors of The TGF-ß Superfamily.

4.7.1. The TGF-ß Family of Receptors.

Most members of the TGFß superfamily bind specifically and with high affinity to a wide variety of cell types, and are known to mediate their signalling through two sets of distinctive transmembrane serine/threonine kinases known as the type I and type II receptors (Wrana *et al.*, 1994).

Both groups of receptor share structural similarities and consist of a small extracellular region containing a 6-9 amino acid cysteine box, a single hydrophobic transmembrane segment and a cytoplasmic serine/threonine kinase domain. Certain features, however, are shared only by the type I receptors, including a characteristic pattern of 7 cysteine residues preceeding the cysteine box in the extracellular domain and a highly conserved glycine/serine rich

region, known as the GS domain, adjacent to the kinase domain (Massague *et al.*, 1994).

TGF-ß initially binds to the type II receptor TßR-II, a constitutively active kinase (Lin and Lodish 1993). This binding is modulated by the TGF-ß type III receptor, a transmembrane proteoglycan. The type I receptor cannot bind TGF-ß directly, but is recruited to the receptor complex following the binding of TGF-ß to the type II receptor. Once the TGF-ß/type I receptor/type II receptor complex is formed, TGF-ßR-I is transphosphorylated by TGF-ßR-II at serine and threonine residues located in the GS domain of the receptor. Transphosphorylation leads to the propagation of a signal to the nucleus via Smad proteins (reviewed in Massague 1998)

4.7.2. The GDNF Family of Receptors.

Initially it was considered the GDNF family of proteins would mediate signalling through a serine threonine kinase receptor system similar to that used by the TGF-ß protein family. However, it has now been established that signalling by the GDNF, neurturin, artemin and persephin occurs through a multicomponent receptor system that comprises the tyrosine kinase Ret and one of four recently identified GPI-linked receptors, GFRα 1-4 (Jing *et al.*, 1996; Treanor *et al.*, 1996; Baloh *et al*, 1997; Buj-Bello *et al.*, 1997; Jing *et al.*, 1997; Klein *et al.*, 1997; Sanicola *et al.*, 1997; 1998; Enokido *et al.*, 1998).

The proposed mechanism of action for this receptor system predicts that activation of Ret, which functions as a signaling component, is mediated by high affinity binding of the GDNF proteins to the specific GFRα receptors. This interaction induces heterotetradimerization of the receptor components, which leads to Ret activation by autophosphorylation on tyrosine residues (Trupp *et al.*, 1996; Durbec *et al.*, 1996; Worby *et al.*, 1996; Robertson and Mason, 1997). Recent experiments have demonstrated that in addition to the GFRα receptors, Ca^{2+} ions may be required for complex formation by Ret and GDNF or neurturin (Nokazi *et al.*, 1998).

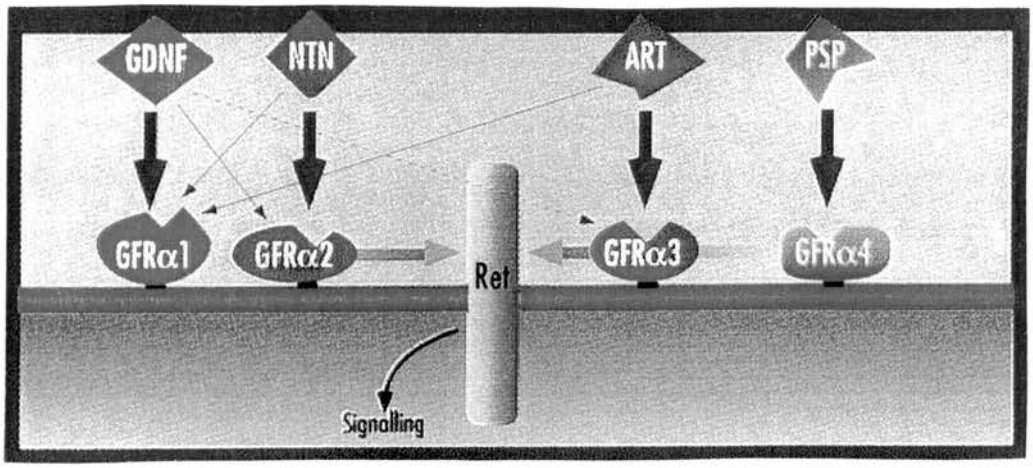
Each GDNF ligand has a preferred GFRα receptor. GDNF interacts preferentially with GFRα-1, whereas neurturin interacts with GFRα-2, artemin interacts with GFRα-3 and persephin interacts with GFRα-4 (Figure 1.3) (Jing *et al.*, 1996; Treanor *et al.*, 1996; Baloh *et al*, 1997; Buj-Bello *et al.*, 1997; Jing *et al.*, 1997; Klein *et al.*, 1997; Sanicola *et al.*, 1997; 1998; Enokido *et al.*, 1998). However, biochemical and cell culture studies have revealed there is some promiscuity in ligand specificity with the GFRα receptors. Equilibrium and competition binding studies using radiolabeled ligand and soluble GFRα components demonstrate high affinity binding of GFRα-1/GDNF and GFRα-2 /neurturin (Klein *et al.*, 1997; Baloh *et al.*, 1997; Jing *et al.*, 1997). Whereas

other studies using the same experimental approach report high affinity binding between GFRα-2/GDNF (Sanicola *et al.*, 1997; Baloh *et al.*, 1997). Although studies of fibroblasts ectopically co-expressing Ret plus the GFRα receptors suggest that GFRα-2 is a preferential receptor for neurturin, GFRα-1 has also been found to be equally effective in mediating responses to GDNF and neurturin (Baloh *et al.*, 1997; Creedon *et al.*, 1997). Furthermore, COS cells co-expressing Ret plus GFRα-2 suggest that GFRα-2 is an equally effective receptor for both ligands (Worby *et al.*, 1998). These results may be explained from studies ectopically co-expressing Ret plus GFRα receptors in SCG neurons. At low concentrations of GDNF and neurturin, neurons co-expressing GFRα-1/Ret confer a survival response to GDNF only, whereas neurons co-expressing GFRα-2/Ret survive only in the presence of neurturin (Buj-Bello *et al.*, 1997). However, at higher concentrations GDNF is equally effective in promoting the survival of GFRα-2/Ret as well as GFRα-1/Ret expressing neurons (Buj-Bello *et al.*, 1997). Similarly, high concentrations of GDNF induce tyrosine phosphorylation of Ret in COS cells co-expressing Ret plus either GFRα-1 and GFRα-2 (Suvanto *et al.*, 1997). In addition, GFRα-1 and GFRα-2 co-expressed with Ret in fibroblasts or cultured neurons do not show a survival response to persephin (Enokido *et al.*, 1998; Milbrandt *et al.*, 1998). Similarly, GFRα-1 and GFRα-2 alone do not interact with artemin, however, in fibroblasts co-expressing GFRα-1 and Ret, artemin can bind and initiate Ret phosphorylation (Baloh *et al.*, 1998).

GFRα-3 and GFRα-4 demonstrate a more clear ligand specificity, interacting with only artemin and persephin respectively (Baloh *et al.*, 1998a; Enokido *et al.*, 1998). Soluble GFRα-3 binds with high affinity to artemin but not to GDNF, neurturin, or persephin. Furthermore, in fibroblasts co-expressing GFRα-3 and Ret, artemin, promotes Ret tyrosine phosphorylation whereas GDNF, neurturin and persephin do not. GFRα-3 can bind GDNF with

low affinity in the presence of Ret, however the significance of this interaction remains to be ascertained (Baloh *et al.*, 1998a; Worby *et al.*, 1998; Trupp *et al.*, 1998). The ectopic expression of GFRα-4 and Ret in cultured SCG neurons has shown that GFRα-4 specifically binds persephin but not other members of the GDNF family (Enokido *et al.*, 1998). Taken together these findings indicate that although each GDNF family member binds one preferred GFRα receptor *in vitro*, there is a degree of promiscuity in their ligand specificities, suggesting receptor/ligand crosstalk among the GFRα family.

Ret and GFRα receptors are structurally distinct. Ret encodes a transmembrane receptor tyrosine kinase consisting of a cytoplasmic domain, similar to that found in FGF receptors, a transmembrane domain, and a cysteine rich extracellular domain homologous to the intermolecular binding region of the cadherin family (Iwamoto *et al.*, 1993; Takahashi *et al.*, 1987; 1989). In contrast, the GFRα receptors lack both cytoplasmic and transmembrane domains, anchoring themselves to the outer cell membrane by a glycosyl-phosphaditylinositol (GPI) linkage (Weisenhorn *et al* .,1999).



Adapted from Rosenthal 1999.

Figure 1.3. Schematic diagram representing the interaction of the GDNF family ligands and their receptors.

Ret was originally described as a proto-oncogene (RE-arranged in Transformation) (Takahashi *et al.*, 1985). Activating mutations of RET are associated with human thyroid carcinomas (PTC), familial medullary thyroid carcinoma (FMTC) and multiple endocrine neoplasia type 2 (MEN2A and MEN2B) (Pasini *et al.*, 1996; Edery *et al.*, 1997). Inactivating mutations of Ret lead to Hirschprung's disease (aganglionic megacolon) characterised by the absence of enteric innervation to the gut (Romeo *et al.*, 1994; Edery *et al.*, 1994).

GFRα-1 was the first member of the GFRα receptor family to be isolated, by virtue of its ability to bind GDNF. Using expression libraries derived from enriched populations of GDNF-responsive rat retinal photoreceptors and midbrain dopaminergic neurons, cDNAs were obtained that encoded GDNF-binding proteins (Jing *et al.*, 1996; Treanor *et al.*, 1996). Both

human and chicken GFRα-1 were subsequently identified by screening cDNA libraries (Jing *et al.*, 1996; Buj-Bello *et al.*, 1997). Partial gene fragments of mouse GFRα-2 and GFRα-3 have been identified based on nucleotide sequence similarity with GFRα-1 following a search of an Expressed Sequence Tag (EST) database. Screening of rat and chick cDNA libraries have led to the isolation of full length rat and chick GFRα-2 cDNAs (Buj-Bello *et al.*, 1997; Baloh *et al* ., 1997; Jing *et al.*, 1997). In addition, two mouse clones similar to GFRα-1, but distinct from GFRα-2, were isolated and used for RACE amplification of a full length cDNA that represented GFRα-3. GFRα-3 is the most divergent member of the GFRα receptor family (Widenfalk *et al.*, 1998; Masure *et al.*,1998). A full length GFRα-4 cDNA was obtained by screening a chicken cDNA library with mouse GFRα-1, discussed in chapter 2 of this thesis (Thompson *et al.*, 1998). The isolation of a putative mammalian GFRα-4 homologue is also discussed in chapter 4 of this thesis.

The predicted protein sequences of the GFRα receptor family include a putative signal peptide sequence at the N-terminus and a hydrophobic C-terminal motif reminiscent of a GPI-anchor signal sequence. Six potential N-glycosylation sites have been found in the predicted protein sequence of GFRα-2, compared to only three sites in the GFRα-1 and GFRα-3 sequences (Jing *et al.*,1997; Masure *et al.*, 1998; Baloh *et al.*, 1998a).

GFRα-1 and GFRα-2 mRNAs are widely distributed in the central and peripheral nervous systems. Northern blotting and *in situ* hybridisation have revealed the presence of GFRα-1 mRNA in several regions of the CNS including, the ventral midbrain, tectum, basal forebrain, hippocampus, hypothalamus, olfactory bulb, cerebellum, thalamus, cerebral cortex and ventral horn of the spinal cord (Trupp *et al.*,1997; 1998; Glazner *et al.*,1998; Yu *et al.*, 1998; Golden *et al.*,1999) In the PNS, GFRα-1 mRNA has

been detected in dorsal root, trigeminal, and superior cervical ganglia. Outside the nervous system GFRα-1 mRNA is expressed in the stomach, intestine, liver, ureteric buds of the kidney, developing tongue papillae, teeth, embryonic smooth and striated muscle, heart, oesophagus, and olfactory epithelium (Treanor *et al.*, 1996; Buj-Bello *et al.*, 1997; Sanicola *et al.*, 1997; Nosrat *et al.*, 1997; Yu *et al.*, 1998). GFRα-2 mRNA also is widely expressed, but not quite to the same extent as GFRα-1 mRNA. In the CNS, GFRα-2 mRNA is expressed in the thalamus, hypothalamus, hippocampus, striatum, olfactory bulb, cortex, substantia nigra, and ventral horn of the spinal cord (Widenfalk *et al.*, 1997; Yu *et al.*,1998; Trupp *et al.*,1998; Golden *et al.*,1999). In the PNS, GFRα-2 mRNA has been found in the dorsal root, trigeminal and superior cervical ganglia (Suvanto *et al.*,1997; Jing *et al.*,1997; Widenfalk *et al.*,1997). Outside the nervous system, GFRα-2 is expressed several tissues, including the lung, spleen, heart, ureter, oesophagus, stomach and intestine (Suvanto *et al.*, 1997; Jing *et al.*,1997; Widenfalk *et al.*,1997; Golden *et al.*,1999). GFRα-3 mRNA expression is more restricted compared to GFRα-1 and GFRα-2. GFRα-3 mRNA expression in the CNS is very limited with low expression in the hypothalamus, thalamus and hippocampus (Trupp *et al.*,1998). In the PNS, GFRα-3 mRNA is expressed in the trigeminal, dorsal root, and superior cervical ganglia. In developing non-neuronal tissues, GFRα-3 mRNA expression shows a widespread distribution in heart, liver, lung, kidney, spleen, ovary, and muscle (Naveilhan *et al.*,1998; Jing *et al.*,1997; Nomoto *et al.*,1998; Trupp *et al.*,1998) GFRα-4 mRNA is widely distributed in the developing CNS and in several non-neuronal tissues, as will be discussed in chapter 2 of this thesis (Thompson *et al.*,1998).

The physiological significance of the GFRα receptors during development has been determined by analysis of transgenic mice deficient in the corresponding receptor genes. GFRα1$^{-/-}$ mice are viable at birth but die soon

after (Cacalano *et al.*, 1998). They exhibit severe deficits in spinal motoneurons and sensory neurons. In addition, agensis of the kidney and deficits of enteric neurons are observed, both of which are characteristic of GDNF$^{-/-}$ and Ret$^{-/-}$ mice (Schuchardt *et al.*, 1994; Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*,1996). Midbrain dopaminergic neurons and motoneurons are largely unaffected in GFRα-1 null mice. The superior cervical and nodose ganglia also show minimal or no neuronal loss. However these peripheral ganglia are severely affected in both Ret$^{-/-}$ and GDNF$^{-/-}$ mice (Cacalano *et al.*, 1998; Enomoto *et al.*, 1998).

GFRα2$^{-/-}$ mice are viable and fertile at birth and their major organs appear normal (Rossi *et al.*, 1999). However, they grow poorly after weaning, possibly due to malnutrition. A reduction in parasympathetic innervation of the tear glands has been observed which cause dryness of the eyes and thickening and drooping of the eyelids. Severe deficits are observed within the enteric and parasympathetic nervous system of GFRα2$^{-/-}$ mice which are similar to those of neurturin$^{-/-}$ mice. In particular, the numbers of submandibular and ciliary ganglia neurons are severely reduced and myenteric neurons of the gut show a decrease in fibre density and cell volume which is consistent with an observed decrease in neuropeptide transmitter production and gut muscle contraction (Rossi *et al.*, 1999).

Mice deficient for the GFRα3$^{-/-}$ gene are viable and fertile (Nishino *et al.*, 1999). Severe deficiencies are observed in the SCG while other peripheral ganglia show no deficits. Rostral migration of the SCG precursor cells is impaired in GFRα-3$^{-/-}$ mice compared to wildtype embryos, resulting in the formation of a smaller ganglion. In addition SCG neurons exhibit progressive cell death postnatally. As a result of these two processes SCG neurons fail to innervate their target organs (Nishino *et al.*, 1999).

The recent generation of mice with targeted disruptions in the GDNF, GFRα-1, neurturin and GFRα-2 genes (Moore *et al.*, 1996; Sanchez *et al.*, 1996; Pichel *et al.*, 1996; Cacalano *et al.*, 1998; Enomoto *et al.*, 1998; Heukeroth *et al.*, 1999) have provided valuable data regarding the ligand receptor interactions among the GDNF family members. Mice deficient in either GFRα-1 or GDNF have remarkably similar defects in kidney and enteric nervous system development (Moore *et al.*, 1996; Sanchez *et al.*, 1996; Pichel *et al.*, 1996). In contrast some peripheral ganglia showing severe neuronal loss in $GDNF^{-/-}$ mice, are only slightly affected in GFRα-$1^{-/-}$ mice. This suggests a stringent pairing of GFRα-1 and GDNF in the kidney and enteric nervous system during development. However, in certain neuronal populations such as the peripheral ganglia there must be a significant degree of cross talk between GDNF and the other GFRα receptors. Mice deficient in GFRα-2 and neurturin also display strikingly similar characteristics with severe deficits in the parasympathetic nervous system. The degree of similarity between GFRα-2 and neurturin-deficient mice indicate GFRα-2 is a physiological receptor for neurturin. The physiological relevance of the specific interactions between GFRα-3 and artemin, and GFRα-4 and persephin will become apparent with the generation of artemin, GFRα-4 and persephin deficient mice.

Although evidence suggests that GFRα proteins are necessary for the activation of Ret by the GDNF ligands, it remains to be established whether GDNF ligands first bind to the GFRα-components and then activate Ret or whether GDNF binds to preformed complexes of Ret and the GFRα-receptors at the cell membrane (Jing *et al.*, 1996; Treanor *et al.*, 1996). Recent *in vitro* experiments co-expressing Ret and GFRα receptors in unstimulated COS cells have suggested interactions between Ret and the GFRα-receptors can occur at the cell membrane independent of ligand (Trupp *et al.*, 1998).

The intracellular signalling pathways triggered by GDNF-dependent Ret activation have started to be characterised. Ret has been shown to bind and phosphorylate the SH2 domain of a variety of adaptor proteins, including Grb7, Grb10, Grb2, Shc and She. The specific tyrosine residues of Ret where the phosphorylation of adaptor proteins is thought to occur have been identified as Tyr^{905}, Tyr^{1015}, Tyr^{1062} and Tyr^{1096} (Ohiwa *et al.*, 1997; see review Airaksinen *et al.*, 1999). Ret has been shown to activate several pathways typical for receptor tyrosine kinase signalling, including the Ras-MAPK, phosphoinositol-3-kinase (PI3K), PLCγ, and Jun N-terminal kinase (JNK) dependent pathways (Worby *et al.*, 1996; van Weering *et al.*, 1997; Chiariello *et al.*, 1998). Ret activation of the JNK pathway occurs via Rho/Rac-related small GTPases such as Cdc42 (Chiariello *et al.*, 1998). More recent studies have demonstrated Ret independent signalling of GDNF via GFRα components. It has been shown GDNF can activate Src-type kinases in the absence of Ret. This in turn leads to the activation of PLCγ production of IP-3 and elevation of intracellular calcium (Airakinsinen *et al.*, 1999).

CHAPTER 2.

CLONING AND CHARACTERISATION OF CHICKEN GFRα-1, GFRα-2 AND GFRα-4.

2.1. Introduction.

GDNF is a distantly related member of the transforming growth factor-ß family isolated from a rat glial cell line (Lin *et al.*, 1993). It is a potent survival factor for cultured midbrain dopaminergic neurons (Lin, 1993) and motoneurons, (Henderson *et al.*, 1994; Zurn *et al.*, 1994; Li *et al.*, 1995; Oppenheim *et al.*, 1995; Yan *et al.*, 1995; Houenou *et al.*, 1996) and protects midbrain dopaminergic neurons from MPTP toxicity (Tomac *et al.*, 1995), 6-hydroxydopamine toxicity (Bowenkamp *et al.*, 1996; Choi-Lundberg *et al.*, 1997; Kearns *et al.*, 1997), and axotomy-induced degeneration (Beck *et al.*, 1995; Lu and Hagg, 1997). GDNF also promotes the survival of several other populations of CNS neurons, including noradrenergic neurons of the locus coeruleus (Arenas *et al.*, 1995), Purkinje cells (Mount *et al.*, 1995) and basal forebrain cholinergic neurons (Williams *et al.*, 1995). Accordingly, GDNF is widely expressed in the brain (Schaar *et al.*, 1993; Stromberg *et al.*, 1993; Springer *et al.*, 1994; Arenas *et al.*, 1995; Nosrat *et al.*, 1997; Pochon *et al* ., 1997; Trupp *et al.*, 1997). In the PNS, GDNF promotes the *in vitro* survival of embryonic sympathetic, parasympathetic, and sensory neurons and is expressed in a wide variety of tissues during development (Sutter-Crazzolara and Unsicker, 1994; Buj-Bello *et al.*, 1995; Trupp *et al.*, 1995; Wright and Snider 1996).

The second member of the GDNF family, neurturin was identified based on its ability to support the survival of sympathetic neurons (Kotzbauer

et al., 1996). Neurturin also maintains many of the neuronal populations dependent on GDNF. In addition to sympathetic neurons, these populations include enteric neurons, neurons of the superior cervical, dorsal root, nodose and trigeminal ganglia (Kotzbauer *et al.*, 1996); and in the CNS, spinal motoneurons and dopaminergic neurons of the ventral midbrain (Klein *et al.*, 1997; Horger *et al.*, 1998). Two additional members of the GDNF family have recently been identified, persephin and artemin. Persephin the third member of the family, was cloned based on homology to neurturin and GDNF (Milbrandt *et al.*, 1998). Persephin shares the biological activity of neurturin and GDNF in central neuronal populations, promoting the survival of ventral midbrain dopaminergic and motoneurons but not peripheral neuronal populations (Milbrandt *et al.*, 1998). Artemin is the most recently identified family member, like GDNF and neurturin, artemin supports the survival of sensory and sympathetic neurons (Baloh *et al.*, 1998b).

The physiological relevance of many of the *in vitro* effects of GDNF and neurturin on neuronal survival has been substantiated by the finding of an appreciable reduction in the number of sensory, motor, sympathetic and enteric neurons in GDNF$^{-/-}$mice; and parasympathetic, ciliary and myenteric neurons in neurturin $^{-/-}$mice.

Interestingly, the kidney fails to develop in GDNF $^{-/-}$mice, indicating a role for GDNF in kidney development, which is consistent with the expression of GDNF in this organ during development (Sutter-Crazzolara and Unsicker, 1994; Buj-Bello *et al.*, 1995; Trupp *et al.*, 1995; Nosrat *et al.*, 1997).

GDNF and neurturin signal via multi-component receptors that consist of the Ret receptor tyrosine kinase plus one of two structurally related glycosylphosphatidylinositol (GPI)-linked receptors termed GFRα-1 and GFRα-2. Ret activation and signalling by GDNF and neurturin require one of these GPI-linked receptors, although there was some controversy over the

specificity of GFRα-1 and GFRα-2 for these ligands. Equilibrium and competition binding studies and the survival responses of neurons ectopically co-expressing Ret plus GFRα-1 and GFRα-2 (Buj-Bello *et al.*, 1997) have shown that GFRα-1 is a specific high affinity receptor for GDNF and that GFRα-2 is a specific high affinity receptor for neurturin. Although studies of Ret phosphorylation in fibroblasts ectopically expressing Ret plus GFRα-1 or GFRα-2 suggest that the Ret/GFRα-2 complex is a preferential receptor for neurturin (Baloh *et al.*, 1997), the Ret/GFRα-1 complex is equally effective in mediating responses to GDNF and neurturin (Baloh *et al.*, 1997; Creedon *et al.*, 1997). Furthermore GDNF has been shown to promote Ret phosphorylation in COS cells co-expressing Ret plus either GFRα-1 or GFRα-2 (Suvanto *et al.*, 1997). Although these studies in fibroblast and COS cell lines have shown a degree of promiscuity in the ligand specificity of GFRα-1 and GFRα-2, the physiological relevance of these findings has yet to be ascertained.

An additional member of the GFRα family, GFRα-3, has recently been identified by searching mouse and human EST databases for sequences homologous to GFRα-1 and GFRα-2 (Jing *et al.*, 1997; Naveilhan *et al.*, 1998). Like GFRα-1 and GFRα-2, GFRα-3 is a GPI-linked protein, with specific high affinity binding for the recently identified GDNF family member, artemin. Although artemin signals via the GFRα-3/Ret receptor complex, it can also activate the GFRα-1/Ret complex (Baloh *et al.*, 1998b).

The most recent member of the GFRα family to be identified, GFRα-4, as will be discussed in this chapter, was isolated based on nucleotide sequence homology with GFRα-1 (Thompson *et al.*, 1998). GFRα-4 shares features characteristic of the GFRα receptors, and has been recently demonstrated to mediate Ret activation and signalling by persephin (Enokido *et al.*, 1998).

GFRα-1, GFRα-2, and GFRα-3 are widely expressed in the central and peripheral nervous systems and in a variety of other tissues and organs during development (Jing *et al.*, 1996; Treanor *et al.*, 1996; Trupp *et al.*, 1996; Vega *et al.*, 1996; Worby *et al.*, 1996., Baloh *et al.*, 1997; Buj-Bello *et al.*, 1997;Jing *et al.*, 1997; Sanicola *et al.*, 1997; Suvanto *et al.*, 1997 Widenfalk *et al.*, 1997; Golden *et al.*, 1999). However, whereas the expression of GFRα-1 and GFRα-2 is maintained in the adult, GFRα-3 expression is barely detectable in adult tissues and organs (Naveilhan *et al.*, 1998). Like members of the GFRα family, Ret is also widely expressed in the nervous system and in other organs and tissues (Pachnis *et al.*, 1993; Schuchardt *et al.*, 1995; Tsuzuki *et al.*, 1995).

In this chapter the cloning of GFRα-1 and GFRα-2 is described which was carried out in collaboration with Anna Buj-Bello. I also describe the cloning of GFRα-4, a novel member of the GFRα family which codes for a protein, that shares significant amino acid homology with GFRα-1, GFRα-2, and GFRα-3. The expression pattern of GFRα-4 in the brain and several other organs and tissues of the developing chick embryo is reported.

2.2. Methods and Materials.

2.2.1. General Methods and Precautions.

All the following procedures described are compiled from my own research and experimentation carried out during my PhD

2.2.1. a) Chemicals.

All chemicals used for the preparation of solutions were of ultra pure grade suitable for molecular genetic manipulations. Water was deionised, double distilled and, if necessary, sterilised by autoclaving.

2.2.1. b) Analytical Methods.

All PCR products and plasmids were analysed using agarose gel electrophoresis.

i) Agarose Gel Electrophoresis.

Concentrations of agarose (Gibco-BRL) and low melting point (LMP) agarose (Gibco-BRL) used to separate amplified and digested DNA varied according to the size of DNA fragment. (these are specified in the appropriate sections). 1.0%, 0.8% and 3.5% were used routinely to separate DNA fragments by electrophoresis, with 1g, 0.8g, and 3.5g respectively being melted in 100mls of 1.0 x TAE buffer (Tris borate ethylenediamine-tetra acetic acid (EDTA) (nbl) by boiling in the microwave for 3 minutes and then cooling to 55^{o}C in a water bath. Ethidium bromide (EtBr) was added (5mg/ml) to the agarose solution. The agarose could then be poured into the appropriate gel casting tray and left for 20 minutes to set. A suitable electrophoresis tank was filled with 1 x TAE and the set gel immersed in the buffer. A 1/5 volume of

loading buffer (20% Ficoll 0.1M EDTA (pH8), 1% SDS, 0.25% bromophenol blue, 0.25 xylene cyanol) was added to each sample before loading into the wells. A 1Kb ladder (BRL) was loaded into one of the wells as a size marker. The gel was left to run at 100 volts or 70 volts for LMP gels for ~ 45 minutes, depending on the size of the gel. The gels could then be photographed using a UVP Image Analyser.

ii) Gel Purification of DNA

All DNA fragments were purified from gels by phenol /chloroform extraction, this allowed quick purification of DNA from residual reagents such as enzymes, dinucleotide triphosphates (dNTPs), primers, and digested DNA, which may have prevented further manipulations. The DNA band was cut from a LMP agarose gel, using a razor blade. The gel slice was then placed in a 1.5 ml eppendorf tube and 3 volumes of STE (100mM NaCl, 10mM Tris pH7.6, 1mM EDTA, 0.1% SDS) added. This was incubated at 65°C for 20 minutes, and then 1 volume of basic phenol added, before centrifugation at 13,000rpm for 10 minutes. Following this, the aqueous phase was removed and transferred to a fresh tube. 0.5 volumes of basic phenol and chloroform were then added. The tube was then vortexed before centrifuging again at 13,000rpm for 10 minutes. The aqueous phase was removed and placed in a 1.5 ml Eppendorf containing 2.5 volumes of ethanol, and 1/20 3M Sodium Acetate pH4.0 at -80°C for 1 hour to the precipitate DNA. To pellet the DNA, centrifugation, was carried out at 15,300rpm for 20 minutes at 4°C. The pellet was then washed three times in 70% ethanol and resuspended in an appropriate volume of dH_2O.

2.2.2. Screening of a cDNA library.

To isolate GFRα-1, GFRα-2, and GFRα-4, an E10 chicken brain cDNA library (provided by Vladimir Buchman) was screened using a 1.5Kb probe which corresponded to the full length coding region of the mouse GFRα-1 cDNA (gift of Arnon Rosenthal). Since only a partial cDNA clone was obtained for GFRα-4, a second E6 chicken brain cDNA library (provided by V. Buchman) was also screened using the partial cDNA of the chicken GFRα-4. These libraries were chosen because hybridisation of a northern blot containing total RNA of various chicken tissues with the mouse GFRα-1 revealed the mRNA transcript to be present in several tissues, including brain.

2.2.2. a) Titering and Plating of the Bacteriophage Lambda ZapII Library.

Serial dilutions were made of the bacteriophage library in order to determine its titer before the screening procedure was carried out. Bacteriophages were plated for each dilution and the appropriate concentration chosen (approximately 100,000 pfu/dish). The library was found to contain $6x10^7$ pfu/ml.

The host bacteria chosen for the bacteriophage Lambda Zap II library was Escherichia coli PKLF'. A culture of the bacteria was grown to an OD_{600} of 1.0 in YT broth containing 100µg/ml tetracycline and 0.2% maltose/10mM $MgSO_4$. 0.5ml of the bacterial culture was transferred to ten 7ml bijou tubes, and 10µl of a 1:100 dilution of bacteriophage stock was added. The tubes were then incubated at 37°C for 20 minutes to allow bacteriophage adsorption to occur. 7mls of 0.7% YT top agarose containing 0.2% maltose/10mM $MgSO_4$ which had been preheated to 48°C was added, this mixture was immediately transferred onto ten previously prepared $10cm^2$ LB agar dishes and dispersed by tilting back and forth. The dishes were incubated at 37°C for

between 6-12hrs, until the plaques covered the dish but were not confluent. The dishes were then stored at 4°C for 1-2hrs before transfer.

2.2.2. b) Transfer of Bactriophage Library.

Bacteriophages were transferred onto Hybond-N-+Nitrocellulose filters (amersham). Replica filters were made for each dish to eliminate the possibility of artifacts. Denaturation was carried out using 0.5N NaOH for 10 minutes, and neutralisation using 1M Tris -HCl pH 7.5 for 1minute, this was followed by incubation in 0.5M Tris-HCl pH 7.5/ 1.5M NaCl for 15 minutes. Bacteriophages were then fixed to the membranes by U.V. crosslinking (120,000 μjoules, Stratagene Stratalinker) and baking at 80°C for 2 hours.

2.2.2. c) Hybridisation of recombinant Bacteriophage DNA.

A 1.5kb insert of mouse GFRα-1 and a 700bp insert of chicken GFRα-4 were radiolabeled by nick translation.

i) Preparation of chicken GFR α-4 insert by restriction digestion

The bluescript clone of GFRα-4 was digested with the restriction endonucleases *EcoRI* (Gibco-BRL) and *Tth1 III* (Promega). 10 units of EcoRI was used for digestion with 0.1 volumes of react 3 buffer, the DNA was incubated at 37°C for 1 hour and 30 minutes. Further digestion was carried out with 10 units of *Tth1 III* in 0.1 volumes of buffer B (Promega), and the DNA was incubated at 65°C for 1 hour and 30 minutes. The cDNA insert was then purified using low melting point agarose gel electrophoresis as described in section 2.2.1.b.i)

ii) ^{32}P labelling of cDNA inserts by nick translation

To a 0.5 ml Eppendorf 1.0µg of DNA, 1mM dNTP mix (5mM dATP, dTTP and dGTP), 0.1 volumes of 10 x nick translation buffer, 5µl [α ^{32}P] dCTP (specific actvity >3000Ci/mmole; 10µCi/µl in aqueous solution), 10 mg DNase and 5 units DNA polymerase I were added. The contents were mixed by gently tapping the side of the tube, and then centrifugated for 1-2 secs. The reaction tube was then placed in a water bath at 15°C and left for 1 hour to allow incorporation of ^{32}P dCTP. To monitor the course of the reaction, an aliquot of the reaction mix was measured with the Geiger counter and then measured again after successive washing with Trichloroacetic acid (TCA). The residual activity remaining gave a measure of the proportion of radiolabel that was incorporated into the DNA. After incubation the reaction was stopped by adding 0.1 volumes of 0.25M EDTA pH8.0, and STE buffer to a volume of 100µl. The labelled probe was purified on a Nick column (sephadex G-50) (Pharmacia Biotech) to remove the unincorporated label and dNTPs according to manufacturers instructions.

iii) Hybridisation of membranes

The filters were prehybridised for 4-6 hours at 59°C in 10cm^2 sterilin dishes that were sealed in plastic bags containing the prehybridisation buffer (4 x SSC, 0.2% SDS, 5mM EDTA, 15mM sodium phosphate pH 7.0, 5 x Denhardts solution and 100µg/ml salmon sperm DNA). After prehybridisation, the buffer was then poured off, and fresh hybridisation buffer added, (same as above solution) which contained the denatured ^{32}P labelled nick translated probe of mouse GFRα-1 cDNA. Hybridisation was carried out for 16-20 hours at 59°C. The filters were then transferred to a plastic box and low stringency washes were carried out using the following conditions: rinse

three times in 2 x SSC, and three times for 15 minutes at 59°C in 2 x SSC/0.2% SDS. The filters were then exposed to Kodak Omat X-ray film for 12 hours. The filters were then washed a second time at higher stringency (wash three times for 15 minutes at 65°C in 2 x SSC/0.2% SDS). Again the filters were exposed to Kodak Omat X-ray film for 20 hours.

Duplicate autoradiographs from the filters were orientated and compared. Hybridisation signals that appeared on both autoradiographs were marked, overlain on the agar dishes, and the corresponding areas that contained the clone of interest were isolated using a Pasteur pipette. The phage cores were then transferred to 1.5ml Eppendorf tubes containing 500µl of SM buffer (100mM NaCl, 10mM $MgSO_4$, 50mM Tris-HCl pH7.5), 5µl chloroform and stored at 4°C until use.

Using the isolated phage suspensions, the entire screening procedure was repeated for the second and third screenings in order to isolate and purify the desired clones. The phage suspension was titred and new dishes with the required density of clones were prepared. The transfer, denaturation and hybridisation procedures were as above. The hybridisation solution containing the probe that was used for the first screening, was used for the second and third screenings. Following hybridisation, the filters were washed with the same high stringency washes as before. Exposure of the filters to the autoradiographs varied depending on the intensity of each signal. At the third screen it was possible to align a well separated plaque with a positive signal, which illustrated isolation of the desired clone.

Screening of the E6 chicken brain cDNA library with the partial chicken GFRα-4 cDNA was carried out as described above. However, screening was carried out at high stringency: prehybridisation was at 60°C, followed by hybridisation at 65°C, and subsequent washes at 65°C.

2.2.2. d) Isolation of Recombinant cDNA.

An *in vivo* excision method was used to isolate the recombinant cDNA (Sambrook *et al* 1989). The excision of the recombinant cDNA from the phage UNI-ZAP XR vector occurs by insertion of the cloned insert into the bluescript plasmid (pBs) using the Exassist helper phage (Stratagene). From a 10ml culture of XL1-Blue MRA host cells ($0D_{600}$=1.0) grown in L-Broth containing 100µg/ml tetracycline, 200µl of cells were transferred to a 1.5ml eppendorf containing 250µl of phage suspension from the tertiary screen. To this, 1µl of exassist helper phage (1×10^{10} pfu/ml) (Stratagene) was added and the mixture was incubated at 37°C for 15 minutes. This step allows the adsorption of phages to the bacteria. 3mls of LB broth was added and the mixture incubated for a further 2-2.5 hours at 37°C. 1ml of the reaction was then incubated at 70°C for 15 minutes, followed by centrifugation at 4000 x g for 15 minutes. The supernatant contained the excised phagemid pBs. To plate the phagemid, 100µl of this stock was transferred to a 1.5ml eppendorf tube containing 200µl of XL1 cells and incubated at 37°C for a further 10 minutes to allow adsorption of the phagemid to the bacteria. Following this, 100µl of the cells were plated on some previously prepared LB agar dishes containing 50µg/ml ampicillin and grown overnight at 37°C. During this time, single stranded plasmid DNA was converted to double-stranded DNA which replicates as a conventional plasmid. Single colonies were picked from each plate and incubated into 10ml cultures of L-Broth containing 100µg/ml ampicillin.

2.2.3. Analysis of Recombinant Plasmids.

Following isolation of the recombinant clones from the bluescript phagemid, cultures were prepared for small scale (Qiagen) and large scale (PEG precipitation) plasmid extraction, which was carried out as described in sections 2.2.3.a) and b).

2.2.3. a) Small Scale Plasmid Extraction.

To allow extraction of the recombinant plasmids from the colonies of selected clones, 10 ml overnight cultures were prepared by inoculating a single colony into L-Broth containing 100µg/ml ampicillin, and incubating overnight at 37°C in an orbital shaker. From these 10 ml cultures, the recombinant plasmid was extracted using the Plasmid Mini DNA Extraction Kit (Qiagen). 2mls of the overnight culture was placed in a microfuge tube and centrifuged at 13,000rpm for 1 minute. The supernatant was removed and the bacterial pellet resuspended in 250µl of buffer P1 (50mM Tris-HCl, 10mM EDTA). 250µl of buffer P2 (0.2M NaOH, 1% SDS) was then added, mixed gently and incubated for 5 minutes at room temperature (RT). Then 350µl of buffer N3 (3M Potassium acetate pH5.5) was added, mixed gently, centrifuged at RT for 15 minutes and the supernatant carefully removed. During centrifugation an appropriate number of Qiaprep spin columns were placed in 2ml collection tubes, and the supernatants from each sample carefully applied to each column by pipetting. Samples were centrifuged for 30-60 seconds, at 13,000rpm and the flow through discarded. Then the columns were washed by adding 750µl of buffer PE and centrifuging for 30-60 seconds at 13,000rpm. The flow-through was discarded and the columns centrifuged for a minute to remove the residual wash buffer. The Qiaprep spin columns were then placed into fresh 1.5ml eppendorf tubes. To elute the DNA, 60µl of dH_2O was added to the

center of each column, left to stand for 1 minute and centrifuged for 1 minute at 13,000rpm, to collect the plasmid DNA.

2.2.3. b) Large Scale Plasmid Extraction: Polyethylene Glycol (PEG) Preparation.

To allow large scale plasmid extraction from the selected clones, overnight cultures were prepared by inoculating 10µl of the previous 10ml cultures into 40mls of L-Broth containing 100µg/ml ampicillin. 40ml cultures were centrifuged at 6000rpm (Jouan fuge) to obtain a bacterial pellet. This was resuspended in 2mls of Solution I (50mM glucose, 25mM Tris pH8.0, 10mM EDTA and 10mg/ml lysozyme). 4mls of Solution II (0.2M NaCl, 1%SDS) was added, and mixed gently. 3mls of Solution III (5M Potassium acetate, pH4.8) was added, mixing immediately, but gently and then left on ice for 30-60 minutes. To remove cell debris and chromosomal DNA, centrifugation was carried out at 4°C for 20 minutes at 11,000rpm. The supernatant was transferred to a fresh 15ml Falcon tube, 5mls of isopropanol was added and this solution was incubated at -20°C for at least 30 minutes. After precipitation, the samples were centrifuged for 10 minutes at 5000rpm in a Backet rotor. The pellet was washed with 70% ethanol and dried in the Speed Vac. The pellets could then be resuspended in 200-500µl of water. To obtain highly pure DNA, PEG precipitation was then carried out on all of the samples. To each sample, 2 volumes of 7.5M ammonium acetate was added, mixed gently and placed at -20°C for 30 minutes. The tubes were then centrifuged at 13,000rpm in a microcentrifuge for 10 minutes at 4°C. The supernatant was transferred to a fresh 1.5ml Eppendorf, 0.56 volumes of isopropanol was added and the samples placed at -20°C for 30 minutes. The samples were centrifuged at 13,000rpm for 10 minutes and the pellets washed with 70% ethanol and vacuum dried. To the pellets, 100µl of dH_2O and 100µl

of PEG-NaCl were added, the contents were mixed gently and the samples placed on ice for 30 minutes. The samples were centrifuged as before and the supernatants transferred to fresh 1.5ml eppendorf tubes. Another 100µl of PEG-NaCl was added to the samples which were then placed on ice for 30 minutes. Following this, the samples were centrifuged as before, the supernatants were discarded, and the pellets washed three times in 70% ethanol. Finally the DNA pellets were vacuum dried and resuspended in 30-50µl of dH_2O.

2.2.3. c) Restriction Analysis of Recombinant cDNA Clones.

In order to ascertain the size of insert in the bluescript clones isolated from the cDNA libraries, endonuclease restriction digestion was carried out using 10 units of the restriction enzymes (*EcoRI / XhoI*) in 0.1 volumes of multicore buffer (Promega). Incubation was carried out on a heating block at 37°C for 1 hour and 30 minutes. The digested plasmid was run on a 1% agarose gel as described in section 2.2.1.b.i), alongside a 1Kb BRL ladder. The gels were viewed using a U.V. transilluminator.

2.2.3. d) Southern Blotting and Hybridisation of recombinant DNA.

Using the agarose gel from the restriction digestion to prepare a Southern Blot, the recombinant clones isolated from the library were hybridised with [α ^{32}P] labelled mouse GFRα-1 and chicken GFRα-4 cDNA probes. This was to confirm that all the clones hybridised with these probes. The agarose gel containing the digested recombinant clones was cut to the appropriate size and placed in a plastic box to incubate on a rocking platform in the following series of solutions: (i) rinsed twice in dH_2O, (ii) 30 minutes in Denaturing solution (1.5M NaCl, 0.5M NaOH) (iii) rinsed twice in dH_2O and (iv) incubated for 30 minutes in neutralising solution (0.5M Tris, 1.5M NaCl pH7.2). While incubating in the neutralising solution a stack of 8-10 cm high 3MM Whatman paper and a piece of HybondN$^+$ (Amersham) transfer membrane were cut to the same size as the gel. The latter was washed in dH_2O and placed in 20 x SSC until needed. On completion of the neutralisation step, the gel was inverted and placed on 3MM Whatman paper on the blotting apparatus. The membrane was then placed on the gel and the air bubbles removed. Two pieces of the 3MM Whatman paper stack were soaked in 2 x SSC and placed on the membrane, again air bubbles were removed and the remainder of the Whatman paper was placed on top with a plastic plate for weight. This was left overnight for capillary transfer of the DNA. After transfer the blot was dismantled and the DNA was U.V. crosslinked to the membrane as described in section (2.2.2.b). The membrane was then rinsed with 2 x SSC and baked at 80^{o}C in an oven.

Prehybridisation of the membrane was carried out in a sealed plastic bag containing prehybridisation buffer at 60^{o}C for 20 minutes. This buffer was discarded and the hybridisation solution containing the α ^{32}P labelled probe was added and the membrane was hybridised for 4-6 hours at 60^{o}C.

Following this, the membrane was placed in a plastic box and washed twice in 2 x SSC, and then three times in 2 x SSC / 0.2%SDS for 15 minutes each time. The membrane was then exposed to Kodak Omat Xray film overnight.

2.2.3. e) DNA Sequencing of The Recombinant Plasmids.

The bluescript clones isolated from the libraries were sequenced using an adaption of Sanger's dideoxy sequencing method to determine the primary structure of the cDNA. The M13 forward and M13 reverse primers which flanked the multiple cloning sites in Bluescript were used for initial sequencing. Further sequencing of the inserts required overlapping sets of primers. The sequencing reaction was carried out using the sequenase 2.0 kit (Amersham Life Sciences Plc/USB, Amersham Place, Little Chalfont, Buckinghamshire.) as follows: ~5µg of plasmid DNA was diluted in a total volume of 20µl with dH_2O, denaturation of the plasmid DNA was carried for ~ 30 minutes by incubating at 37°C with 0.1 volumes of 2M NaOH/2mM EDTA (0.8g NaOH, 0.0064g EDTA in 10mls dH_2O). The DNA was then precipitated at -80°C using 2.5 volumes of ethanol and 3M Sodium acetate pH5.2. After centrifugation, the DNA pellet was washed twice in 70% ethanol, air dried and resuspended in 7µl of dH_2O. To this, 2µl of sequenase reaction buffer was added plus 1-2 pmol of primer and left at 37°C for 30 minutes. To each tube containing the annealed template primer, the following solutions were added: 1µl of 0.1 M DTT, 2µl of 1.5 labelling mix (USB kit), 0.5µl of ^{35}S-dATP (12.5 mCi/ml), 2µl of 1.8 Sequenase DNA polymerase version 2.0 (UBS kit). During this procedure the tube was always kept in wet ice. The mixture was then mixed gently and incubated at room temperature for 2 minutes. Finally the tubes were centrifuged for 15 seconds to spin down the solution. Four 0.5ml Eppendorf tubes were labelled A, T, C, and G, 2.5µl of each termination mix ddATP, ddTTP, ddCTP, and ddGTP (80µM) were added

to each of four Eppendorf tubes. These tubes were pre-warmed at 37°c. Subsequently 3.5µl of labelling reaction was transferred to each termination mix tube. The mixture was incubated for 5 minutes at 37°c. Then the reaction was stopped by the addition of 4µl of stop solution (95% Formamide, 20mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene CyanolFF) (UBS) To each termination tube followed by mixing. Samples were heated at 80°C for 20 minutes and snap cooled on ice.

The sequencing products were electrophoresed on denaturing 6% polyacrylamide gels in TBE buffer (100mMTris-HCl pH8.0, 100mM Boric acid, 0.1MEDTA pH8.0) using the Biorad sequencing apparatus. Electrophoresis of the samples was carried out at 55W constant power for approximately 3-4 hours. After this, the gels were fixed by incubating in 10% acetic acid for 30 minutes and then by baking at 80°C for 1 hour. Sequencing gels were then exposed to Kodak Biomax X-ray films overnight.

2.2.4. Expression Analysis by Northern Blotting.

The developmental expression pattern of GFRα-4 in embryonic chicken was analysed by northern blot hybridisation of various chicken tissues. Since GFRα-4 is homologous to GFRα-1, GFRα-2 and GFRα-3. The GFRα-4 cDNA probe was prepared from the 5' untranslated region to prevent cross hybridisation of the probe between other GFR α mRNAs.

2.2.4. a) Preparation of a ^{32}P labelled chick GFR α -4 probe.

This was carried out as described in section (2.2.2.c i)

2.2.4. b) Total RNA Extraction of Chicken Tissues.

All of the solutions were prepared with (Diethylpyrocarbonate) DEPC - treated dH_2O to remove any contaminating ribonucleases. Various neuronal and non-neuronal tissuses were dissected from White Leghorn chicken embryos. These tissues were immediately frozen and stored at -80°C until use. Total RNA was extracted using guanidium isothiocyanate by a single step method (Chomczynski *et al.,* 1987). The tissues were defrosted and homogenised through a 1ml needle in denaturing solution D (4M guanidium thiocyanate, 25mM tri-sodium citrate pH7.0, 0.5% N-laurylsarcosine, 0.1M 2-ß-mercaptoethanol). Approximately 1ml of solution D was used per gram wet weight of tissue. After homogenisation, 0.1 volumes of 2M Sodium Acetate pH4.0 was added, and mixed thoroughly by inversion, followed by 1 volume of water-saturated acidic phenol and 0.1 volumes of chloroform/isoamyl alcohol (24:1). Samples were then vortexed for 10-15 seconds and placed on ice for 15 minutes. The samples were centrifuged for 15 minutes at 14,000rpm at 4°C. The aqueous phase was transfered to a fresh 1.5ml Eppendorf at -20°C to precipitate for 1-2hrs. Following centrifugation, the pellets were washed in 70% ethanol and dried in a speed vacuum. The samples were then resuspended in solution D and incubated at 37°C for 10 minutes. 0.1 volumes of 2M sodium acetate pH 4.0 and 2.5 volumes of 100% ethanol were added and the samples left to re-precipitate at -20°C. The samples were centrifuged and the RNA pellet was washed twice in 70% ethanol and then dried in a speed vacuum. The RNA pellet was resuspended in 15µl 1 x MOPS buffer/100mg of starting material and 2 volumes of formamide: formaldehyde (3:1) were added to the samples. The RNA was then denatured by incubating at 65°C for 15 minutes and then placed on ice. Before electrophoresis of the RNA samples

4.5µl of bromophenol/ethidium bromide (30/1)/100mg of starting tissue was added to the samples.

2.2.4. c) Denaturing Gel Electrophoresis of Total RNA.

1.2% BRL agarose and 3-[N-morpholino]propanesulfonic acid (MOPS)/2.2 M formaldehyde denaturing gels were used to resolve RNA. The gels were prepared and poured in a fume hood, and electrophoresis was carried out at 100-150mA for 3-4 hours in a Gibco-BRL tank filled with 1 x MOPS buffer. Before loading RNA samples onto the gel, the wells were filled with 5 x MOPS/Formaldehyde (1:3) to prevent degredation of the RNA during electrophoresis. After the initial electrophoresis, each sample was allocated a score depending on the intensity of its ethidium bromide staining, this enabled normalisation of the all the RNA samples before running a second gel for blotting.

2.2.4. d) Preparation of Northern Blot.

After separation of the total RNA by gel electrophoresis, the RNA was transferred from the agarose gel to Hybond N^+ membrane. Following electrophoresis, the gel was transferred to a plastic box containing 250mls of 10 x SSC and incubated for 30-60 minutes. While the gel was soaking, a stack of Whatman 3MM paper and a piece of Hybond N^+ membrane was cut to exactly the same size as the agarose gel. The membrane was left to soak in dH_2O and then left in 10 x SSC until needed. The transfer apparatus for northern blotting was the same as described for the Southern blotting.

2.2.4. e) Hybridisation of Northern Blot.

Prehybridisation of the membrane was carried at 40°C overnight in sealed plastic bags containing the following buffer: 50% formamide, 5 x SSC, 30 mM sodium phophate pH 7.0, 10mM EDTA, 0.5% SDS, 5 x Denhardt's solution and 250µg/ml salmon sperm DNA. When prehybridisation was complete, the buffer was discarded and hybridisation was carried out at 42°C for 48 hours in the same solution as above plus the radiolabelled chicken GFRα-4 cDNA probe prepared as described in section (2.2.2.c.ii). Following hybridisation, the filters were washed three times in 2 x SSC at room temperature and then three times at 65°C in 2 x SSC/0.2% SDS for 15 minutes each time. The membranes were then exposed to Kodak Omat x-ray film.

To compare the relative levels of RNA, the GFRα-4 cDNA probe was stripped off the filters by boiling in 0.1 x SSC/0.1 SDS for 10 minutes, and the filters were subsequently hybridised with a GAPDH cDNA probe. The membranes were prehybridised at 42°C overnight and hybridised at 42°C for 48 hours. After hybridisation filters were washed three times at 68°C for 15 minutes with 2 x SSC /0.2% SDS before being exposed to X-ray film.

2.2.5. Expression Analysis by *In Situ* Hybridisation.

In situ hyridisation was used to study the spatial expression of GFRα-4 mRNA in sagital and transverse cross sections of E18 chicken embryos.

2.2.5. a) Preparation of E18 Chicken Embryo Cryosections.

E18 chicken brain and trunk were dissected and embedded in OCT (Tissue Tek) and immediately frozen in liquid nitrogen. Using a cryostat, 15µm to 30µm sections were cut, mounted on poly-L-lysine coated slides and stored at -80°C until further use.

2.2.5. b) Subcloning of GFRα-4 into pGEM-T vector.

PCR amplification was used to prepare an 800bp DNA fragment of GFR α-4 using the primers 5'-CCCGAAAGAAGTTCCTTGTC-3' and 5'-TGCCTGGCACCGCTGGACTCC-3'. The PCR reaction was carried out in sterile 0.5ml Eppendorf tubes. Two reaction tubes were prepared: one for the amplified product and the other a negative control. To each tube, 5µl of 10 x PCR reaction buffer (Promega), 2.5µl of 25mM $MgCl_2$ (Promega), 2.0µl of 25mM dNTPs, 15pmol of each primer, and 20.5µl of dH_2O were added and mixed by pipetting. Centrifugation was carried out briefly, and 1ng/µl of pBs/GFRα-4 was added to the amplification tube: Both tubes were then overlain with 50µl of mineral oil followed by the addition of 0.5µl of Taq DNA polymerase (Promega). Amplification reactions were carried out in a Hybaid Omnigene thermal cycler. The PCR cycling parameters were as follows: denaturing at 95°C for 1 minute, annealing at 55°C for 30 seconds and extension at 72°C for 1minute for 35 cycles. The amplified product was then run on a 1% LMP agarose gel and purified as described in section 2.2.1.b i). This was then ligated into pGEM - T easy vector (Promega). For the ligation reaction, 100ng of purified PCR product was ligated with 50 ng of vector. The reaction was set up as follows: 1µl of vector (50ng) (Promega), 4µl of PCR amplified DNA (100ng), 1µl ligase buffer (Promega), 1µl T4 DNA ligase (3 units/µl) (Promega) and dH_20 to a final volume of 10µl. The reaction tube was then left to incubate at 4°C for 12 hours.

Following ligation, competent cells XL1 were transformed with ligated reaction mix. To a tube containing 100µl of XL1 cells 3µl of ligation reaction mix was added, to another tube containing 100µl of XL1 cells 1µl of dH_20 was added (negative control). The tubes were placed on wet ice for 1 hour, heat shocked at 42°C, for 90 seconds and placed back on ice for 5 minutes.

900µl of L-Broth was added to the cells, and the mixture was transferred to a 25ml tube, which was then incubated at 37°C for 1 hour. After incubation, the cells were plated on previously prepared LB-agar dishes containing 100µg/ml ampicillin. The dishes were then incubated at 37°C overnight, after which selected colonies were grown in culture for plasmid extraction.

2.2.5. c) Linearization of GFRα-4 Subclone.

The pGEM-T plasmid containing the 800bp (1b) insert was linearized using the restriction endonucleases *Sal I* and *SacII*. Two separate reaction tubes were prepared and plasmid DNA was digested with 20 units of each enzyme and 0.1 volumes of buffer C (Promega). Incubation was carried out at 37°C for 1 hour and 30 minutes respectively.

2.2.5. d) Synthesis of Dioxygenin (DIG)-Labelled Sense and Antisense cRNA.

For the synthesis of GFRα-4 sense and antisense cRNA, the plasmid insert was linearised with *Sal I* and *SacII,* respectively, and the transcription enzyme used was T7 and SP6 RNA polymerase, respectively. The run off transcription reactions were prepared as follows: 10µl of 5 x Transcription buffer (DIG RNA labelling kit, (Boehringer)), 4µl of 0.1M DTT (Gibco-BRL), 4µl of 10 x DIG RNA labelling mix (Boehringer), 1µl (38 units) of ribonuclease inhibitor (RNA guard, Pharmacia), 5µl (1µg) of linearized plasmid, 1.5µl of T7 or Sp6 RNA polymerase (75 units) (Promega) and dH_2O were added to a final volume of 50µl. The tubes were incubated at 37°C for 2 hours. After 1 hour, a 2µl aliquot was removed from each tube and analysed on a 1% agarose gel to monitor the progress of the reaction. After 2 hours, 2µl of ribonuclease-free *DNase I* was added and incubated for 15 minutes at 37°C. This allowed digestion of the template plasmid DNA, which may otherwise

have prevented hybridisation of the cRNA probe. The sense and antisense cRNA probes were precipitated by the addition of 100µl of TE (50mM Tris-HCl, 1mM EDTA, pH 8.0), 10µl 4M lithium chloride and 300µl of ethanol. The tubes were then agitated and left at -20°C for 30 minutes to precipitate the RNA. The RNA was pelleted by centrifugation at 15,300rpm for 10 minutes at 4°C. The pellet was then washed twice in 70% ethanol and dried in a speed vacuum. The cRNA was then resuspended in an appropriate amount of dH_2O and stored at -80°C until needed.

2.2.5. e) *In Situ* Hybridisation.

i) Pretreatment of cryosections on slides

The slides were removed from storage at -80°C, and placed in Coplin jars at 4°C containing chilled 4% paraformaldehyde/PBS pH7.5 for 5 minutes. They were then rinsed twice in 1xPBS and incubated in 0.1% Triton-X-100/PBS pH7.5 for 5 minutes at 4°C, and then in 4% paraformaldehyde/PBS pH7.5 for 5 minutes at 4°C. The slides were then transferred to 0.1M triethanolamine/PBS pH8.0 with acetic anhydride and incubated for 10 minutes at room temperature. Next the slides were immersed for 2 minutes each time in 70% ethanol, 96% ethanol, and 100% ethanol. Then they were incubated in chloroform for 5 minutes, and immersed in 100% ethanol and 96% ethanol for 2 minutes, before being air dried in a flow hood.

ii) Hybridisation of sections with GFRα-4 cRNA probes

Sections were hybridised with both sense and antisense probes. These were prepared by adding approximately 1µg of each cRNA to 1ml of *in situ* hybridisation buffer (50% formamide, 5 x SSC, 0.5% triton -X-100, 5% CHAPS, 5mg/ml yeast tRNA, 5mM EDTA pH 8.0, 2.5mg/ml heparin, and distilled water to 50mls). To the appropriately labelled slides, a few drops of

either sense or antisense probe was added to cover the surface of the section. Each section was then covered with parafilm, to avoid evaporation of the solution, and placed in a humidified box to hybridise at 55°C for 24 hours.

iii) Post hybridisation washes

After hybridisation, the slides were removed from the humidified chamber and washed sequentially in Coplin jars containing solutions 1, 2, 3, and 4 at 55°C for 5 minutes: solution 1 (50% formamide, 5xSSC, 0.1% Triton -X- 100, 0.5% CHAPS), solution 2 (75% solution 1/2 x SSC), solution 3 (50% solution 1/2 x SSC), and solution 3 (25% solution 1/2 x SSC). The slides were then washed twice in 2 x SSC/0.1% CHAPS. and 0.2% x SSC / 0.1% CHAPS, and twice at room temperature in TBT (50mM Tris-HCl pH7.5, 150mM NaCl, 0.1% Triton x 100) for 5 minutes and then 20 minutes. Following this, the slides were washed in 1% Blocking reagent / TBT for 30 minutes at room temperature, with two further 5 minute washes in TBT at room temperature.

iiii) Incubation of sections with Anti- DIG antibody

150µl of a 1:250 dilution of anti-DIG-AP, Fab fragments (Boehringer) in TBT/2% sheep serum (Sigma) was then carefully pipetted onto the surface of the slides to cover the section. The slides were then placed in a humidified chamber and left to incubate at room temperature for 2 hours and 30 minutes.

v) Post antibody washes

Following incubation of the sections, the residual antibody was removed by washing twice for 5 minutes in TBT, and then three times for 5 minutes in NTMT (100mM NaCl, 100mM Tris - HCl, 50mM $MgCl_2$ pH 9.5) at room temperature.

vi) Detection

Finally the sections were developed with 3.5µl of NTB solution (Boehringer) and 3.5µl of bovine calf intestinal phosphatase (B-Cip) in 1ml of NTMT. A few drops of this solution were placed on the slides, which were then placed in a humidified box in the dark for the colorimetric reaction to proceed. When complete, the slides were mounted with a glycerol/PBS mounting solution (AFI) (Citifluor), and kept at 4°C until needed.

2.3. Results.

2.3.1. Full Length Sequences of GFRα-1, GFRα-2, and GFRα-4 cDNA's.

To obtain the GFRα-1, GFRα-2 and GFRα-4 cDNA's, an E10 chicken brain cDNA library was screened at low stringency using the full length mouse GFRα-1 as a probe. From the primary screen, thirty one plaques were found to hybridise with the mouse GFRα-1 probe. Of these, thirteen were chosen for further analysis. Rescue of the thirteen clones from the bacteriophage into bluescript, was carried out by *in vivo* excision. On sequencing, eight of the thirteen clones obtained were found to be overlapping cDNAs spanning a region of 3Kb that corresponded to GFRα-1. Four clones were found to be overlapping cDNAs spanning a region of 2.5-2.9Kb that corresponded to GFRα-2. One clone contained a 2.6kb cDNA that corresponded to a partial sequence of GFRα-4. To isolate of the full length GFRα-4, a second E6 chicken brain library was screened at high stringency. From the primary screen, two plaques hybridised with the partial chicken GFRα-4 probe. Both were analysed further, and the bluescript clones isolated by *in vivo* excision. On sequencing, the first clone was found identical to the partial 2.6kb GFRα-4 cDNA previously isolated. The second clone however, contained a 4.3kb insert that corresponded to the full coding sequence of GFRα-4.

The eight overlapping clones corresponding to GFRα-1 were sequenced on both DNA strands. This revealed a cDNA of 3.0Kb, of which the nucleotide sequence is illustrated in the 5'to 3' direction (Figure 2.1). The 5' untranslated region had a high G/C content and was 739bp long. This was followed by a 1.4Kb open reading frame open reading frame (ORF) that terminated at a stop codon TAG at nucleotide position 2149. The 3' untranslated region was 1887bp long and was A/T rich.

Sequencing of the four clones corresponding to GFRα-2 revealed a cDNA of 2.9Kb. The cDNA was sequenced on both strands in the 5'to 3' direction and the nucleotide sequence is shown in (Figure 2.2). The cDNA has a short 5' sequence followed by an ORF of 1.4Kb that starts at nucleotide 38 and extends to a stop codon TAG at nucleotide position 1435. The 3' untranslated region is 1.5Kb in length and A/T rich.

The clone obtained for GFRα-4 contained a cDNA of 3.8Kb. Due to the length of this cDNA, exonuclease deletion clones were prepared to facilitate sequencing. Both DNA strands were sequenced in the 5'to3' direction and the nucleotide sequence is shown in (Figure 2.3). In this cDNA, the 5' untranslated sequence is short. This is followed by an ORF of 1.3Kb that extends from nucleotide 15 to a stop codon TGA at nucleotide position 1307. The termination codon is followed by a long A/T rich 3' untranslated region of ~2.5Kb.

Figure 2.1. The 5' to 3' chicken GFRα-1 cDNA nucleotide sequence. The open reading frame is shown in uppercase and nucleotide numbers are denoted on the right.

```
cgagcgcgccccctgaggcagcggggctcctcctgaggcgcgggcagcgc     50
accgccgtctcagtcaccgggctgcggctacccgtcgggagcccccctgc    100
tgcgcccgacctgccccgccggagcagctccctgcaggtgacggagctcc    150
ctcccagtttgcccggctaaactttcccagctgccggcagcacctgtgcg    200
tatctcccgggcgagcggcacgaccgcctccgcgcgaagaaataaagttg    250
cggctttgaggaggggacgaatcccttcctgcagggctcgggcactcgcg    300
gggagccccgtaggtgcgtgcggggcgtcagcgctcttcccccacccct    350
ccccgtgagcgctcggtccctccccgggcacaggctgctgccacccaagg    400
aggcgcccggctgctgccccgctgccgccgctccgtgcacacacacagac    450
acacaaatacggtgcgttgcgcctccgggagtcagcgcctccgccttcag    500
gttgctcagacctgaaatctacggggaatctcagcgttcttgtcgccgct    550
cctgccggtggaagcggtgaagaggagagattttgatcattatcattatt    600
attgttatttccccttcttatatcaatggatcggaacttggagtctttg    650
cacctcggcggttttggaatatctacatgctgagcctctttgttgtggca    700
catcggctcagttcgggggaaccatccggagacggcacgATGTTCCTCGC    750
GCTCCTCTACTTGGCTCTGCCCTTAGCGGACGTACTTCTGTCGGCAGAAG    800
TCAGCGGGCTGCCCGGAGGGGACCGCCTCGACTGCGTGAAAGCCAGCGAT    850
CAGTGTCTCAAGGAGCAGAGCTGTAGTACTAAGTACAGGACACTGAGGCA    900
GTGTGTAGCCGGCAAAGAGAGCAACTTCAGCCGGGCGACGGGCCTGGAGG    950
CGAAGGATGAATGCAAAAGCGCCATGGAGGCTCTCAAGCAGAAATCTCTG   1000
TACAACTGCCGCTGCAAGAGGGGCATGAAGAAGGAGAAAAACTGCCTGCG   1050
CATTTACTGGAGCATGTACCAGAGCTTACAGGGAAATGACTTGCTTGAGG   1100
ATTCTCCCTATGAACCAGTTAACAGCAGGCTATCAGACATATTCAGGCTA   1150
GCACCGATTGTATCAGTGGAGCCAGTACTATCAAAGGGGAACAACTGCTT   1200
GGATGCAGCAAAAGCTTGTAACCTAAATGATACCTGCAAGAGGTTTAGAT   1250
CTGCTTACATAACCCCCTGCACCAGCAGCACGTCTAATGAAATCTGTAAC   1300
AAGCGGAAGTGTCATAAGGCCCTCCGGCTATTTTTTGACAAAGTTCCCCC   1350
AAAGCACAGCTACGGGATGCTCTTCTGCTCCTGTCGAGACGTAGCCTGTA   1400
CAGAAAGGAGGCGGCAGACTATTGTTCCTGTGTGTTCATATGAGGACAGG   1450
GAGAAACCAAACTGCCTGAATTTACAAGAATCCTGCAAGAAGAATTACAT   1500
CTGCAGATCTCGCCTTGCAGATTTTTTCACAAACTGCCAGCCTGAGTCAC   1550
GCTCTGTTAGTAGCTGTCTGAAGGAGAACTACGCTGACTGCCTCCTCGCT   1600
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TACTCGGGGCTCATTGGCACAGTGATGACACCGAACTACATAGACTCAAG 1650

TAGTCTCAGCGTTGCCCCATGGTGTGACTGCAGCAACAGTGGTAATGACA 1700

TAGATGAATGCCGGAAATTTCTGAATTTCTTCCAGGACAACACATGCCTT 1750

AAAAATGCAATTCAGGCTTTCGGCAATGGTACTGATGTGAATGTGTGGCA 1800

GCCAATATTACCGGTACAGACCACTACAGCCACAACTACAACAGCTTCCA 1850

GACTTAAAAACACAGGTTCAGAGACCACCAACAATGAAATACCCACCCAC 1900

AATGATTCACCAGCATGTGCGAACCTGCAGGCACAGAAGAAGCGGAAATC 1950

CAATGAATCTGTAGATACAGAACTCTGTCTTAATGAGAATGCTATTGGGA 2000

AAGACAACACACCAGGAGTCTCCACCAGCCACATATCCTCGGAGAATTCT 2050

TTTGCCCTTCCTACAAGCTTCTATCCAAGCACACCACTCATTCTGATGAC 2100

AATTGCACTCTCACTCTTTTTGTTCCTAAGCTCATCAGTCGTCTTGTAGc 2150

tgcattgcaaaaggacatgtaaaaaaatctgtttcctgtcctctgttgtt 2200

tatctggaattccaggtctgggggctaagctgaggcacttctgctagaac 2250

agtttgtcagctggaaaaatttttctctctaaaaaagcttcttgtgata 2300

tttagaggctttgtgaatacttggtgcagtgctacattccaaacccaaga 2350

ggcttttgggcatgcagtgttttgaagagacagtgtataaatttgcctgt 2400

aaagagatctggttggattattttaataattatattaattctggccttta 2450

cctgagaaggaggatggcagttttcttaagatcctatttatctcattgga 2500

tggttttggttttcaaattgatcaaactccagattatcaaggatgtcagg 2550

cttttgtctaatggtgaatgttctcccagagagtggactttatgaaactt 2600

cttcatttgataaattgctactgatgttaaactctttcagtgtattagca 2650

ttttcctctttaaatgtttacgtactgtaagtgattctgcgttccctgct 2700

gagaagccattctaattcacatacaggtgtaacgtatgtctttcagttaa 2750

attcttatagagtgtggcatagaactttctaacaaaacatttatctttta 2800

attataatcatctagccttaacgagggtgaagattctttaaattaacaag 2850

aagcagccattgtgaaagctccgtaaacgtacatttcataaactttgagg 2900

atgaacagtagaaaaaaattttgctgcagggtttcagctgtacagtcacc 2950

ggtcttctgtgcttccattgtgacaatatagtccaattattatactgttc 3000

attcaataaaaaagatatgcattttacttgctctact 3037

Figure 2.2. The 5' to 3' chicken GFRα-2 cDNA sequence. The open reading frame is shown in uppercase and nucleotide numbers are denoted on the right.

```
cctaaacgcacgtggaggaactcgtggatgtactaacATGATTTTGGCCA     50
ACGCCTTCTGCATCGTCCTCTTTGTAGATGAGACCCTCCGCTCGCTGGCC    100
GCCCCCCCGTCCCCCCCCGGGCAGGACCTGCAGGGCTGGCGGGTGCCGGT    150
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cggggaaaataaacatgtcacgtctctcgccgg 2933

Figure 2.3. The 5' to 3' chicken GFRα-4 cDNA sequence. The open reading frame is shown in uppercase and nucleotide numbers are denoted on the right.

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catggttgggatgggtgctaatattgtgctggaaaaaaaaaaaaaaatca 3850

aaaaa 3855

2.3.2. Protein sequence of chicken GFRα-1, GFRα-2 and GFRα-4 in comparison with mouse GFRα family members.

Comparison of chicken GFRα-1, GFRα-2 and GFRα-4 with protein alignments of the recently identified GFRα-family members demonstrates varying degrees of amino acid identity between the proteins (Figure 2.4.).

The chicken GFRα-1 cDNA encodes a protein of 496 amino acids and shares ~80% amino acid identity with the rodent GFRα-1 homologues (Jing *et al.*, 1996, Treanor *et al.*,1996). The nucleotide sequence of chicken GFRα-2 encodes a protein of 466 amino acids. This protein shares ~80% amino acid identity with the rodent GFRα-2 homologues, and ~48% identity with mouse and chicken GFRα-1. Both chicken GFRα-1 and GFRα-2 include all 30 cysteine residues at the same relative positions to those found in their homologues In contrast, chicken GFRα-1 and GFRα-2 show less homology to mouse GFRα-3 which contains only 28 of the 30 conserved cysteine residues and shares approximately 30% identity.

The nucleotide sequence of chicken GFRα-4 predicts a 431 amino acid protein that is more closely related to GFRα-1 and GFRα-2 than to GFRα-3. Whereas GFRα-4 has approximately 40% amino acid identity with both mouse and chicken GFRα-1 and GFRα-2, it has only 27% identity with mouse GFRα-3. Of the 30 cysteines that are present in GFRα-1 and GFRα-2, only 28 are conserved in GFRα-4.

Chicken GFRα-1, GFRα-2 and GFRα-4 possess an N-terminal hydrophobic domain of 13 amino acids, that corresponds to a putative signal peptide for secretion, and a cysteine rich domain. This is followed by a C-terminal hydrophobic region which is characteristic of GPI-linked proteins, and contains a carboxy-terminal hydrophobic domain separated by a hydrophilic linker region from a cleavage/binding consensus sequence for GPI

linkage (GFRα-1 Ser_{428}, Thr_{429}, Ser_{430}, GFRα-2 Ala_{443} Gly_{444} Ser_{445}, GFR α-4 Gln_{400}, Thr_{401}, Ser_{402}) (Gerber *et al* 1992).

Figure 2.4. Aligned Protein sequences of mouse GFRα-1, GFRα-2, and GFRα-3; and chicken GFRα-1, GFRα-2 and GFRα-4. Conserved cysteines are shown in red, and amino acids in GFRα-1, GFRα-2, and GFRα-3; that are identical to those in GFRα-4 are shown in blue. The N-terminal, hydrophobic, putative signal peptides are underlined in yellow, the C-terminal hydrophobic domain is underlined in red, and the putative binding/cleavage consensus sequences for GPI linkage are enclosed in red boxes.

```
mGFRα1   MFLATLYFVLPLLDLL--MSAE-VS---GGDRL-------DCVKASDQCLKEQSCSTKYR   47
cGFRα1   MFLALLYLALPLADVLL--SAE-VSGLPGGDRL-------DCVKASDQCLKEQSCSTKYR   50
mGFRα2   MILANAFCLFFFLDETLR-SL-ASPSSPQGSELHGWRPQVDCVRANELCAAESNCSSRYR   58
cGFRα2   MILANAFCIVLFVDETLR-SLAAPPS-PPGQDLQGWRVPVDCIRANKLCAAEGSCSSRYR   58
mGFRα3   MGLSWSPRPPLLMILLLVLSLWLPLGAGNSLATEN-RFVNSCTQARKKCEANPACKAAYQ   59
cGFRα4   MRGILYFCTLILLEG---M-AEAVSSSR----------DCLQAGESCTNDPICSSKFR   44

mGFRα1   TLRQCVAGKETNFSLTSGLEA-KDECRSAMEALKQKS-LYNCRCKRGMKKEKNCLRIYWS  105
cGFRα1   TLRQCVAGKESNFSRATGLEA-KDECKSAMEALKQKS-LYNCRCKRGMKKEKNCLRIYWS  108
mGFRα2   TLRQCLAGRDRN----TML-ANK-ECQAALEVL-QESPLYDCRCKRGMKKELQCLQIYWS  111
cGFRα2   TLRQCLAGRDRN----TML-ANK-ECQAALEVL-QESPLYDCRCKRGMRKEIQCLQVYWS  111
mGFRα3   HLGSCTSSLSRPLPLEES--AMSADCLEAAEQLRN-SSLIDCRCHRRMKHQATCLDIYWT  116
cGFRα4   TLRQCIAGNGANKL---GPDA-KNQCRSTVTALLS-SQLYGCKCKRGMKKEKHCLSVYWS   99

mGFRα1   MYQSL-QGNDLLEDSPYEPVNSRLSDIFRAVPFISDVFQQVEHISKGNNCLDAAKACNLD  164
cGFRα1   MYQSL-QGNDLLEDSPYEPVNSRLSDIFRLAPIVSV---EPVL-SKGNNCLDAAKACNLN  163
mGFRα2   IHLGLTEGEEFYEASPYEPVTSRLSDIFRLASIFSGTGADPVVSAKSNHCLDAAKACNLN  171
cGFRα2   IHLGLAEGEEFYEASPYEPITSRLSDIFRLASIFSG--MDPATNSKSNHCLDAAKACNLN  169
mGFRα3   VHPARSLGDYELDVSPYEDTVTSKPWKMNLSKLNML--KPD-----SDLCLKFAMLCTLH  169
cGFRα4   IHHTLMEGMNVLESSPYEPFIRGFDYV-RLASITA---GSENEVTQVNRCLDAAKACNVD  155

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cGFRα1   DTCKRFRSAYITPCTSSTS-NEICNKRKCHKALRLFFDKVPPKHSYGMLFCSC--RDVAC  220
mGFRα2   DNCKKLRSSYISICNREISPTERCNRRKCHKALRQFFDRVPSEYTYRMLFCSC--QDQAC  229
cGFRα2   DNCKRLRSGYISTCSKEISATEHCSRRKCHKALRQFFDNVPSEYTYRLLFCSC--KDQAC  227
mGFRα3   DKCDRLRKAYGEACSGI-----RCQRHLCLAQLRSFFEKAAESHAQGLLLCPCAPEDAGC  224
cGFRα4   EMCQRLRTEYVSFCIRRLARADTCNRSKCHKALRKFFDRVPPEYTHELLFCPC--EDTAC  213

mGFRα1   TERRRQTIVPVCSYEER-ERPNCLNLQDSCKTNYICRSRLADFFTNCQPESRSVSNCLKE  280
cGFRα1   TERRRQTIVPVCSYEDR-EKPNCLNLQESCKKNYICRSRLADFFTNCQPESRSVSSCLKE  279
mGFRα2   AERRRQTILPSCSYED-KEKPNCLDLRSLCRTDHLCRSRLADFHANCRASYRTITSCPAD  288
cGFRα2   AEPRRQTIVPFCSYEDDKEKPNCLDLRNVCRADHLCRSRLADFHANCQASFQSLTSCPGD  287
mGFRα3   GERRRNTIAPSCALPSVT--PNCLDLRSFCRADPLCRSRLMDFQTHCHP-MDILGTCATE  281
cGFRα4   AERRRQTIVPACSYE-SKEKPNCLAPLDSCRENYVCRSRYAEFQFNCQPSLQTASGCRRD  272

mGFRα1   NYADCLLAYSGLIGTVMTPNYIDSSSLSV--APWCDCSNSGNDLEDCLKFLNFFKDNTCL  338
cGFRα1   NYADCLLAYSGLIGTVMTPNYIDSSSLSV--APWCDCSNSGNDIDECRKFLNFFQDNTCL  337
mGFRα2   NYQACLGSYAGMIGFDMTPNYVDSNPTGIVVSPWCNCRGSGNMEECEKFLKDFTENPCL  348
cGFRα2   NYQACLGSYTGLIGFDMTPNYVDASTTSITISPWCSCKGSGNLEECEKFLRDFTENPCL  347
mGFRα3   QS-RCLRAYLGLIGTAMTPNFISKVNTTVALS--CTCRGSGNLQDECEQLERSFSQNPCL  338
cGFRα4   SYAACLLAYTGIIGSPITPNYIDNSTSSI--APWCTCNASGNRQEECESFLHLFTDNVCL  330

mGFRα1   KNAIQAFGNGSDVTMW--QPAPPVQTTTAMTTTAFRIKNKPLGPAGSENEIPTHV--LPP  394
cGFRα1   KNAIQAFGNGTDVNVW--QPILPVQTTTATTTTASRLKNTGSETTN--NEIPTHND-SPA  392
mGFRα2   KNAIQAFGNGTDVNMSPKGPTFSATQAPRVEKTPSLPDDLSDS-TSLGTSVITT------  401
cGFRα2   RNAIQAFGNGTDVNLSPKNPSPPITMLPKVEKSPALPDDINDSNTMYDTSIITT------  401
mGFRα3   VEAIAAKMRFHRQLFSQDWADSTFSVVQQQNSNPALRLQPRLPILSFSILPLILLQTLW   397
cGFRα4   QNAIQAFGNGTYLNAATA-PSISPTTQMYKQERNANRAAATLSENIFEHLQPTKVAGEER  389

mGFRα1   CANLQA--QKL--KSNVSGSTHLCLSDNDYGKDGLAGAS-SHITTKSMAAPPSCGLSSLP  448
cGFRα1   CANLQA--QKKR-KSNESVDTELCLNENAIGKDNTPGVSTSHISSENSFALPTS--FYPS  446
mGFRα2   CTSI----QEQGLKANNSKELSMCFTELTTNISPGSKKVIKLYSGSCRARLSTALTALPL  456
cGFRα2   CTSI----QEHGQKLNKSKEQSLCYSETQLTTDTMPDQKTFVDQKAA-GSRHRAAR-ILP  454
cGFRα4   LLRGSTRLSSETSSPAAPCHQAASLLQLWLPPTLAVLSHFMM                    431

mGFRα1   VMVFTALAALLSVSLAETS          468
cGFRα1   TPLILMTIALSLFLFLSSSVVL       469
mGFRα2   LMVTLA                       463
cGFRα2   AVPIVLLKLLL                  466
```

2.3.3. Expression analysis of GFRα-4 mRNA in the developing chicken embryo.

Northern blot hybridisation was used to analyse the expression of GFRα-4 in the chicken embryo during development. A transcript of ~3.8Kb was present in the nervous system and various other non-neuronal tissues.

Northern blot hybridisation revealed the GFRα-4 transcript in the kidney, skeletal muscle, skin, intestine and lung of E10 embryos. In the kidney, the tissue with the highest level of expression by far, there was evidence of an additional minor transcript of~4.5Kb. In contrast, GFRα-4 was barely detectable in the liver and heart of E10 embryos. Within the CNS, the spinal cord expressed the highest level of GFRα-4 mRNA; the cerebellum, midbrain, medulla oblongata and pons, expressed lower levels and within the forebrain only very low levels were detectable (Figure 2.5.).

Within the CNS, the level of GFRα-4 mRNA expression in the spinal cord increased to reach a maximum at E14 and then gradually decreased from E14-E18. In the hindbrain, there was a gradual increase in mRNA expression with age from E10-E18 (Figure 2.6.). In the cerebellum, barely detectable levels of mRNA expression were observed from E10-E14, however, from E14-E18, there was a dramatic increase in mRNA expression. In contrast, high levels of GFRα-4 mRNA expression in the forebrain were observed from E10-E14 (Figure 2.6).

Developmental changes in the expression of GFRα-4 mRNA were clearly observed in some tissues. In the skin, there was gradual increase in the level of mRNA expression from E6-E18. In contrast, there was a marked decrease in the level of GFRα-4 mRNA expression in muscle from E6-E18 (Figure 2.7).

Figure 2.5. GFRα-4 mRNA expression in the E10 chicken embryo. Total RNA from neuronal and non-neuronal tissues were transferred to a filter and hybridised with the GFRα-4 probe. Bands corresponding to the 3.8Kb transcript are indicated. An additional 4.5kb band was observed in the kidney. To compare the relative levels of total RNA present in each sample an ethidium bromide stained gel of the RNA is shown

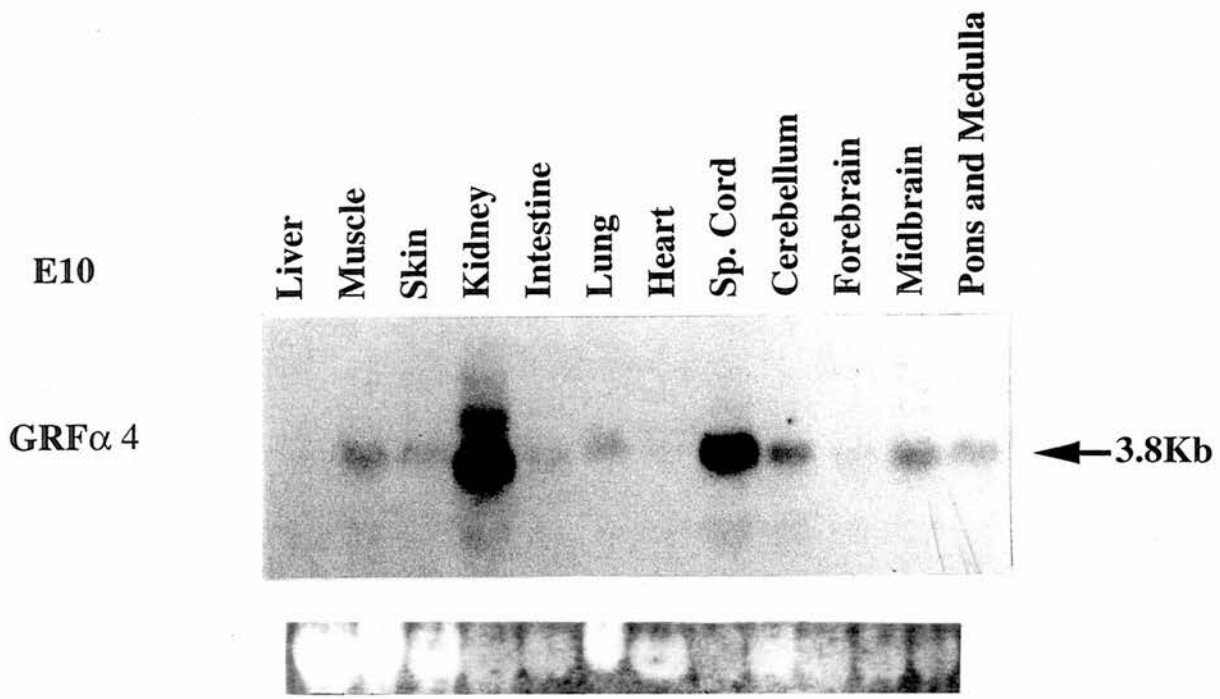


Figure 2.6. Developmental changes in GFRα-4 mRNA expression in the CNS of the chicken embryo. Northern blots of total RNA from CNS tissues: spinal cord, forebrain, hindbrain, and cerebellum. Filters were hybridised with a GFRα-4 probe. Bands corresponding to the 3.8Kb transcript are indicated. To compare the relative levels of total RNA present in each sample an ethidium bromide stained gel of the RNA is shown.

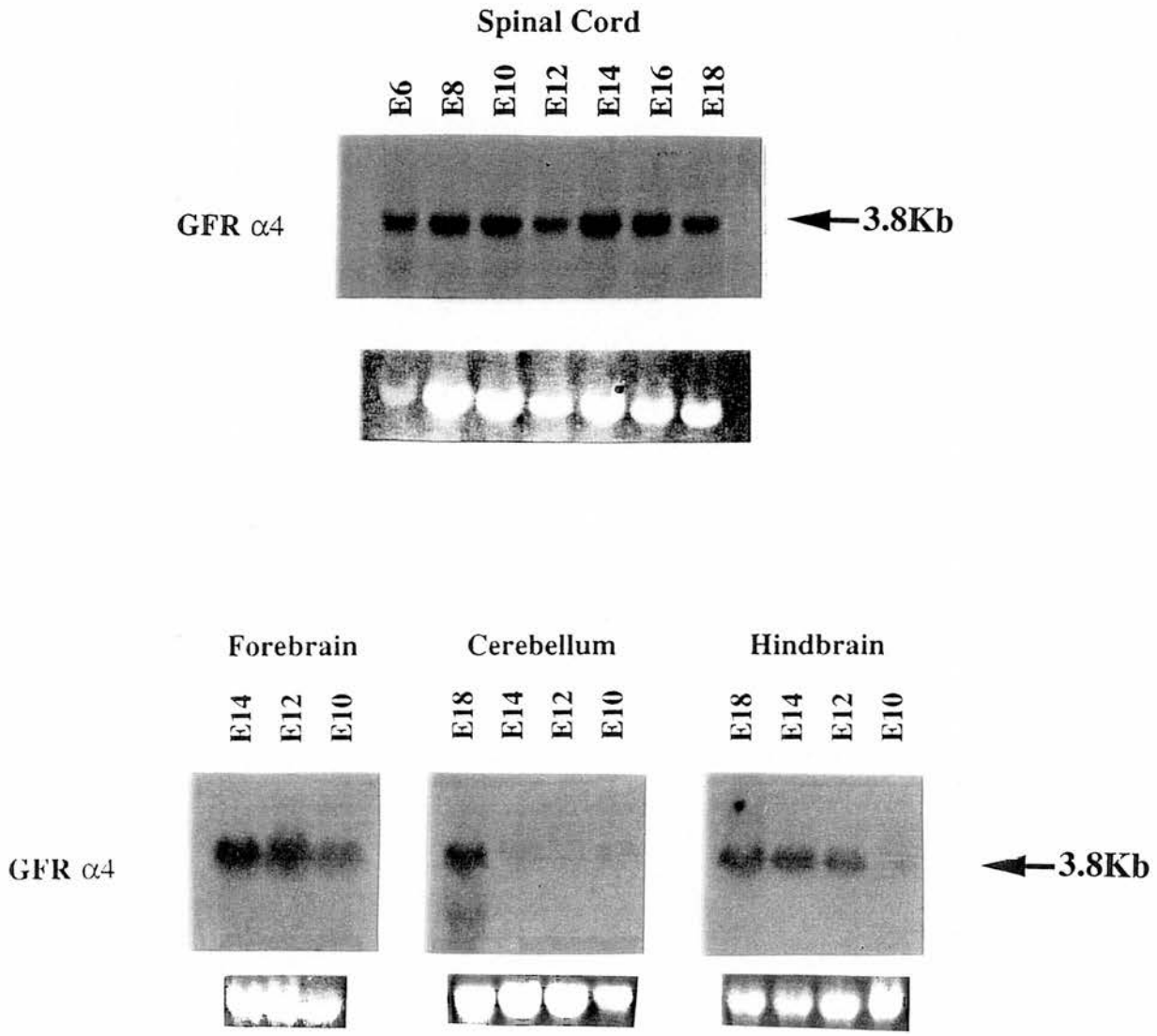
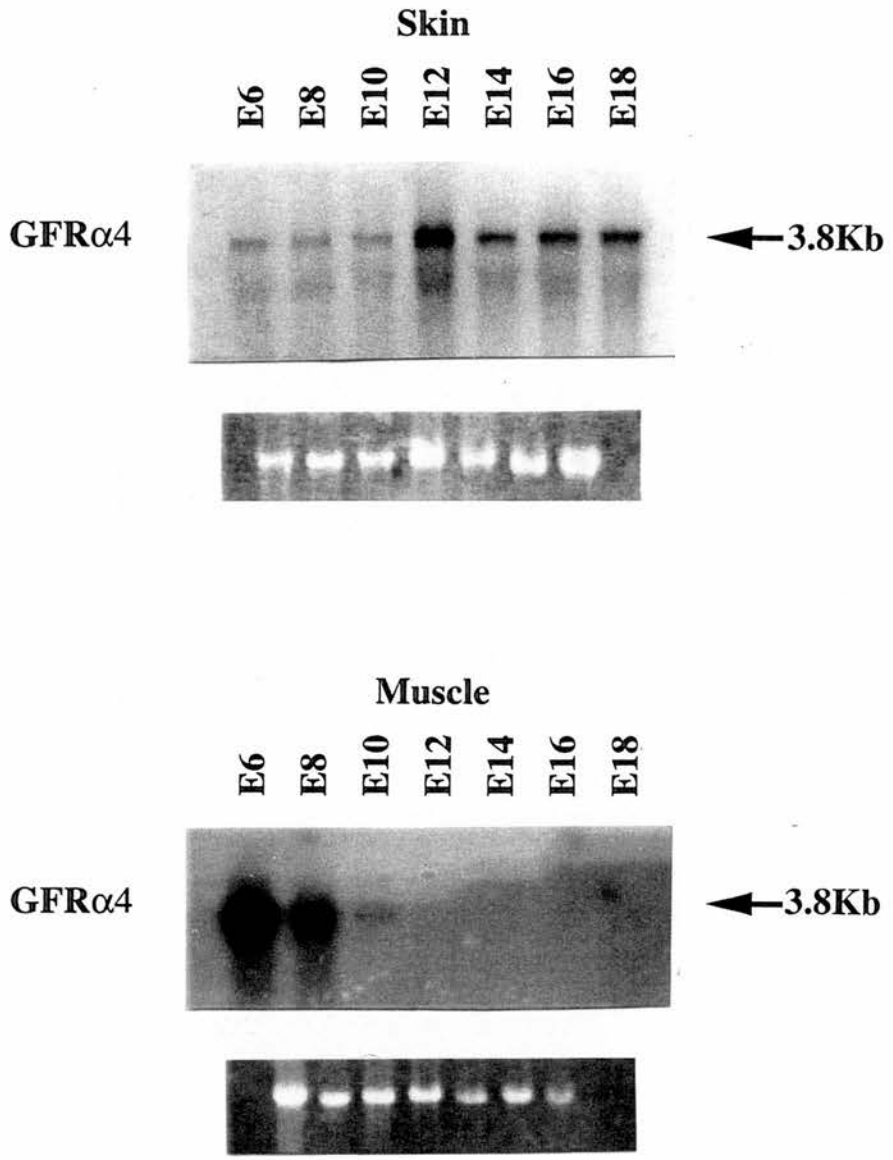


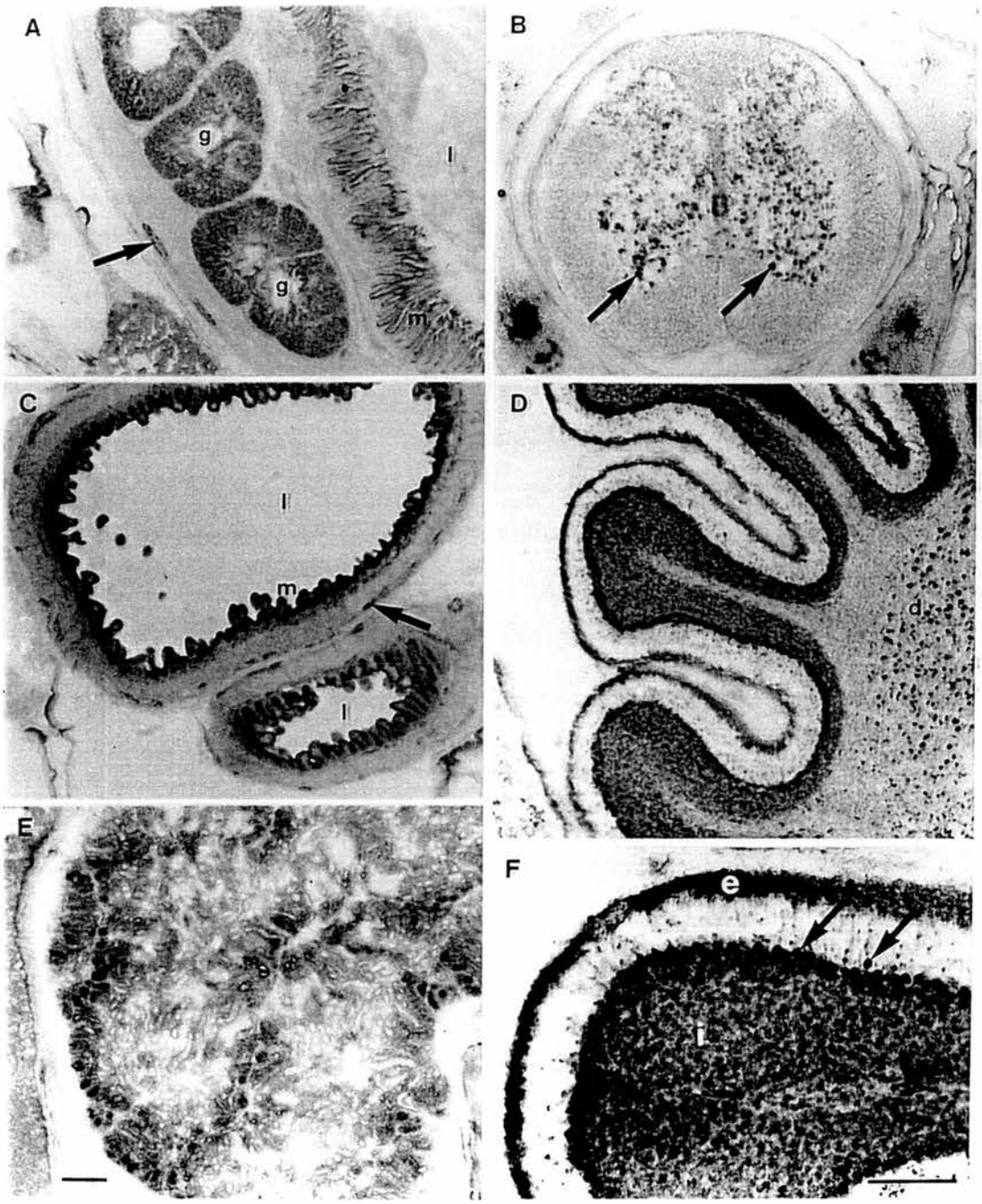
Figure 2.7. Developmental changes in GFRα-4 mRNA expression in peripheral tissues of the chicken embryo. Northern blots of total RNA from non-neuronal tissues: skin and muscle. Filters were hybridised with a GFRα-4 probe. Bands corresponding to the 3.8Kb transcript are indicated. To compare the relative levels of total RNA present in each sample an ethidium bromide stained gel of the RNA is shown.



2.3.4. Expression Analysis of GFRα-4 by *In situ* hybridistaion.

In situ hybridisation was used to localise the expression of GFRα-4 at the cellular level in the E18 chicken embryo. Prominent expression of GFRα-4 mRNA was observed in several neuronal populations of the developing CNS. This included neurons of both the dorsal and the ventral gray columns of the spinal cord, the Purkinje cells, granule cells, and neurons of the deep nuclei of the cerebellum. The pattern of hybridisation in the intestine was consistent with expression of GFRα-4 mRNA in the myenteric plexus and mucosal epithelium. Prominent expression was also observed in the proventricular glands of the stomach and in the developing kidney. No staining was observed in sections hybridised with a sense probe (Figure 2.8).

Figure 2.8. Localisation of GFRα–4 mRNA in tissue sections of E18 chick embryos by *in situ* hybridisation. (A) Section through the wall of the proventriculus of the stomach showing prominent labeling of the proventricular glands (p), mucosa (m), and ganglia of the myenteric plexus (arrow). The lumen of the proventriculus (l) lies to the right. (B) Transverse section through the lumbar spinal cord showing labeled neurons throughout the gray matter, although labeling is more intense among the motorneurons of the anterior gray column (arrows). (C) Section through the intestine showing prominent labeling in the mucosa and ganglia of the myenteric plexus (arrow). (E) Section through the kidney showing labeling in the developing renal tubules and glomeruli. (D and F) Sections through the cerebellum showing very prominent labeling of Purkinje cells (arrows) and neurons of the deep cerebellar nuclei (d). Granule cells in the external granular layer (e) and internal granular layer (i) are also prominently labeled. Bars, 200μm (the bar in E applies to A-E).



2.4. Discussion.

In this chapter the cloning and characterisation of chicken GFRα-1, GFRα-2 and GFRα-4 is presented, as well as the expression of GFRα-4 during embryonic development. Members of the chicken GFRα receptor family have been identified on the basis of their sequence homology to mouse GFRα-1, by screening embryonic brain cDNA libraries. At the time this work was carried out the sequence of only GFRα1 in the mouse was known. Although homologues of chicken GFRα-1 and GFRα-2 have since been isolated in rat, mouse, and human (Jing*et al* 1996; Treanor *et al.*, 1996; Klein *et al.*, 1997; Baloh *et al.*, 1997), no mammalian homologue of chicken GFRα-4 has so far been reported. In contrast, the third member of the receptor family, GFRα-3, has only been cloned in mouse (Jing *et al.*, 1997; Worby *et al.*, 1998; Nomoto *et al.*, 1998; Masure *et al.*, 1998). Comparison of the primary amino acid sequence of chicken GFRα-1, GFRα-2 and GFRα-4 with mouse GFRα-1, GFRα-2 and GFRα-3 reveal that these proteins form a novel family of structurally related receptors. The nucleotide sequences of chicken GFRα-1 and GFRα-2 predict proteins of 469 and 466 amino acids respectively. Chicken GFRα-4 predicts a 431 amino acid protein that is more closely related to GFRα-1 and GFRα-2 than to GFRα-3. Chicken GFRα-1 and GFRα-2 share ~48% amino acid identity between them, and whereas GFRα-4 shares ~40% amino acid identity with both mouse and chicken GFRα-1 and GFRα-2, it shares only 27% amino acid identity with mouse GFRα-3. All members of the GFRα receptor family display features that are characteristic of GPI-linked proteins including an N-terminal, hydrophobic, putative signal peptide for secretion and a C-terminal hydrophobic domain separated by a hydrophilic linker region from a cleavage/binding consensus sequence for GPI-linkage.

From the sequence alignment of the GFRα receptors shown in the results, it can be seen that the relative spacing of the cysteine residues is conserved between each protein. Of the 30 cysteines that are conserved between mouse and chicken GFRα-1 and GFRα-2, 28 are conserved in mouse GFRα-3 and chicken GFRα-4. This indicates that GFRα receptors have similar 3-dimensional structures. Regions of similarity between the primary amino acid sequences of these receptors are shown in discrete segments throughout the central portion of the protein. It has been proposed that these represent 3 cysteine rich domains each ~100 amino acids long which are bound together by hinge regions and are necessary for the correct folding and 3-dimensional conformation of the protein (Suvanto *et al.*, 1997).

Recent *in vitro* and *in vivo* experiments have clearly shown that the GDNF family of ligands mediate their cellular responses through a multi-component receptor complex comprising a shared Ret tyrosine kinase component and one of the four GFRα receptors. Furthermore, it has been established that the GFRα receptors play a key role in ligand discrimination. Analysis of the interaction of GDNF and neurturin with GFRα-1 and GFRα-2 *in vitro* has yielded conflicting data. Initial studies in cell lines not expressing Ret suggested that GDNF only interacted with GFRα-1 and neurturin only interacted with GFRα-2. However, in the presence of Ret, GFRα-2 can also bind GDNF if it is present at sufficiently high concentrations (Sanicola *et al.*, 1997). Similarly, sympathetic neurons expressing exogenous GFRα-2 can survive in the presence of GDNF, although neurturin is more effective at promoting their survival (Buj-Bello *et al.*, 1997). GDNF and neuturin both support the *in vitro* survival of mouse embryonic nodose and parasympathetic neurons. Comparison of the survival of these neurons from GFRα-1 and wildtype mice strongly suggest that neurturin promotes the *in vitro* survival of

nodose neurons by activating GFRα-1, and GDNF promotes the *in vitro* survival of parasympathetic neurons by activating GFRα-2 (Cacalano *et al.*, 1998). However, more recent experiments with sensory neurons from GFRα-$2^{-/-}$ mice demonstrate that GFRα-2 is the primary receptor for neurturin (Rossi *et al.*, 1999). Furthermore, analysis of GFRα-1, GFRα-2 and GFRα-3 mRNA expression in the trigeminal ganglion of wild type and $GDNF^{-/-}$ mice revealed a marked decrease in GFRα-1 positive neurons, but no change in the number of neurons expressing GFRα-2 and GFRα-3. This suggests that GFRα-1 is the only physiologically relevant GFRα receptorfor GDNF in trigeminal neurons (Naveilhan *et al.*, 1998)

The preferred receptors for artemin and persephin have recently been identified as GFRα-3 and GFRα-4, respectively (Baloh *et al.*, 1998a; Enokido *et al.*, 1998). However, the specificity of ligand interaction with GFRα-3 and GFRα-4 has not been thoroughly studied and remains to be established. Comparison of GFRα-3 and artemin and GFRα-4 and persephin knockout mice when they are generated will be very informative.

The analysis of GFRα-4 mRNA expression by northern blotting has revealed that the gene is widely expressed in both neuronal and non-neuronal tissue of the developing chicken. A single 3.8Kb transcript is expressed in most tissues. In the chicken CNS, GFRα-4 mRNA expression is found in all of the regions analysed. At E10, the spinal cord expresses the highest level of GFRα-4 mRNA, followed by lower levels in cerebellum, midbrain, medulla oblongata and pons, and very low levels in the forebrain. Similarly, the level of GRFα-1 mRNA is also higher in the spinal cord than in the brain of E10 chicken embryos (Buj-Bello *et al.*, 1997). Interestingly, GFRα-4 mRNA appears to be expressed at higher levels than in other regions of the CNS throughout the period E6-E18. Consistent with this, *in-situ* hybridization

revealed that the GFRα-4 transcript is localised to motoneurons of the ventral grey column in the chick spinal cord at E18. GFRα-1 and GFRα-2 mRNAs have also been reported to be expressed at high levels in the ventral region of the developing mouse spinal cord throughout development. (Golden *et al.*, 1999). However, in the adult spinal cord, GFRα-2 transcripts appear to be located in the dorsal horn (Klein *et al.*, 1997). In the cerebellum, GFRα-4 mRNA shows a marked increase in expression from E10-E18. Accordingly, *in situ* hybridization reveals strong labelling of the GFRα-4 transcript in Purkinje cells, granule cells and neurons of the deep cerebellar nuclei at E18. In contrast, mouse GFRα-1 and GFRα-2 mRNAs are prominantly expressed in the cerebellum throughout development (Golden *et al.*, 1999). In the hindbrain, GFRα-4 mRNA expression shows a developmental increase from E10-E18. GFRα-3 has a more distinctive expression pattern compared to GFRα-1, GFRα-2 and GFRα-4 with a limited distribution in the CNS. By *in situ* hybridization, GFRα-3 mRNA has been detected in the thalamic parafascicular nucleus, the amygdalohippocampal anterolateral nucleus, and the medial preoptic nucleus of the hypothalamus (Trupp *et al.*, 1998; Worby *et al.*, 1998; Baloh *et al.*, 1998a).

Northern blot analysis of GFRα-4 mRNA expression in non-neuronal tissues of the chick embryo at E10 revealed high level expression in the kidney, with the presence of an additional 4.5Kb transcript. Moderate levels of expression were detected in the lung, intestine, skin and skeletal muscle, with barely detectable expression in the heart and liver. In contrast, northern analysis of GFRα-1 and GFRα-2 mRNA expression at this age in the chick embryo revealed the highest levels of expression in the intestine and liver, respectively (Buj-Bello *et al.*, 1997). *In situ* hybridization revealed the expression of GFRα-4 mRNA in the myenteric plexus and mucosal epithelium

of the E18 embryonic chicken. The most prominent expression was observed in the proventricular glands of the stomach and in the developing kidney. In agreement with these findings, RT/PCR analysis of expression carried out by a colleague Epaminondas Doxakis has shown high expression of GFRα-4 mRNA throughout development in the intestine and kidney (Thompson *et al.*, 1998).

The expression of GFRα-4 mRNA was also analysed at successive stages of development in non-neuronal tissues of the developing chick. Northern blotting revealed a gradual increase in GFRα-4 expression in skin from E6 to E18 and a marked decrease in expression in muscle from E6 to E18. RT/PCR analysis has also revealed the same developmental changes in the expression of GFRα-4 in skeletal muscle and skin in the embryonic chick (Thompson *et al.*, 1998). Northern blot analysis of GFRα-1 and GFRα-2 mRNA expression in skin has shown the same gradual increase from E6 to E18 (Buj-Bello *et al.*, 1997).

Several studies have revealed the widespread overlapping and complementary expression pattern of Ret and the GFRαmRNAs in both neuronal and non-neuronal tissues. During embryogenesis, the expression of Ret mRNA has been found in several region of the CNS, including the mesencephalon and pons, the glomerular layer of the olfactory bulb, thalamus, hypothalamus, cerebellum, and the ventral horn of the spinal cord (Trupp *et al.*, 1998, Golden *et al.*, 1999). Within the PNS, Ret mRNA has been found in the dorsal root, trigeminal, sympathetic and enteric ganglia (Yu *et al.*, 1998; Tsuzuki *et al.*, 1995). Ret mRNA has also been detected in several non-neuronal tissues, including the liver, kidney, intestine, spleen, thymus, ovary, lung and lymph nodes (Tsuzuki *et al.*, 1995).

To explore the relationship between Ret and the GRFα's in the formation of specific receptors for GDNF family ligands, several studies have

analysed the co-expression of these receptors. Ret appears to follow a similar pattern of expression as the GFRα receptors throughout development. For example, in the developing chick embryo, Ret and the GFRα receptors show a complementary change in expression in the brain, muscle and intestine with age (Thompson *et al.*, 1998). In agreement with the expression of GDNF mRNA in the striatum of the developing rat, both GFRα-1 and Ret mRNA show expression in the same area (Nosrat *et al.*, 1997). Ret and GFRα-1 mRNA have also been co-localised to cells of *Auerbach's plexus* and *Meissner's plexus*, with complementary expression of GDNF in the surrounding muscle layers of the intestine in developing rat (Nosrat *et al.*, 1997). Furthermore, complementary expression of GFRα-1, Ret, and GDNF has been observed in the epithelial buds of the Wolffian duct branches and peripheral structures of the developing rat kidney (Nosrat *et al.*, 1997). The complementary patterns of expression seem to support the suggested function of Ret as a signal transducing protein mediating GDNF actions via GFRα-1 (Durbec *et al.*, 1996; Jing *et al.*, 1996).

Although in general the expression of Ret and the GFRα's appear to be complementary throughout development, there are some notable exceptions. For example, high levels of GFRα-1 mRNA expression in the adult rat have been found in areas where there is no Ret expression, such as neuronal populations of the lateral geniculate nucleus, superior colliculus, and extensive regions of the cerebral cortex (Trupp *et al.*, 1997). Similarly, the expression of GFRα-4 mRNA is most prominent in the kidney at E10 and E18 of the developing chick embryo. However, it has been shown that the expression of Ret in the chick embryo is very low at the same stages of development (Thompson *et al.*, 1998).

Expression of the GFRα receptors in the absence of Ret raises the possibility that these receptors may interact with as yet unidentified Ret-like

signal transducing molecules. The GFRαreceptors could act *in trans* to mediate ligand signalling via Ret expression on neighboring cells. It is also possible they may have roles of their own existing as membrane bound or truncated soluble forms that regulate the availability of GDNF ligands in the extracellular space.

CHAPTER 3.

CLONING AND EXPRESSION OF A PUTATIVE MOUSE GFRα-1 SPLICE VARIANT.

3.1. Introduction.

GFRα-1 was initially identified in the rat and has since been cloned in several other species (Jing *et al.*, 1996; Treanor *et al.*, 1996; Buj-Bello *et al.*, 1997). Equilibrium and competition binding studies and experiments examining phosphorylation of Ret in response to ligand binding have indicated that GFRα-1 is the preferred receptor for GDNF. The high affinity binding of GDNF to GFRα-1 mediates Ret dimerisation and autophosphorylation (Jing *et al.*, 1996; Treanor *et al.*, 1996). Within the CNS, GFRα-1 mRNA is expressed in motoneurons of the hypoglossal and facial nuclei, dopaminergic neurons of the subtantia nigra and the ventral tegmental area, the reticular and ventro medial nucleus of the thalamus, the hypothalamus, hippocampus, zona incerta, amygdala and retina. In the PNS, GFRα-1 mRNA is expressed in Schwann cells and trigeminal, superior cervical, enteric and dorsal root ganglia (Nosrat *et al.*, 1997; Yu *et al.*, 1998; Golden *et al.*, 1999). In non-neuronal tissues, GFRα-1 mRNA has been detected in the kidney, heart, muscle, intestine, stomach and liver (Nosrat *et al.*, 1997; Sanicola *et al.*, 1997; Yu *et al.*, 1998). The demonstration that GFRα-1 and Ret form a functional receptor for GDNF and also for neurturin in some cell types together with expression studies showing the widespread distribution of GFRα-1 throughout the nervous system suggests an important role for this receptor during development.

The existence of splice variants of various growth factor receptors such as TrkA, TrkB, TrkC and the fibroblast growth factor receptors has been

clearly demonstrated (Barker *et al.*, 1993; Meakin *et al.*, 1997; Tsoulfas *et al.*, 1996). These alternatively spliced isoforms of the Trks, show differences in specificities and affinities to their cognate ligands (Strohmaier *et al.*, 1996; Tsoulfas *et al.*, 1996; Ninkina *et al.*, 1997; Baxter *et al.*, 1997). Two groups have identified variant isoforms of GFRα-1: RETL1 in human and GDNFß in mouse (Sanicola *et al.*, 1997; Dey *et al.*, 1998). These variant isoforms have been shown to carry a deletion in exon five of the gene (Angrist *et al.*, 1998). In contrast to Trk splice variants, RETL1 binds GDNF with a similar high affinity to normal GFRα-1 and phosphorylates Ret efficiently (Sanicola *et al.*, 1997).

In this chapter the cloning of a novel variant of mouse GFRα-1 (GFRα-1 sv) homologous to human RETL1 is described. To extend previous data on RETL1 expression, the expression of GFRα-1sv mRNA has been determined in neuronal and non-neuronal tissues of the developing and adult mouse. In addition a preliminary functional analysis of the GFRα-1sv receptor is described.

3.2. Methods and Materials.

3.2.1. Screening of a cDNA library.

Screening of an E13 mouse trigeminal ganglion cDNA library for mouse GFRα-4 led to the identification of the mouse GFRα-1sv. An 800bp region of the chicken GFRα-4 cDNA was used as a probe to screen the cDNA library and was carried out as described in section 2.2.2. Conditions for titering, plating and transfer of the bacteriophage were as before. However, screening of the library was carried out at medium stringency, with prehybridisation at 58°C, followed by hybridisation at 60°C, and subsequent washing at 60°C. Positive recombinant cDNA clones isolated from the tertiary screen were rescued as described in section 2.2.2.d.

3.2.2. Analysis of Recombinant Plasmids.

Following isolation of the recombinant clones from the bluescript phagemid, cultures were prepared for small scale (Qiagen) and large scale (PEG Precipitation) plasmid extraction, which was carried out as described in sections 2.2.3.a) and b). Plasmid DNA was then analysed by restriction digestion and Southern blot hybridisation, carried out as described in section 2.2.3. c) and d). Southern blot hybridisation conditions were prehybridisation at 58°C for 20 minutes and hybridisation at 58°C for 4 hours. The primary structures of the selected cDNA clones were determined by DNA sequencing as described in (section 2.2.3. e). None of the clones obtained from screening the library were found to be mouse GFRα-4, however, a cDNA clone encoding GFRα-1sv was identified. GFRα-1sv was sequenced on both strands with overlapping primers.

3.2.3. Polymerase Chain Reaction.

DNA sequencing of the mouse GFRα-1sv cDNA clone revealed that a small region of the 5' end was absent. To obtain the remaining 5' end of the mouse GFRα-1sv cDNA, a forward primer was designed to the known 5' nucleotide sequence of normal mouse GFRα-1 and a reverse primer to the region of the splice site. Using first strand cDNA already prepared from adult mouse heart, amplification was carried out using the primers SV1 5' CACGCTGAGCTCTCTCCGCGAG 3' and SV2 5'GTTCCACTGATATGAACGGGAC 3'. A 50µl reaction mix containing: 5µl cDNA, 1 x PCR Buffer (Promega), 2.5mM $MgCl_2$ (Promega), 0.1M dNTPs (Helena Biosciences), 15pmol SV1 and SV2, and 5 units of *Taq* Polymerase was set up and overlaid with 50µl of mineral oil (Gibco-BRL). Amplification reactions were carried out in a Hybaid Omnigene thermal cycler. The PCR cycling parameters for primers SV1 and SV2 were as follows: denaturing at 95°C for 45 seconds, annealing at 60°C for 45 seconds, extension at 72°C for 1 minute (40 cycles).

3.2.4. Expression Analysis by Reverse Transcription Polymerase Chain Reaction.

3.2.4. a) Total RNA Extraction of Mouse Tissues.

Various neuronal and non-neuronal tissues were dissected from embryonic, post natal and adult CD1 mice. These tissues were immediately frozen and stored at -80°C until use. Total RNA was extracted using guanidium isothiocyanate by a single step method (Chomczynski *et al.*, 1987) as described in section 2.2.4 b). Following ethanol precipitation, the RNA

pellets were resuspended in an appropriate volume of dH_2O depending on the size of each RNA pellet.

3.2.4. b) Reverse Transcription / Polymerase Chain Reaction.

First strand cDNA was synthesised by reverse transcription of total RNA using random hexanucleotides (Promega). A 50µl reaction mix was setup containing: 1µg of total RNA, 1 x Superscript RT buffer (Gibco -BRL), 0.5mM dNTPs (Helena Biosciences), 10µM random hexanucleotides (Promega), 10µM DTT and 200 units of RNase inhibitor. The RT reaction mix was overlaid with 50µl of mineral oil before being heated to 90°C for 3 minutes (to remove RNA secondary structure) and being allowed to cool to 37°C. When the reaction had reached 37°C, 2µl of Superscript reverse transcriptase was added and the reaction was incubated at 37°C for 1 hour. Once first strand synthesis was complete, cDNA from each reaction tube was amplified using the primers SV3 5'-ATTCCGGGCAGTCCCGTTCAT-3' and SV4 5'-AGGTGTCATCCAGGTTGCAGG-3'. PCR was carried out in a 50µl reaction containing the following: 5µl cDNA, 1 x PCR Buffer (Promega), 2.5mM $MgCl_2$ (Promega), 0.1M dNTPs (Helena Biosciences), 15pmol SV3 and SV4, and 5 units of *Taq* Polymerase. The PCR mix was overlaid with 50µl of mineral oil (Gibco-BRL). Amplification reactions were carried out in a Hybaid Omnigene thermal cycler. After denaturing at 90°C for 2 minutes, 29 cycles consisting of denaturing at 90°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1 minute 30 seconds were carried out. A final extension at 72°C for 10 minutes was carried out. The amplification products for GFRα-1 and GFRα-1sv were 107bp and 92bp respectively. These were resolved by agarose gel electrophoresis.

3.2.4. c) Agarose Gel Electrophoresis.

The amplification products of GFRα-1 and GFRα-1 splice variant were analysed using ethidium bromide stained 4% LMP agarose gels (Biomax), prepared as described in section 2.2.1. b.i).

3.2.5. Microinjection of mouse GFRα-1sv.

3.2.5.a) Preparation of neuronal cell cultures and microinjection.

To investigate whether GFRα-1sv showed altered ability to mediate a survival promoting signal to GDNF and neurturin compared to full length GFRα-1, microinjection was used to introduce pRc/cmv or pMex expression plasmids containing mouse GFRα-1sv cDNA, plus Ret cDNA (from Arnon Rosenthal) or mouse GRFα-1 cDNA plus Ret cDNA into SCG neurons (work carried out in collaboration with Luzia Pinon). Previous studies have shown that sympathetic neurons of the SCG of P4 mice are not naturally responsive to either GDNF or neurturin (Buj-Bello *et al.,* 1997). Therefore the survival of microinjected neurons in the presence of GDNF and neurturin reflects the efficacy of GFRα-1sv in binding these ligands and participating in transducing a signal into the cell.

SCG were dissected from P4 CD1 mice. The ganglia were dissociated and the neurons were separated from non-neuronal cells by differential sedimentation. The resultant neuronal suspensions were plated on 60mm diameter dishes (Nunc, Gibco) pre-coated with poly-DL-ornithine (0.5mg/ml, in 0.15M borate buffer, over night at RT) and laminin (20μg/ml, in F-14 medium, 4 hours at 37°C). A grid containing nine 4mm x 4mm squares was

enscribed onto the bottom of the dishes with a scapel blade. The cell density plated was 100-150 neurons per grid. The neurons were cultured for 12 hours with 2ng/ml NGF in a defined medium containing Ham's F-14 supplemented with glutamine (containing pathyocyte-4-BSA, putrescine, progesterone, L-thyroxine, sodium selenite, tri-iodothyronine). After 12 hours in culture, the neurons were washed 3 times with F12 +10% heat-inactivated horse serum (HIHS) to remove the NGF. The neurons were then microinjected with the appropriate vector at a concentration of 100µg/ml into their nuclei (approximately 100 cells for each condition). The neuronal cultures were then supplemented with GDNF or neurturin at concentrations of 5ng/ml or 50ng/ml. After 3 hours post-injection the injected and non-injected neurons were counted. By this time, any neurons that were likely to die from the effects of the injection would have done so. The number of surviving neurons was counted after 48 hours in culture. Survival is expressed as a percentage of the number of injected neurons minus non-injected neurons.

3.2.5. b) Cloning of Mouse GFRα-1sv into PRc/cmv Expression Vector.

A 2.2kb ApaI/XbaI mouse GFRα-1sv cDNA fragment from the bluescript clone was subcloned into the expression vector PRc/cmv that had been linearised with ApaI/XbaI. The ligation reaction was prepared as follows: 1µl of 10xT4 DNA ligase Buffer, 1µl GFRα-1sv cDNA fragment (50ng), 2µl ApaI/XbaI linearised PRc/cmv vector (10ng), 5µl dH_20 and 1µl T4 DNA ligase (New England Biolabs). The ligation reaction was incubated at 4°C overnight. The ligation product was transformed into E. Coli XL1 cells and grown in culture. 100-200µl of this was spread on LB agar plates containing 100µg/ml and incubated overnight at 37°C. Selected clones were incubated and grown as 10ml cultures. Plasmid DNA was purified using a Qiagen mini

prep kit, and digested with the restriction enzymes ApaI/XbaI. Constructs for GFRα-1 and Ret were prepared previously. Each construct was diluted to a concentration of 100μg/ml for microinjection.

3.3. Results.

3.3.1. Full length Sequence of Mouse GFRα–1sv.

An E13 mouse trigeminal ganglion cDNA library was screened at medium stringency using an 800bp region of the chicken GFRα-4 cDNA as a probe, with the intention of isolating a full length mouse GFRα-4 cDNA. From the primary screen, fifteen plaques were found to hybridise with the chicken GFRα-4 probe. Of the fifteen clones, four were chosen for further analysis. Rescue of the four clones from the bacteriophage into bluescript was carried out by *in vivo* excision. On sequencing, three of the four clones obtained were found to correspond to mouse GFRα-1, the remaining clone corresponded to mouse GFRα-1sv.

The two DNA strands of the mouse GFRα-1sv clone were sequenced in the 5' to 3' direction. This revealed a 267bp region of the cDNA clone was absent at the 5' end. To obtain the remaining 5' sequence forward and reverse primers were designed to amplify an overlapping cDNA. The forward primer was designed to recognise a region of the 5' untranslated sequence of normal mouse GFRα-1, and the reverse primer was designed bind to the splice site of the deleted region GFRα-1sv.

The nucleotide sequence of the 5'to3' strand is shown in (Figure 3.1). The length of the GFRα-1sv cDNA is ~1.4Kb. It has a short 5' region which is followed by a single ORF which extends from nucleotide 77 to the termination codon TAG at nucleotide position 1469. This is followed by a short 3' untranslated region.

Figure 3.1. The 5' to 3' mouse GFRα-1sv cDNA sequence. The open reading frame is shown in uppercase and nucleotide numbers are denoted on the right.

```
cacgctgagctctctccgcgagatccggtggcggctttggattttggggg     50
ggcggggaccagctgcgcggcggcaccATGTTCCTAGCCACTCTGTACTT    100
CGTGCTGCCACTCCTGGATTTGCTGATGTCGGCCGAGGTGAGTGGTGGGG    150
ACCGCCTGGACTGTGTGAAAGCCAGTGATCAGTGCCTGAAGGAACAGAGC    200
TGCAGCACCAAGTACCGCACACTGAGGCAGTGCGTGGCGGGCAAGGAAAC    250
CAACTTCAGCCTGACATCCGGCCTCGAGGCCAAGGATGAGTGCCGCAGCG    300
CTATGGAGGCCTTGAAGCAGAAGTCTCTCTACAACTGCCGCTGCAAGCGG    350
GGCATGAAGAAAGAGAAGAATTGTCTGCGTATCTACTGGAGCATGTACCA    400
GAGCCTGCAGGGAAATGACCTACTGGAAGATTCCCCATACGAGCCGGTTA    450
ACAGCAGGCTGTCAGATATATTCCGGGCAGTCCCGTTCATATCAGTGGAA    500
CACATTTCCAAAGGGAACAACTGCCTCGATGCAGCCAAGGCCTGCAACCT    550
GGATGACACCTGCAAGAAGTACAGGTCCGCCTACATCACCCCCTGTACCA    600
CCAGCATGTCCAATGAAGTCTGCAACCGCCGCAAGTGCCACAAAGCCCTC    650
AGGCAGTTCTTCGACAAAGTTCCAGCCAAGCACAGCTACGGGATGCTCTT    700
CTGCTCCTGCCGGGACGTCGCCTGCACCGAGAGGCGGCGACAGACTATCG    750
TCCCTGTGTGCTCCTATGAAGAACGAGAGAGGCCCAACTGCCTGAATCTG    800
CAAGACTCCTGCAAGACAAATTACATCTGCAGATCTCGCCTTGCAGATTT    850
TTTTACCAACTGCCAGCCAGAGTCAAGGTCTGTCAGCAACTGTCTTAAGG    900
AGAACTACGCAGACTGCCTCCTGGCCTACTCGGGACTGATTGGCACAGTC    950
ATGACTCCTAACTACATAGACTCCAGCAGCCTCAGTGTGGCGCCGTGGTG   1000
CGATTGCAGCAACAGTGGCAATGACCTGGAAGATTGCCTGAAGTTTCTGA   1050
ATTTTTTTTAAGGACAATACGTGTCTCAAAAATGCAATTCAAGCCTTTGGC   1100
AATGGCTCGGATGTGACCATGTGGCAACCAGCCCCCCCAGTCCAGACCAC   1150
CACTGCCACGACTACCACTGCCTTCCGGATCAAGAACAAGCCTCTAGGGC   1200
CAGCAGGCTCTGAGAATGAGATTCCCACACACGTTTTACCACCGTGTGCT   1250
AATTTGCAGGCACAGAAGCTGAAATCCAATGTATCGGGCAGTACACATCT   1300
CTGTCTTTCTGATAATGATTACGGAAAGGATGGTCTCGCTGGTGCCTCCA   1350
GCCACATAACCACAAAATCAATGGCTGCTCCTCCCAGCTGCGGTCTGAGC   1400
TCACTGCCGGTGATGGTGTTCACCGCTCTGGCTGCCCTGTTGTCTGTATC   1450
ATTGGCAGAAACATCGTAGctgcatccagtatga                   1484
```

3.3.2. Protein Alignments of mouse GFRα-1, mouse GFRα-1sv and RETL1.

To allow a comparison to be made of the mouse and human splice variants with mouse GFRα-1 the amino acid sequences of each protein were aligned. Mouse GFRα-1 and GFRα-1sv encode predicted proteins of 468 and 463 amino acids respectively. RETL1 encodes a protein of 460 amino acids.

Sequence comparison of GFRα-1sv with RETL1 the human homologue of this splice variant, and full length mouse GFRα-1 reveal that the mouse and human splice variants both have a deletion of 5 amino acids in exon 5 of the gene. The mouse GFRα-1 and GFRα-1sv amino acid sequences otherwise are identical. Both GFRα-1sv and RETL1 display the characteristic features of GPI-linked proteins including, the N-terminal, hydrophobic putative signal peptide, the C-terminal hydrophobic domain and the putative binding/cleavage consensus sequences for GPI linkage.

Figure 3.2. Aligned protein sequences of mouse GFRα-1, GFRα-1sv, and RETL1. Conserved cysteines are shown in red. Amino acids in GFR α-1 and RETL1 that are identical to those in GFRα-1sv are shown in blue. The five amino acids that are missing are highlighted in yellow. The N-terminal, hydrophobic putative signal peptides are underlined in green and the C-terminal hydrophobic domain is underlined in red, and the putative binding/cleavage consensus sequences for GPI linkage are enclosed in red boxes.

```
mGFRα1 MFLATLYFVLPLLDLL--MSAE-VS---GGDRL-------DCVKASDQCLKEQSCSTKYR   47
mGFRsv MFLATLYFVLPLLDLL--MSAE-VS---GGDRL-------DCVKASDQCLKEQSCSTKYR   47
RETL1  MFLATLYFALPLLDLL--LSAE-VS---GGDRL-------DCVKASDQCLKEQSCSTKYR   47

mGFRα1 TLRQCVAGKETNFSLTSGLEA-KDECRSAMEALKQKS-LYNCRCKRGMKKEKNCLRIYWS  105
mGFRsv TLRQCVAGKETNFSLTSGLEA-KDECRSAMEALKQKS-LYNCRCKRGMKKEKNCLRIYWS  105
RETL1  TLRQCVAGKETNFSLASGLEA-KDECRSAMEALKQKS-LYNCRCKRGMKKEKNCLRIYWS  105

mGFRα1 MYQSL-QGNDLLEDSPYEPVNSRLSDIFRAVPFISDVFQQVEHISKGNNCLDAAKACNLD  164
mGFRsv MYQSL-QGNDLLEDSPYEPVNSRLSDIFRAVPFIS-----VEHISKGNNCLDAAKACNLD  159
RETL1  MYQSL-QGNDLLEDSPYEPVNSRLSDIFRVVPFIS-----VEHIPKGNNCLDAAKACNLD  159

mGFRα1 DTCKKYRSAYITPCTTSMS-NEVCNRRKCHKALRQFFDKVPAKHSYGMLFCSCRD--VAC  221
mGFRsv DTCKKYRSAYITPCTTSMS-NEVCNRRKCHKALRQFFDKVPAKHSYGMLFCSCRD--VAC  216
RETL1  DICKKYRSAYITPCTTSVS-NDVCNRRKCHKALRQFFDKVPAKHSYGMLFCSCRD--IAC  216

mGFRα1 TERRRQTIVPVCSYEER-ERPNCLNLQDSCKTNYICRSRLADFFTNCQPESRSVSNCLKE  280
mGFRsv TERRRQTIVPVCSYEER-ERPNCLNLQDSCKTNYICRSRLADFFTNCQPESRSVSNCLKE  275
RETL1  TERRRQTIVPVCSYEER-EKPNCLNLQDSCKTNYICRSRLADFFTNCQPESRSVSSCLKE  275

mGFRα1 NYADCLLAYSGLIGTVMTPNYIDSSSLSV--APWCDCSNSGNDLEDCLKFLNFFKDNTCL  338
mGFRsv NYADCLLAYSGLIGTVMTPNYIDSSSLSV--APWCDCSNSGNDLEDCLKFLNFFKDNTCL  333
RETL1  NYADCLLAYSGLIGTVMTPNYIDSSSLSV--APWCDCSNSGNDLEECLKFLNFFKDNTCL  333

mGFRα1 KNAIQAFGNGSDVTMW--QPAPPVQTTTAMTTTAFRIKNKPLGPAGSENEIPTHV--LPP  394
mGFRsv KNAIQAFGNGSDVTMW--QPAPPVQTTTAMTTTAFRIKNKPLGPAGSENEIPTHV--LPP  389
RETL1  KNAIQAFGNGSDVTVW--QPAPPVQTTTAMTTTALRVKNKPLGPAGSENEIPTHV--LPP  389

mGFRα1 CANLQA--QKL--KSNVSGSTHLCLSDNDYGKDGLAGAS--SHITTKSMAAPPSCGLSSL  448
mGFRsv CANLQA--QKL--KSNVSGSTHLCLSDNDYGKDGLAGAS--SHITTKSMAAPPSCGLSSL  443
RETL1  CANLQA--QKL--KSNVGSNTHLCISNGNYEKEGL-GAS--SHITTKSMAAPPSCGLSPL  443

mGFRα1 PVMVFTALAALLSVSLAETS        468
mGFRsv PVMVFTALAALLSVSLAETS        463
RETL1  LVLVVTALSTLL--SLTETS        460
```

3.3.3. RT/PCR analysis of mouse GFRα-1 and GFRα-1sv mRNA expression in developing and adult mouse.

The developmental expression patterns of mouse GFRα-1 and GFRα-1sv were examined by RT/PCR in various tissues of embryonic (E14 and E18), post natal (P4) and adult mice. Total RNA was prepared from tissues of the CNS and peripheral organs as described 3.2.3. The amount of RNA was standardised in each tube by diluting the RNA pellets in an appropriate amount of dH_2O. The concentration of RNA and first strand cDNA was measured by spectrophotometry. To distinguish between the GFRα-1 and GFRα-1sv cDNA fragments, primers were designed to span the deleted region in the open reading frame.

GFRα-1 expression was observed in all tissues examined at all ages, whereas GFRα-1sv mRNA was found predominantly in peripheral organs. At E14, relatively high levels of GFRα-1 mRNA were detected in the spinal cord, hindbrain and stomach, with relatively lower levels in the cerebellum, liver, intestine, skin, forebrain, midbrain, lung, kidney and heart. Only GFRα-1 mRNA was present in the spinal cord, midbrain, hindbrain, and cerebellum. Whereas both GFRα-1 and GFRα-1sv mRNAs were present in the other tissues examined. In tissues expressing both GFRα-1 and GFRα-1sv the relative levels of each transcript were approximtely the same (Figure 3.3). At E18, only the full length GFRα-1 mRNA was expressed in the CNS. Although GFRα-1 expression in the CNS appeared to be higher than in peripheral tissues, caution must be exercised in interpreting the data from such non-quantitative RT/PCR assays. The expression of both GFRα-1 and GFRα-1sv mRNAs were detected in the liver, lung, heart, intestine, stomach, skin and kidney. However, the expression of GFRα-1sv in the liver heart and stomach was significantly less than GFRα-1 (Figure 3.3). The expression pattern was

similar at P4, only GFRα-1 mRNA was expressed in the CNS, whereas both GFRα-1 and GFRα-1sv mRNAs were detected in the heart, liver, intestine, stomach, skin, kidney and lung. Interestingly, in heart, the level of the GFRα-1sv transcript was higher than GFRα-1 (Figure 3.4). Although the full length GFRα-1 mRNA was the predominant transcript detected in the adult CNS, the forebrain, midbrain, hindbrain and cerebellum also expressed low levels of GFRα-1sv mRNA. Both GFRα-1 and GFRα-1sv mRNAs were expressed in the liver, stomach, intestine, lung, heart, skin and kidney.

Figure 3.3. Representative picture of RT/PCR analysis of GFRα-1 and GFRα-1sv mRNA expression in the central nervous system and various peripheral organs of E14 and E18 mouse. Amplified GFRα-1 and GFRα-1sv transcripts correspond to the RT/PCR products of 107bp and 92bp respectively.

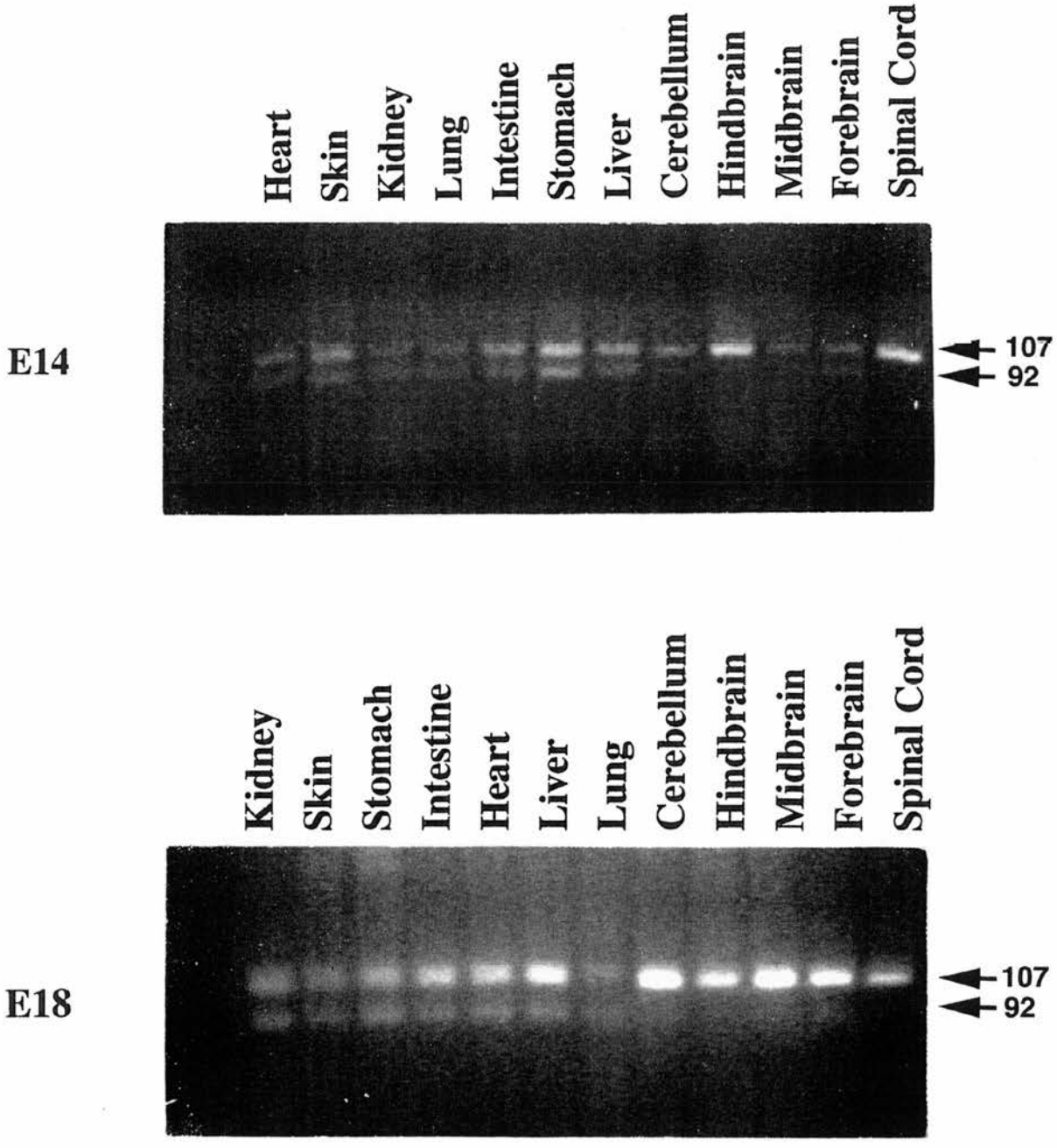
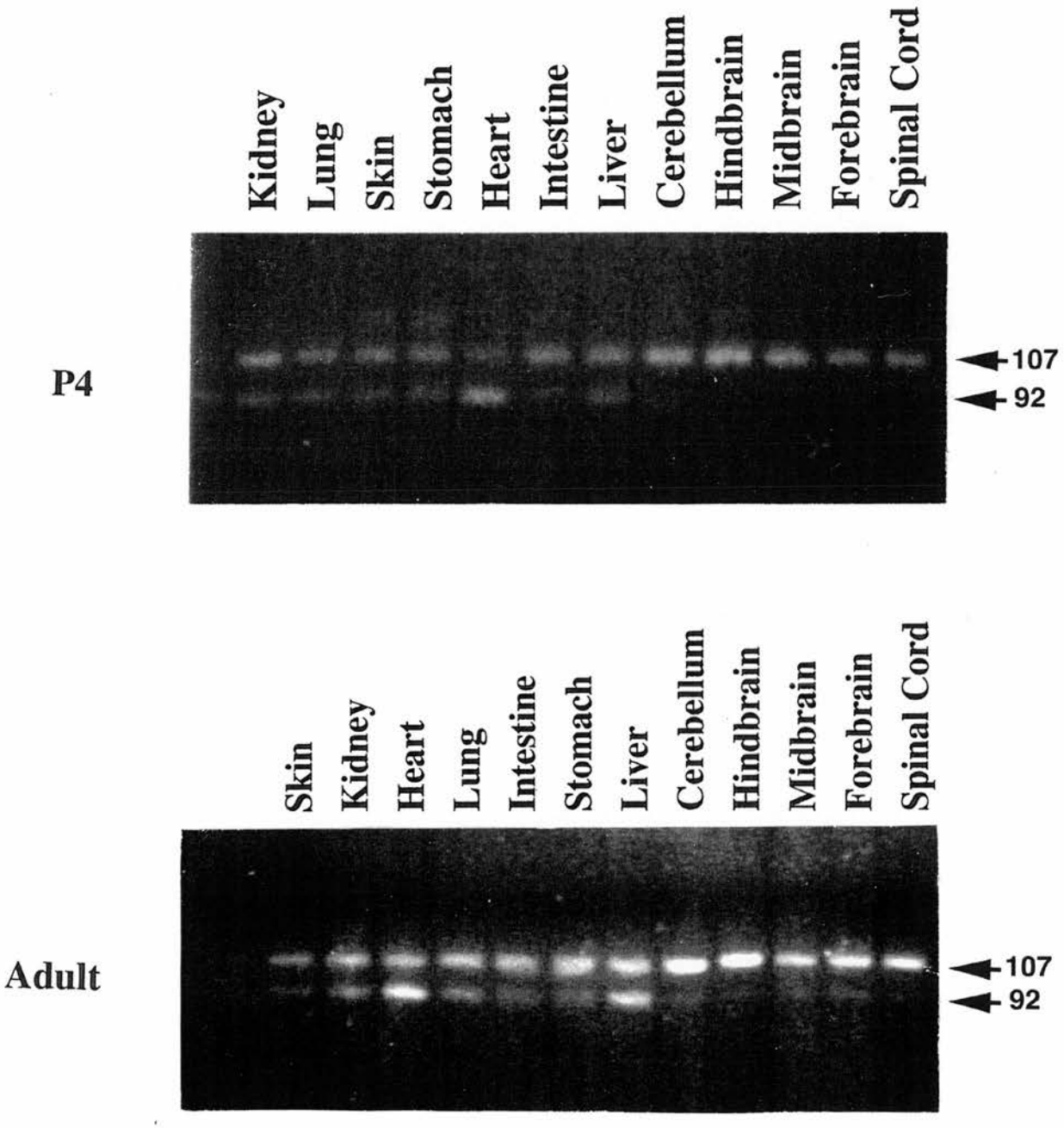


Figure 3.4. Representative picture of RT/PCR analysis of GFRα-1 and GFRα-1sv mRNA expression in the central nervous system and various peripheral organs of P4 and Adult mouse. Amplified GFRα-1 and GFRα-1sv transcripts correspond to the RT/PCR products of 107bp and 92bp respectively.



3.3.4. Ectopic Expression of GFRα-1sv and Ret on SCG Neurons.

To investigate functional differences between GFRα-1 and GFRα-1sv, low density dissociated cultures of P4 mouse SCG neurons were established. These neurons were microinjected with expression plasmids containing GFRα-1 plus Ret or GFRα-1sv plus Ret. The ectopic expression of Ret and GFRα-1 in SCG neurons had a negligible effect on the number of neurons surviving in medium without added factors (Figure 3.5 and 3.6).

Approximately 2% of SCG ectopically expressing GFRα-1 and Ret survived in the absence of added trophic factors. Neurons co-expressing GFRα-1 plus Ret in the presence of 5 ng/ml GDNF showed a significantly enhanced survival response of approximately 15% compared to these control cultures ($p< 0.01$, t-test) (Figure 3.5). However, neurons co-expressing GFRα-1 plus Ret showed significantly greater neuronal survival compared to neurons co-expressing GFRα-1sv and Ret ($p = 0.05$, t-test) (Figure 3.5).

Approximately 10% of SCG neurons co-expressing GFRα-1 plus Ret survived in cultures supplemented with 5ng/ml neurturin (Figure 3.6). Unlike GDNF supplemented neurons co-expressing GFRα-1sv plus Ret, which showed a reduced survival response compared to those co-expressing GFRα-1 plus Ret, neurturin supplemented neurons co-expressing GFRα-1sv plus Ret showed a significantly enhanced neuronal survival compared to neurturin supplemented cultures co-expressing GFRα-1 plus Ret (Figure 3.6) ($p= 0.05$, t-test).

These results were obtained from preliminary microinjection experiments investigating the functional differences between GFRα-1 and GFRα-1sv. However further experiments using appropriate controls will be necessary, such as SCG neurons injected with empty vector, or GFRα-1 and Ret alone, grown in the presence of GDNF and neurturin.

Figure 3.5. Bar chart showing the percent survival of P4 mouse SCG neurons 48hrs after microinjection with expression plasmids for GFRα-1, GFRα-1sv and Ret grown either with or without 5ng/ml GDNF. ** $p< 0.01$, * $p= 0.05$.

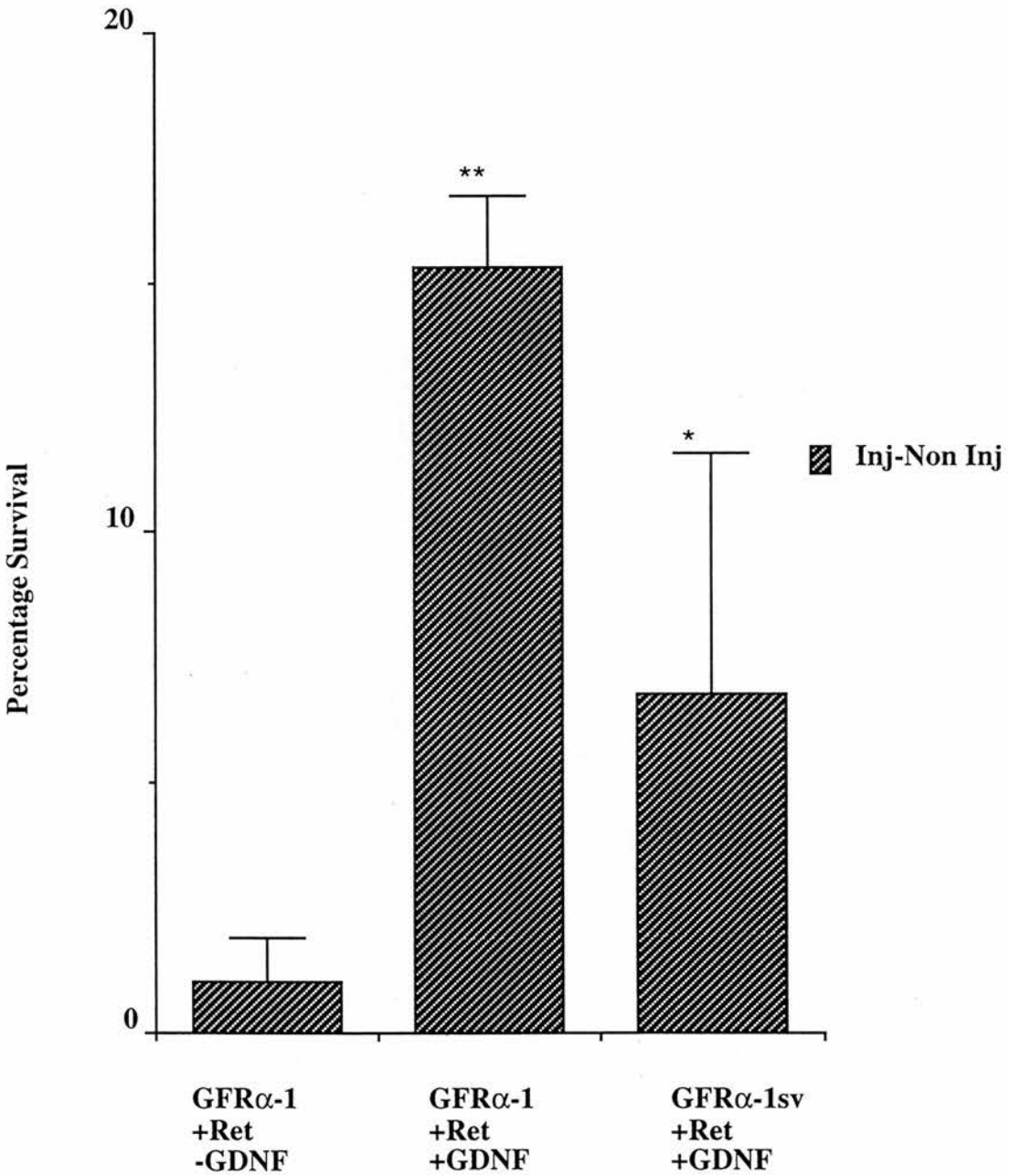
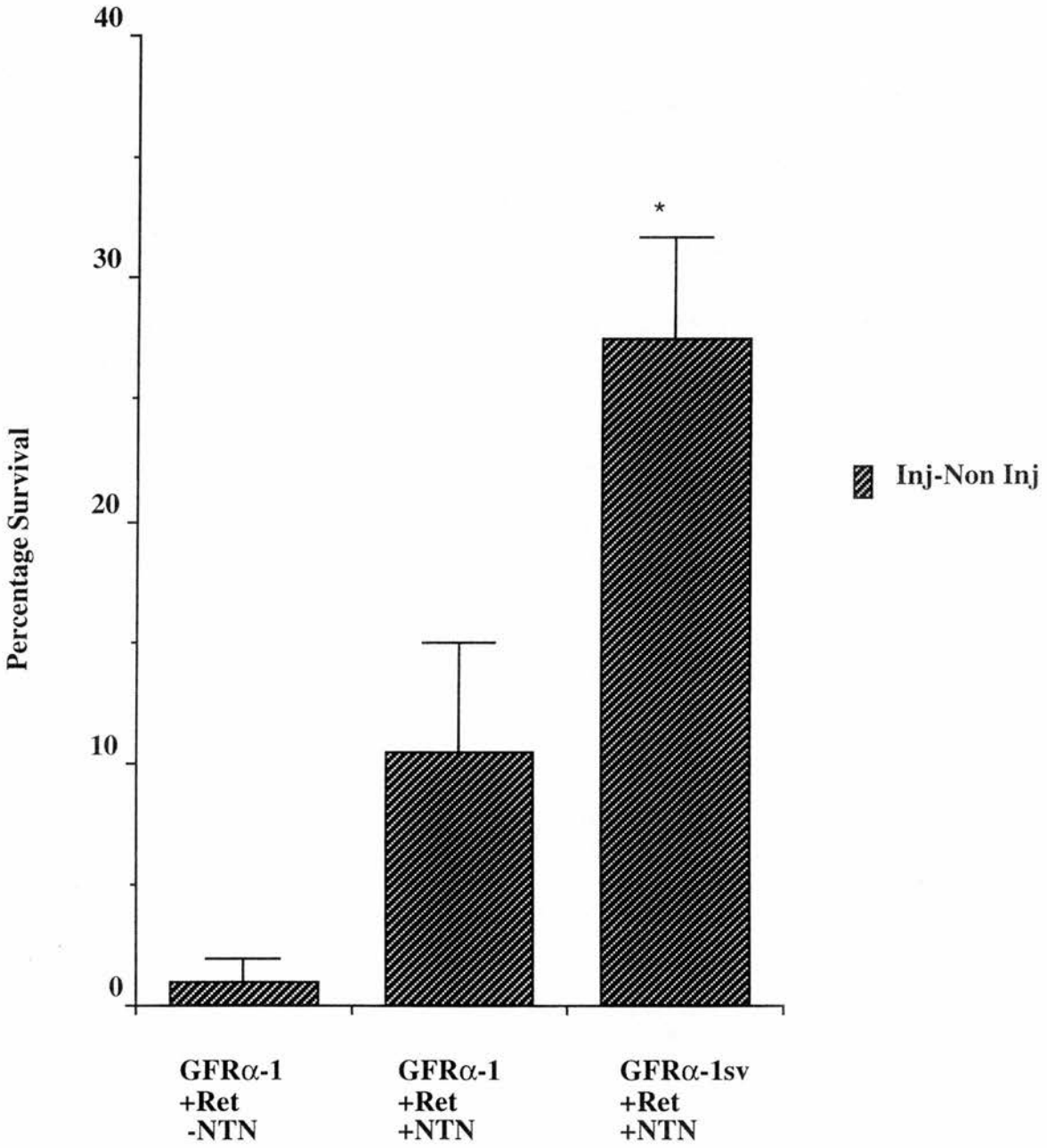


Figure 3.6. Bar chart showing the percent survival of P4 mouse SCG neurons 48hrs after microinjection with expression plasmids for GFRα-1, GFRα-1sv and Ret grown with or without 5ng/ml neurturin. * p= 0.05.



3.4. Discussion.

This chapter reports the cloning and characterisation of a novel mouse GFRα-1 isoform, GFRα-1sv. The GFRα-1sv receptor is homologous to the recently identified human RETL1 and mouse GDNFRβ (Sanicola *et al.,* 1997; Dey *et al.*, 1998). Sequence alignment of GFRα-1sv and RETL1 with mouse GFRα-1 reveal a deletion of 5 amino acids in the same region of each ORF from amino acid position 139 to 144. The nucleotide sequence of GFRα-1sv encodes a protein of 463 amino acids that shares 94 ~% amino acid identity with RETL1 and ~98% identity with GFRα-1.

To examine the expression of GFRα-1sv in the developing and adult mouse, total RNA from neuronal and non-neuronal tissues was analysed by RT/PCR. GFRα-1sv and GFRα-1 transcripts were co-expressed in nearly all peripheral tissues including, liver, stomach, intestine, heart, lung, kidney and skin. Interestingly, high levels of GFRα-1sv mRNA were expressed in the heart of P4 and adult mice compared with lower levels of GFRα-1 mRNA. In the heart it appears that the expression of GFRα-1sv mRNA increases relative to the full length GFRα-1mRNA with age. Consistent with this pattern of expression, Northern blotting has shown that RETL1 mRNA displays a marked increase in the rat heart with age, reaching its highest level of expression in the adult (Sanicola *et al.*, 1997). GDNF and neurturin mRNAs are not expressed in the heart at this stage (Golden *et al.*, 1999). The high levels of GFRα-1sv mRNA expression suggest a potentially important role for this receptor in the development of the heart. However the lack of GDNF and NTN mRNA expression in this organ raises the possibility that GFRα-1sv may bind to an unidentified ligand or be functionally redundant. The phenotype of GFRα-3 null mice suggests that this receptor may be functionally redundant in some ganglia of the PNS. Although GFRα-3 shows widespread expression in

the developing and adult PNS, the only peripheral ganglia ganglia displaying cell loss in GFRα-3$^{-/-}$ mice is the SCG (Nishino *et al.*, 1999). It is possible of course that GFRα-3 signalling effects subtle changes rather than neuronal survival in these other ganglia. In contrast to the expression of GFRα-1mRNA in the postnatal heart, very low levels of GFRα-1sv and GFRα-1 mRNA expression were observed in the lung at E18. In accordance with these findings, northern blotting and *in situ* hybridisation have revealed very low levels of RETL1 and GFRα-1 expression in the lung at E18 respectively (Yu *et al.*, 1998; Sanicola *et al.*, 1997). In the mouse CNS, expression of GFRα-1sv mRNA was very limited. Low levels of the GFRα-1sv transcript were observed in the forebrain and cerebellum at E14, and in the forebrain, midbrain, hindbrain, and cerebellum of the adult. In contrast, RETL1 mRNA is expressed at high levels throughout all regions of the human brain except for the cerebellum (Sanicola *et al.*, 1997). These differences in the localisation of GFRα-1sv transcripts in the CNS may reflect different roles for GDNF and neurturin in the developing brains of humans and rodents. Since GFRα-1sv can transduce signals from neurturin so well (see below), perhaps its human homologue partially replaces the function of GFRα-2 in the human CNS. A comparison of the relative expression of the variant isoforms of GFRα-1 and GFRα-2 in human and mouse may be informative in this respect.

To compare directly the ability of GFRα-1sv and GFRα-1 to bind GDNF and neurturin and transduce a survival-promoting signal through Ret, P4 SCG neurons ectopically expressing either GFRα-1 and Ret or GFRα-1sv and Ret were cultured in the presence of GDNF or neurturin. Whereas GFRα-1sv was less effective than GFRα-1 in mediating the survival promoting effect of GDNF, GFRα-1sv was more effective than GFRα-1 in mediating the survival promoting effects of neurturin. One interpretation of this is that

GFRα-1sv binds GDNF with a lower affinity and neurturin with a higher affinity than GFRα-1. Previous studies have demonstrated that the binding of GDNF to both GFRα-1 and GFRα-2 is competable by GDNF and neurturin, but the binding of neurturin to these receptors is only affected by excess neurturin (Jing *et al.*, 1997). This suggests the presence of two distinct binding sites in the GFRα receptors: one that binds only neurturin and another that can harbour both GDNF and neurturin. In GFRα-1sv it is possible that the GDNF binding site has been disrupted by the 5 amino acid deletion and the disrupted site has increased affinity for neurturin, as reflected by the increased neurturin promoted survival of cell expressing GFRα-1sv. Previous studies investigating the ability of GDNF and neurturin to promote the survival of P4 SCG neurons ectopically co-expressing GFRα-1 and Ret and GFRα-2 and Ret, respectively (Buj-Bello *et al.*, 1997; Enokido *et al.*, 1998), have reported a higher percentage of surviving than in the preliminary experiments described in this chapter. Therefore, further experiments will be necesssary to fully elucidate the survival response conferred by ligand binding to GFRα-1sv. However, the preliminary results presented here suggest that GFRα-1sv binds neurturin more effectively than it binds GDNF. GDNF and neurturin both support the survival of early embryonic nodose neurons *in vitro* (Buj-Bello *et al.*, 1995; Kotzbauer *et al.*, 1996; Forgie *et al.*, 1999). However, the survival response to both factors is lost in GFRα-1 deficient nodose neurons, suggesting that GFRα-1 mediates the *in vitro* survival promoting effect of neurturin (Cacalano *et al.*, 1998). It will be interesting to see whether nodose ganglion neurons express GFRα-1sv at high levels compared to other peripheral neurons.

Alternative splice variants for many growth factor receptors have been reported, including FGFR1, TrkA, TrkB, TrkC, (Tsoulfas *et al.*, 1993; Barker

et al., 1993; Wang *et al.*, 1995; Ninkina *et al.*, 1997; Meakin *et al*, 1997; Elkabes *et al.*, 1995). When alternative splicing occurs near the 5' end of a gene, ligand binding domains are often affected resulting in altered ligand specificity. For example, a splice variant of the TrkB receptor lacking in exon 9 shows reduced binding affinity for, NT-4/5 and NT-3, but not BDNF (Strohmaier *et al.*, 1996). Furthermore, a splice variant of Trk A containing an extra 18bp in the extracellular domain of the gene binds NT-3 with increased affinity (Clary and Reichardt, 1994). Additionally, splice variants of TrkB lacking either two or all three leucine rich motifs in the extracellular domain do not bind BDNF, NT-4/5 or NT-3, demonstrating that leucine rich repeats are essential for ligand binding (Ninkina *et al.*, 1997).

Splice variants of Ret and GFRα-2 have previously been reported (Ivanchuck *et al.*, 1997; Wong *et al.*, 1998). Several of the Ret isoforms result from exon skipping in the extracellular ligand binding domain. Similarly, both splice variants of GFRα-2 display deletions in the N-terminal putative ligand binding domain. As a consequence, both receptors may display altered affinities and or specificities for a particular ligand. Several studies have shown that receptor-ligand cross talk can occur between GDNF family ligands and GFRα receptors other than their preferred receptors (Jing *et al.*, 1998;Baloh *et al.*, 1997; Creedon *et al.*, 1997; Buj-Bello *et al.*, 1997; Naveilhan *et al.*, 1998; Baloh *et al.*, 1998; Trupp *et al.*, 1998). The degree of cross talk appears to depend to a certain extent on the cellular context in which GDNF family receptors are expressed. A study of GFRα-1 deficient mice has shown that in some neuronal cell types at certain developmental stages, the GFRα-1/Ret complex can quite clearly act as an effective receptor for neurturin, therefore GDNF must promote *in vitro* survival through activating a receptor complex other than GFRα-1/Ret (Cacalano *et al.*, 1998). Ligand

receptor cross talk may have important implications not just for determining the survival of certain neuronal populations but also in determining their phenotype and functional characteristics. It is possible that alternative splicing of GFRαreceptors in different neuronal populations governs the ability of these neuronal populations to respond to different ligands of the GDNF family.

CHAPTER 4.

CLONING AND EXPRESSION OF A PUTATIVE MOUSE GFRα-4.

4.1. Introduction.

Persephin is a recently identified member of the GDNF family of growth factors (Milbrandt *et al.*, 1998). In the CNS, persephin exhibits biological activities similar to GDNF and neurturin, the founder members of the GDNF family of growth factors promoting the survival of embryonic midbrain dopaminergic neurons and motoneurons and inducing branching of the ureteric bud (Henderson *et al.*, 1994; Yan *et al.*, 1995; Sanio *et al.*, 1997; Milbrandt *et al.*, 1998). However, in contrast to GDNF and neurturin, which support the survival of autonomic and sensory neurons, (Buj-Bello *et al.*, 1995; Ebendal *et al.*, 1995; Trupp *et al.*, 1995; Kotzbauer *et al.*, 1996; Klein *et al.*, 1997; Forgie *et al.*, 1999) persephin does not promote the survival of peripheral neurons (Milbrandt *et al.*, 1998). Persephin is widely expressed at low levels throughout the nervous system and has been detected within non-neuronal tissues including skeletal muscle, skin and adrenal glands (Milbrandt *et al.*, 1998; Jaszai *et al.*, 1998).

The GDNF family of ligands signal through a heteromeric receptor complex consisting of the tyrosine kinase Ret and a GPI-linked GFRα component (Jing *et al.*, 1996; Treanor *et al.*, 1996; Trupp *et al.*, 1996; Baloh *et al.*, 1997; Klein *et al.*, 1997). Ret transduces the signal whereas ligand binding and specificity are obtained through interaction with one of the GFRα receptors. GDNF and neurturin bind GFRα-1 and GFRα-2, respectively, although some cross talk can exist (Buj-Buj-Bello *et al.*, 1997; Creedon *et al.*, 1997; Baloh *et al.*, 1997; Jing *et al.*, 1998; Naveilhan *et al.*, 1998; Sanicola *et al.*, 1997; Trupp *et al.*, 1998; Worby *et al.*,

1998). Artemin, the third member of the family, binds GFRα-3 (Baloh *et al.*, 1998b)

A fourth member of the GFRα receptor family GFRα-4 has recently been cloned from chick (Thompson *et al.*, 1998; discussed in chapter 2 of this thesis) and has been shown to mediate the trophic effects of persephin in conjunction with Ret (Enokido *et al.*, 1998). This chapter describes the cloning of a putative mouse homologue of chick GFRα-4. In addition, it describes the expression of mouse GFRα-4 in various neuronal and non-neuronal tissues of the developing and adult mouse.

4.2 Methods and Materials.

4.2.1. Searching an EST Database.

To isolate mouse GFRα-4, the full length chicken GFRα-4 cDNA was used as a query sequence to perform a Basic Local Alignment Search Tool (BLAST) search of the mouse Expressed Sequence Tag (EST) database. One EST was identified and obtained from the Japanese Collection of Research Bioresources Genebank (JCRB-Genebank, accession number AU35938). The EST was a partial gene fragment of approximately 1Kb that showed significant homology to chicken GFRα-4.

4.2.2. Screening of a cDNA Library.

To isolate the remaining 5' sequence, an adult mouse brain cDNA library was screened using a 1Kb partial mouse GFRα-4 cDNA as a probe. This library was chosen because hybridisation of a Northern blot containing total RNA of various mouse tissues with chicken GFRα-4 revealed the mRNA transcript to be present in several tissues including brain.

Screening of the cDNA library was carried out as described in section 2.2.2. Serial dilutions were made of the bacteriophage library to determine its titre. Bacteriophages were plated at approximately 100,000 pfu/dish. The host bacteria chosen for the bacteriophage Lambda Zap II library was Escherichia coli MRF'. Conditions for plating and transfer of the bacteriophage were as before. However, screening of the library was carried out at high stringency, with prehybridisation at 65°C, followed by hybridisation at 65°C, and subsequent washing at 65°C. Fifteen recombinant cDNA clones isolated from the tertiary screen were rescued as described in section 2.2.2.d

4.2.3. Analysis of Recombinant Plasmids.

Analysis of the clones was carried out as described in section 2.2.3. Conditions for Southern blot hybridisation were: prehybridisation at 65°C for 20 minutes followed by hybridisation at 65°C for 6 hours. The membrane was washed briefly three times in 2xSSC at room temperature and three times in 2xSSC/0.1%SDS at 65°C for 15 minutes. The primary structures of the selected cDNA clones were determined by DNA sequencing. Five of the clones obtained were found to be mouse GFRα-4, however only one of these clones contained additional 5' sequence compared to the partial mouse GFRα-4 used to screen the library.

4.2.4. 5' Rapid Amplified cDNA Ends (RACE).

In an attempt to isolate the remaining 5' GFRα-4 sequence, 5' RACE was carried out using a mouse brain Marathon RACE library (Clontech). Three nested reverse primers were designed to an appropriate 5' region of the mouse GFRα-4 sequence RACE 1 5'-CGCTCGCAGCGCTCCAGGGGCTCCAGGCAAG-'3, RACE 2 5'-GCATTCTTCGCGCCGGTTTCCACTG-'3, and RACE 3, 5'-CCCAAGCAGGGGTTCCTTGTAAAGAGCTTGC-'3. Using the most 3' reverse primer and the adaptor primer AP1 from the Marathon RACE kit (Clontech), the first round of PCR amplification was carried out. A 50µl PCR reaction mix was set up containing: 5µl mouse brain cDNA library (Clontech), 1 x cDNA PCR Buffer (Clontech), 50mM dNTPs (Helena Biosciences), 10µM RACE 3 and AP1 primers, and 1µl 50 x Advantage 2 Polymerase Mix (Clontech). The reaction was overlain with 50µl of mineral oil (Sigma). A negative control was prepared as above in which 5µl of dH_2O was added instead of the mouse brain cDNA template. Amplification reactions were carried out in a Program thermal cycler (PTC)-100 (MJ Research), and the PCR cycling parameters for primers RACE 3 and AP1 were

as follows: an initial denaturing step at 94°C for 1 minute followed by, 40 cycles of denaturing at 94°C for 30 seconds, and extension at 68°C for 4 minutes. After the first round of amplification, 5µl of PCR product was analysed on a 1% agarose gel as described in section (2.2.1.b.i). Following this, 5µl of the first round PCR product was used as a template for a second round of nested PCR amplification with the primers RACE 2 and AP2. PCR conditions were the same as those for first round amplification. The second round PCR product was cloned into pGEM-T vector as described in section (2.2.5.b). Following transformation and small scale plasmid extraction the clone was sequenced as described in section (2.2.3.e).

4.2.5. Expression Analysis by Northern Blotting.

The expression pattern of GFRα-4 in various developing and adult mouse tissues was analysed by Northern blot hybridisation. A 1Kb mouse GFRα-4 cDNA probe was labelled with ^{32}P by nick translation and used for hybridisation (2.2.4.a). Several neuronal and non-neuronal tissues were dissected from CD1 mice including: forebrain, midbrain, hindbrain, cerebellum, liver, stomach, intestine, kidney, lung, heart and skin. Total RNA was extracted from these tissues and the samples were electrophoresed on a MOPS/formaldehyde denaturing gel. Preparation of a Northern blot to allow transfer of the RNA samples to the Hybond N^+ membrane was carried out as described in section (2.2.4.d). Conditions for hybridisation of the Northern blot membrane were prehybridisation at 42°C overnight and hybridisation at 58°C for 48 hours. This was followed by washing the membrane three times in 2 x SSC at room temperature and three times in 2 x SSC/0.2% SDS, at 58°C. The membranes were then exposed to Kodak Omat x-ray film.

4.3 Results.

4.3.1. Partial nucleotide sequence of mouse GFRα-4.

A BLAST search of a mouse EST database in the DDBJ Genebank, using the full length chicken GFRα-4 cDNA as a query, identified one EST. Alignment of this sequence to chicken GFRα-4 revealed the EST encoded a partial cDNA of approximately 1.Kb, that showed significant homology to full length chicken GFRα-4 but was missing approximately 600bp from the 5' end. To try to obtain the 5'cDNA sequence of GFRα-4, an adult mouse brain cDNA library was screened at high stringency using the partial mouse GFRα-4 as a probe. From the primary screen, sixteen plaques were found to hybridise with the mouse GFRα-4 probe. Eight were chosen for further analysis. On sequencing, one of the clones was found to be an overlapping cDNA that corresponded to GFRα-4. However, the region of overlap was only approximately 30 amino acids. 5' RACE was used to try and isolate the remaining 5' sequence. PCR amplification yielded a 1.3Kb DNA fragment that was cloned in pGEM-T vector. On sequencing, the cloned insert was found not to be mouse GFRα-4. While screening the mouse brain cDNA library for the remaining 5' nucleotide sequence of GFRα-4, a mouse genomic library was screened in parallel by a colleague using the 1Kb mouse GFRα-4 cDNA as a probe. From the genomic clones isolated, DNA sequencing revealed an additional 300bp of 5' GFRα-4 nucleotide sequence.

The overlapping clones corresponding to GFRα-4 were sequenced on both DNA strands. This revealed a cDNA of ~0.95Kb, of which the nucleotide sequence is shown in the 5' to 3' direction (Figure 4.1). Relative to the length of the other GFRα nucleotide sequences, the 5' sequence starts approximately 0.4Kb into the ORF. The ORF is approximately 0.8Kb long and extends to the stop

codon TGA at nucleotide 843. The termination codon is followed by a short 3'untranslated region (Figure 4.1).

Figure 4.1. The 5' to 3' partial mouse GFRα-4 cDNA sequence. The open reading frame is shown in uppercase and nucleotide numbers are denoted on the right.

GGGTCTGCGAGCTTTACCGACGGGAATCGCTGCGTGGACGCGGCCGAGGC 50

GTGTACAGCAGACGAGCGGTGCCAGCAGCTGCGCTCTGAGTACGTGGCAC 100

GATGCCTGGGCCGGGCAGCGCCCGGGGGCAGGCCGGGACCCGGGGGCTGC 150

GTGCGCTCCCGCTGCCGCCGAGCCCTGCGCCGCTTCTTCGCGCGTGGGCC 200

TCCGGCGCTCACGCATGCGCTGCTCTTCTGCGGCTGCGAAGGCTCCGCGT 250

GCGCCGAGCGCCGGCGCCAGACTTTCGCGCCCGCCTGCGCGTTCTCCGGC 300

CCGGGGTTGGTGCCGCCCTCTTGCCTGGAGCCCCTGGAGCGCTGCGAGCG 350

CAGCCGCCTGTGCCGGTGCGTGCGTGCGGGGCGGGCTGGGCCGGCTCACC 400

CGCGTCCGGGCGCGCGCAGGCCCCGTCTCCTTGCCTTCCAGGCCTCATGC 450

GCTCCCGCGCCCGGCTCCCGCGACCGCTGCCCGGAGGAGGGGGGCCCGCG 500

TTGTCTGCGCGTCTACGCAGGCCTCATGGGCACCGTGGTCACCCCTAACT 550

ACCTGGACAACGTGAGCGCGCGCGTTGCGCCCTGGTGCGGCTGTGCGGCC 600

AGTGGAAACCGGCGCGAAGAATGCGAAGCCTTCCGCAAGCTCTTTACAAG 650

GAACCCCTGCTTGGGTGAGGGGGCCTGGAGGTCCCGGGGAACCACGGATG 700

TCTGTGGCCCAATCAAGCTGCCTGGCCCGTGGGTCTTATTTACGTCGCAT 750

CATGTTTGGTGTGGGCTATGGACAGTGTGCACATGCCAATGGTACCTTGG 800

GTTGGAAGTTAAGCGTTTAAAACCTTGTTCCAATGGCCTTTGAaagttgg 850

cctccctttttgacacttaatgggggtgggccctttctttcccatgtggt 900

ggccccaacttaacttttggctgggtcttggccttcttgggttgggaaat 950

4.3.2. Protein Alignment of mouse GFRα-4 with mouse GFRα-1 and chicken GFRα-4.

Comparison of mouse GFRα-4 with the primary structures of mouse GFRα-1 and chicken GFRα-4 reveal significant homology between the proteins. All cysteine residues from the available sequence are conserved (Figure 4.2).

The mouse GFRα-1 and chicken GFRα-4 cDNAs share approximately 40% amino acid identity and encode proteins of 468 and 431 amino acids, respectively. From the available sequence, mouse GFRα-4 shares approximately 40% amino acid identity with chicken GFRα-1 and GFRα-4.

Figure 4.2. Aligned Protein sequences of mouse GFRα-1, GFRα-4, and chicken GFRα-4; conserved cysteines are shown in red, and amino acids in mouse GFRα-1, and chicken GFRα-4 that are identical to those in mouse GFRα-4 are shown in blue. For mouse GFRα-1 and chicken GFRα-4, the N-terminal, hydrophobic, putative signal peptides are underlined in yellow, the C-terminal hydrophobic domain is underlined in red, and the putative binding/cleavage consensus sequences for GPI linkage are enclosed in red boxes.

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GRFα1 MFLATLYFVLPLLDLL--MSAE-VS---GGDRL-------DCVKASDQCLKEQSCSTKYR   47
GRFα4 MRGILYFCTLILLEG---M-AEAVSSSR-----------DCLQAGESCTNDPICSSKFR   44
GRFα4 ------------------------------------------------------------

GRFα1 TLRQCVAGKETNFSLTSGLEA-KDECRSAMEALKQKS-LYNCRCKRGMKKEKNCLRIYWS  105
GRFα4 TLRQCIAGNGANKL---GPDA-KNQCRSTVTALLS-SQLYGCKCKRGMKKEKHCLSVYWS   99
GRFα4 ------------------------------------------------------------

GRFα1 MYQSL-QGNDLLEDSPYEPVNSRLSDIFRAVPFISDVFQQVEHISKGNNCLDAAKACNLD  164
GRFα4 IHHTLMEGMNVLESSPYEPFIRGFDYV-RLASITA---GSENEVTQVNRCLDAAKACNVD  155
GRFα4 ------------------------------------GSASFTDG-NRCVDAAEACTAD   21

GRFα1 DTCKKYRSAYITPCTTSMS-NEV-----CNRRKCHKALRQFFDKVPAKHSYGMLFCSCRD  218
GRFα4 EMCQRLRTEYVSFCIRRLARADT-----CNRSKCHKALRKFFDRVPPEYTHELLFCPCED  210
GRFα4 ERCQQLRSEYVARCLGRAAPGGRPGPGGCVRSRCRRALRRFFARGPPALTHALLFCGCEG   81

GRFα1 VACTERRRQTIVPVCSYEER---ERPNCLNLQDSCKTNYICRSRLADFFTN---------  266
GRFα4 TACAERRRQTIVPACSYE---SKEKPNCLAPLDSCRENYVCRSRYAEFQFN---------  258
GRFα4 SACAERRRQTFAPACAAFSGPGLVPPSCLEPLERCERSRLCRCVRAGRAGPAHPRPGAPR  141

GRFα1 -------CQPESRSVSNCLKENYADCLLAYSGLIGTVMTPNYIDSSSLSVAPWCDC--SN  317
GRFα4 -------CQPSLQTASGCRRDSYAACLLAYTGIIGSPITPNYIDNSTSSIAPWCTC--NA  309
GRFα4 LLAFQASCAPAPGSRDRCPEEGGPRCLRVYAGLMGTVVTPNYLDNVSARVAPWCGCRRAA  201

GRFα1 SGNDLEDCLKFLNFFKDNTCLKNAIQAFGNGSDVTMW--QPAPPVQTTTAMTTTAFRIKN  375
GRFα4 SGNRQEECESFLHLFTDNVCLQNAIQAFGNGTYLNAATA-PSISPTTQMYKQERNANRAA  368
GRFα4 SGNRREECEAFRKLFTRNPCLGEGAWRSRGTTDVCGPIKLPGPWVLFTSHHVWCGLWTVC  271

GRFα1 KPLGPAGSENEIPTHV--LPPCANLQA--QKL--KSNVSGSTHLCLSDNDYGKDGLAGAS  429
GRFα4 ATLSENIFEHLQPTKVAGEERLLRGSTRLSSETSSPAAPCHQAASLLQLWLPPTLAVLSH  428
GRFα4 TCQWYLGLEVKRLKPCSNGL                                          291

GRFα1 SHITTKSMAAPPSCGLSSLPVMVFTALAALLSVSLAETS                       468
GRFα4 FMM                                                          431
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4.3.3. Expression analysis of GFRα-4 in developing and adult mouse.

Expression of GFRα-4 in the developing (E18) and adult mouse was analysed using Northern blot hybridisation. At both ages, a transcript of ~1.4Kb was present only in the CNS. However, at E18, there appeared to be an additional transcript minor of ~1.6Kb. At E18, GFRα-4 mRNA was detected in the forebrain, midbrain, hindbrain and cerebellum. Within the CNS, the midbrain and cerebellum revealed the highest levels of GFRα-4 mRNA expression, with moderate levels detectable in the forebrain and hindbrain (Figure 4.3). In contrast, GFRα-4 was barely detectable in non-neuronal tissues of the E18 embryo, including the liver, stomach, intestine, kidney, lung, heart and skin (Figure 4.3).

In the adult mouse a similar pattern of expression was observed. Within the CNS, the forebrain and hindbrain revealed high levels of GFRα-4 mRNA expression with no expression observed in the midbrain and cerebellum (Figure 4.4). GFRα-4 mRNA was barely detectable in non-neuronal tissues of the adult (Figure 4.4)

Figure 4.3. GFRα-4 mRNA expression in the E18 mouse embryo. Total RNA from neuronal and non-neuronal tissues were transferred to a filter and hybridised with the GFRα-4 probe. Bands corresponding to the 1.4Kb transcript are indicated. To compare the relative levels of total RNA present in each sample an ethidium bromide stained gel of the RNA is shown below the autoradiogram.

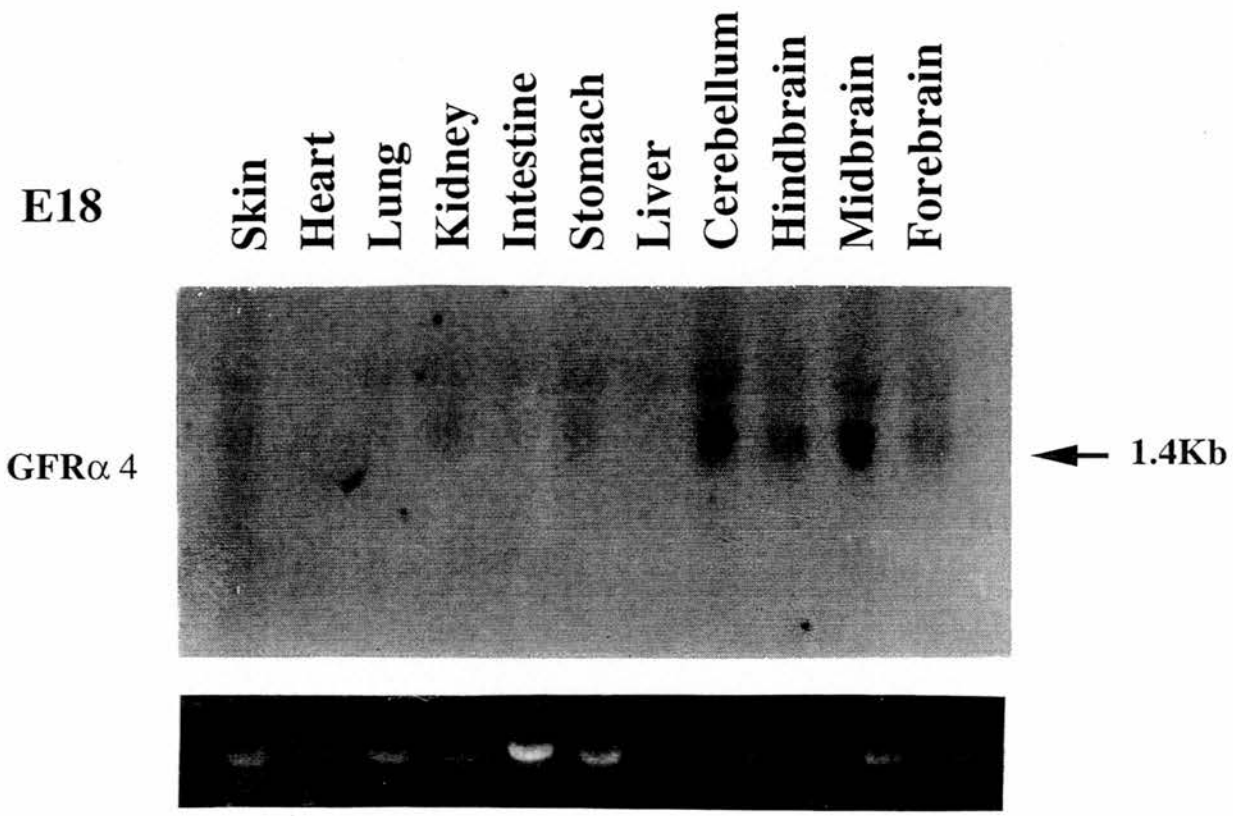
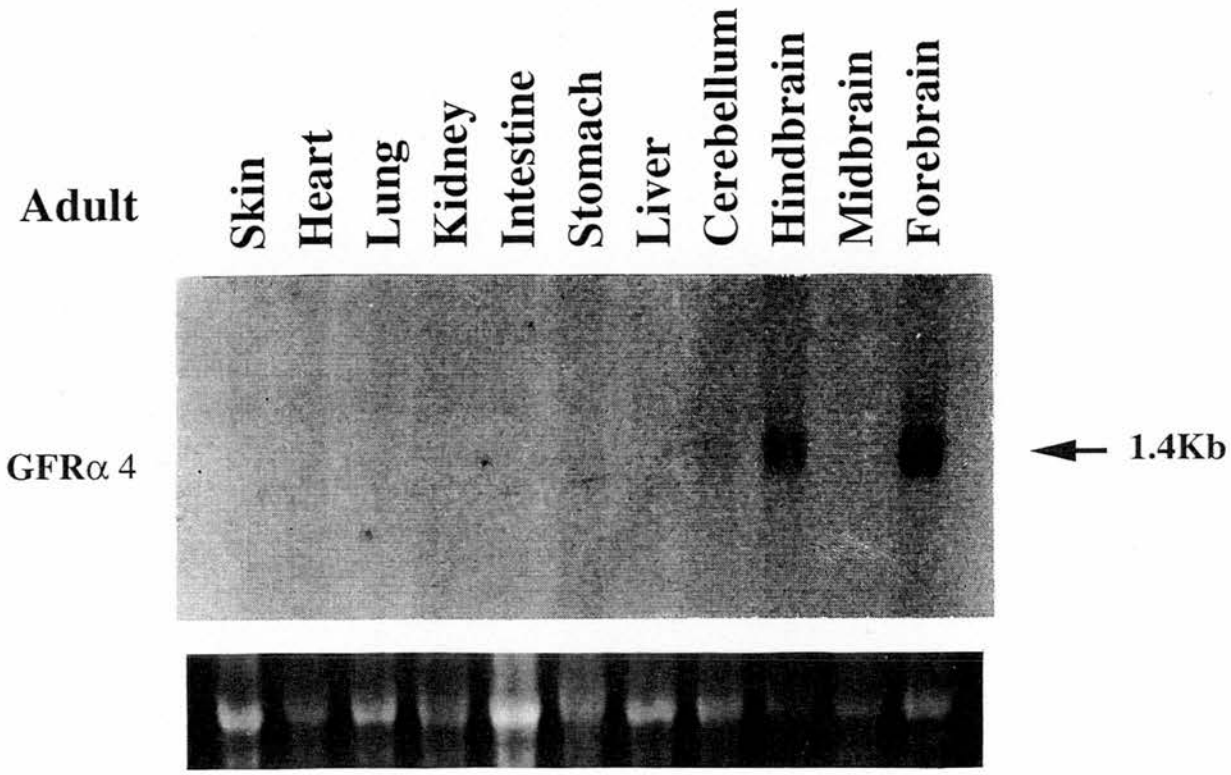


Figure 4.4. GFRα-4 mRNA expression in the adult mouse. Total RNA from neuronal and non-neuronal tissues were transferred to a filter and hybridised with the GFRα-4 probe. Bands corresponding to the 1.4Kb transcript are indicated. To compare the relative levels of total RNA present in each sample an ethidium bromide stained gel of the RNA is shown below the autoradiogram.



4.4. Discussion.

The results presented in this chapter report the cloning and initial characterisation of a partial mouse GFRα-4 cDNA. In addition, the expression of GFRα-4 mRNA has been investigated by Northern blot hybridisation in the developing and adult mouse. Mouse GFRα-4 represents a new member of the mammalian GFRα receptor family that is homologous to the recently identified chicken GFRα-4 (Thompson *et al.*, 1998, discussed in chapter 2 of this thesis). Identification of the same partial GFRα-4 cDNA has recently been reported, mapping to a homologous region on a BAC clone containing the mouse mahogany locus (Gunn *et al.*, 1999). The primary structure of the GFRα-4 protein displays similarities with the other GFRα-4 family members. From the available sequence, all cysteine residues are conserved, however full characterisation of the protein remains to be determined.

The analysis of mouse GFRα-4 expression by Northern blot hybridisation reveals that the gene has a distinctive pattern of expression, being restricted to the CNS. At E18, a transcript of approximately 1.4Kb is predominantly expressed in the midbrain and cerebellum, with lower levels in the forebrain and hindbrain. GFRα-4 mRNA is also expressed at high levels in the cerebellum of the E18 chick, although expression levels are higher in the forebrain. GFRα-4 is not the only member of the GFRα family to be expressed in the developing midbrain and cerebellum. GFRα-1 mRNA is expressed in the midbrain and cerebellum of E18 mouse embryos and the midbrain of E18 chick (Golden *et al.*, 1999; Buj-Bello unpublished data). Similarly, GFRα-2 is expressed at high levels in the midbrain of E18 chick, although its expression is limited in this region of E18 mouse (Golden *et al.*, 1999; Buj-Bello *et al.*, 1997). In contrast, analysis of the expression of GFRα-3 mRNA by in situ hybridisation has revealed limited

expression in the CNS of both developing and adult mouse (Widenfalk *et al.*, 1997).

At E18, GFRα-4 mRNA expression was barely detectable in mouse non-neuronal tissues including, liver, stomach, intestine, heart, kidney, lung and skin. This is in marked contrast to GFRα-1, GFRα-2, and GFRα-3 which are widely expressed in non-neuronal tissues (Nosrat *et al.*, 1997; Buj-Bello *et al.*, 1997; Widenfalk *et al.*, 1997; Yu *et al.*, 1998). This is also in marked contrast to the expression pattern of chick GFRα-4, which is expressed in all chick peripheral tissues throughout development (Thompson *et al.*, 1998; discussed in chapter 2 of this thesis). However, the expression of GFRα-4 mRNA at E18 in the chick was determined with the much more sensitive technique of RT/PCR. The restricted expression of GFRα-4 mRNA in the periphery of the developing mouse may therefore partly reflect the use of Northern blotting, as opposed to RT/PCR, to measure mRNA level. However, since chick GFRα-4 was readily detectable using Northern blotting in various non-neuronal tissues of the E6 chick, it does appear as if GFRα-4 is expressed at significantly lower levels in the periphery of the developing mouse compared to chick. Of course, mRNA expression levels do not necessarily reflect protein levels and it may be that mouse GFRα-4 mRNA is translated more efficiently in embryonic mouse peripheral tissues than in chick tissues. It is also possible that over the course of evolution GFRα-4-persephin signalling has become redundant in inductive and morphological processes in peripheral tissues of the mouse. Perhaps persephin can signal through an alternative GFRα receptor to GFRα-4 in some tissues. Further studies will be needed to determine whether this is the case.

In the adult mouse, high levels of GFRα-4 mRNA expression were observed in the forebrain and hindbrain. In the midbrain and cerebellum GFRα-4 mRNA expression was not detected. In contrast, *in situ* hybridisation has shown

GFRα-1 and GFRα-2 mRNA expression is widespread in adult CNS. GFRα-1 mRNA has been observed in the substantia nigra, hippocampus, dentate gyrus, superior colliculus, lateral septum, and medial habenular nucleus (Nosrat *et al.*, 1997). GFRα-2 mRNA is expressed in the cingulate cortex, dentate gyrus, zona incerta, reticular thalamic nucleus and hypothalamus (Widenfalk *et al.,* 1997). GFRα-3 mRNA has a more limited expression in the adult CNS, only being detected in the olfactory tubercle, cerebellum and hippocampus (Masure *et al.*, 1998).

GFRα-4 mRNA expression was not detectable in non-neuronal tissues of the adult mouse such as liver, stomach, intestine, heart, kidney, lung and skin. Consistent with this, GFRα-1 and GFRα-2 mRNAs were not detected in the liver, intestine, heart, lung, skin and kidney of the adult mouse by in situ hybridisation, although low levels of expression were present in the stomach (Golden *et al.*, 1999). Similarly, Northern blot hybridisation has shown GFRα-3 mRNA expression is not detectable in non-neuronal tissues of the adult mouse (Worby *et al.*, 1998; Nomoto *et al.*, 1998). The expression of chicken GFRα-4 mRNA has not been determined in the adult. Further analysis of GFRα-4 expression will be required using a more sensitive technique than Northern blotting to clarify the pattern of mRNA expression in the developing and adult mouse.

Persephin promotes the survival of midbrain dopaminergic neurons and motoneurons in vitro and has recently been shown to form a functional receptor complex with GFRα-4 and Ret (Milbrandt *et al.*, 1998; Enokido *et al.*, 1998). A recent study examining the expression of PSP mRNA by RT/PCR, has revealed widespread distribution throughout the nervous system. Persephin is expressed in all regions of the CNS analysed including: the cerebral cortex, hippocampus, striatum, diencephalon, dorsal and ventral mesencephalon, pons, medulla oblongata, cerebellum and spinal cord (Jaszai *et al.*, 1998). Recently, persephin

has been shown to be expressed both in the spinal motoneurons and to a lesser extent in the muscle, suggesting that it does not function as a classic target derived neurotrophic factor for motoneurons (Jaszai *et al.*, 1998).

Persephin mRNA is expressed in many peripheral tissues including: muscle, heart, liver, kidney and skin. However, persephin does not appear to support the survival of peripheral sympathetic and enteric neurons *in vitro*. Persephin may, however, affect other aspects of peripheral neuron physiology and phenotype. A recent RT/PCR analysis of GFRα-4 mRNA expression in chick peripheral neurons has revealed that all peripheral neurons do in fact express GFRα-4 mRNA, suggesting that persephin may indeed have some role in the development or function of peripheral neurons (N.Doxakis, personal communication). Analysis of the expression of mouse GFRα-4 in peripheral neurons may help further clarify the trophic actions of persephin during development. Furthermore, the generation of persephin and GFRα-4 knockout mice will ascertain the physiological relevance of these interactions in the nervous system.

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