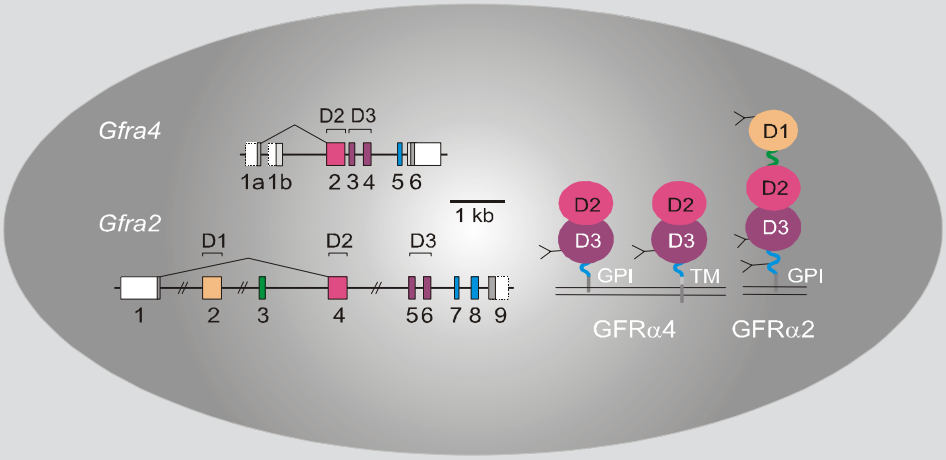

Non-Neuronal Roles for GDNF and Novel GDNF Family Receptors

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Dissertationes Biocentri Viikki Universitatis Helsingiensis

21/2004

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Academic dissertation

To be presented for public criticism, with the permission of the Faculty of Biosciences, University of Helsinki, in auditorium 1041 at Viikki Biocenter (Viikinkaari 5), on September 17th, 2004, at 12 o´clock.

Helsinki 2004

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ISBN 952-10-1942-5 (print)
ISBN 952-10-1943-3 (ethesis, PDF)
Press: Yliopistopaino, Helsinki 2004

“Bättre sent än aldrig”

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SELECTED ABBREVIATIONS

aa	amino acid
ARTN	artemin
bp	base pair
cDNA	complementary DNA
CNS	central nervous system
C-cells	clear cells
cAMP	cyclic adenosine monophosphate
CT	calcitonin
E	embryonic day
ENS	enteric nervous system
ERK	extracellular signal-regulated kinase
ES	embryonic stem cell
FMTC	familial medullary thyroid carcinoma
GDNF	glial cell line-derived neurotrophic factor
GFL	GDNF family ligand
GFRα	GDNF family receptor α
GPI	glycosylphosphatidylinositol
HSCR	Hirschsprung´s disease
kb	kilobase pair
kDa	kilodalton
K_d	equilibrium dissociation constant
KO	knockout
MAPK	mitogen-activated protein kinase
MEN2	multiple endocrine neoplasia type 2
MTC	medullary thyroid carcinoma
mRNA	messenger RNA
NRTN	neurturin
6-OHDA	6-hydroxydopamine
PCR	polymerase chain reaction
PI3-K	phosphatidylinositol 3-kinase
PI-PLC	phosphoinositide-specific pospholipase
PNS	peripheral nervous system
PSPN	persephin
PTC	papillary thyroid carcinoma
PTH	parathyroid hormone
RACE	rapid amplification of cDNA ends
RET	**re**arranged during **t**ransfection
RT-PCR	reverse transcription PCR
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFK	Src family kinase
TGF-β	transforming growth factor-β
WT	wild type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals (I-IV) and, on unpublished results presented in the text.

I **Lindahl*, M**., Timmusk*, T., Rossi, J., Saarma, M., and Airaksinen, M.S. (2000). Expression and alternative splicing of mouse *Gfra4* suggest roles in endocrine cell development. *Mol. Cell. Neurosci*. 15, 522-533.

II **Lindahl*, M.**, Poteryaev*, D., Yu., L., Arumäe, U., Timmusk, T., Bongarzone, I., Aiello, A., Pierotti, M.A., Airaksinen, M.S. and Saarma, M. (2001). Human glial cell line-derived neurotrophic factor receptor α4 is the receptor for persephin and is predominantly expressed in normal and malignant thyroid medullary cells. *J. Biol. Chem*. 276, 9344-9351.

III Hiltunen, P.H., **Lindahl, M.**, Rossi, J., Saarma, M. and Airaksinen, M.S. (2004). Ablation of persephin receptor GFRα4 impairs calcitonin production in young mice. Submitted manuscript.

IV Meng, X., **Lindahl*, M.**, Hyvönen*, M.E., Parvinen, M., de Rooij, D.G., Hess, M.W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., Pichel, J.G., Westphal, H., Saarma, M., and Sariola, H. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* 287, 1489-1493. (Supplementary material for publication IV, http://www.sciencemag.org/feature/data/1046816.shl, web Figures 1-5).

*Equal contribution to the publication

Cover image

Structure of the mouse *Gfra4* gene (top, left) compared to the mouse *Gfra2* gene (bottom, left). Exon colors refer to the homologous Cys-rich domains (D1-D3) and hinge regions in the domain model. N- and C-terminal signal sequences are in grey. Proposed domain structures of the mouse GFRα4 (middle) and GFRα2 receptor (right) (Airaksinen et al., 1999; Leppänen et al., 2004).

ABSTRACT

The glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) comprise four related molecules GDNF, neurturin (NRTN), persephin (PSPN) and artemin (ARTN) that support the survival of many neurons *in vitro* and show different biological actions on distinct neuronal populations *in vivo*. Outside the nervous system, GDNF is crucial for the development of the embryonic kidney. Secreted GFLs activate the transmembrane tyrosine kinase receptor RET through a GPI-linked co-receptor GFRα. GDNF binds preferentially to GFRα1, NRTN to GFRα2, ARTN to GFRα3 and PSPN to chicken GFRα4. Activating mutations in the *RET* gene cause tumors in organs of neuroendocrine origin such as the human medullary thyroid and adrenal medulla. Inactivating mutations in *RET* are associated with Hirschsprung´s disease or congenital megacolon.

In this work, we have characterized the mouse and human GFRα4 receptor that, upon PSPN binding activates Ret. In mouse, *Gfra4* is predominantly expressed in endocrine organs, namely the thyroid, adrenal and pituitary glands. Tissue specific alternative splicing of the mouse *Gfra4* gene produces functional GPI-anchored GFRα4 isoforms mainly in juvenile thyroid gland. In human, *GFRA4* is expressed together with *RET* in thyroid calcitonin producing C-cells, and in medullary thyroid carcinomas (MTC) originating from C-cells. This suggests that GFRα4 may be involved in the pathogenesis of MTC, as expression of other GFRα-receptors is absent in these tumors.

We generated GFRα4-deficient (-/-) mice which are viable and fertile and show no obvious defects in the nervous system or other organs. However, although the number of C-cells is normal in *Gfra4*-/- mice compared to wild-type littermates, calcitonin production is significantly reduced in young but not adult *Gfra4*-/- mice compared to wild-type littermates. It has been shown that extracellular calcium affects intracellular pathways leading to calcitonin production by activating G-protein coupled Ca-sensing receptor in C-cells. Thus, GFRα4/Ret signaling may be important for calcitonin production possibly through the Ca-sensing receptor. Calcitonin is used therapeutically to treat diseases, such as osteoporosis, characterized by increased bone resorption. As the functional GFRα4 continues to be expressed in adult human C-cells, GFRα4 may be relevant in osteoporosis.

GDNF is produced by Sertoli cells, and its receptors GFRα1 and Ret by spermatogonia in the testis. To investigate the roles for GDNF in the testis, we produced transgenic mice overexpressing GDNF specifically in testis. We also analyzed spermatogenesis in testes from heterozygous GDNF-deficient mice. Overexpression of GDNF favored self-renewal of spermatogenic stem cells resulting in depletion of spermatids, whereas low dosage of GDNF in GDNF+/- mice resulted in depletion of undifferentiated stem cells. Therefore we concluded that the GDNF dosage in testis is crucial for the regulation of cell fate decision of undifferentiating spermatogonia. The heterozygous GDNF-deficient and overexpressing transgenic mouse lines may thus serve as useful tools for studying the pathogenesis of male infertility. Furthermore, signaling pathways activated by GDNF may act as potential targets for developing male contraceptives.

Key words: GFRα4, GDNF, PSPN, Ret, thyroid, MTC, calcitonin, testis, spermatogenesis

1. REVIEW OF THE LITERATURE

1.1 Introduction to neurotrophic factors

The development of the nervous system is a complex process involving numerous proteins implicated in cell fate decision of neural progenitors, migration of neural crest cells to proper targets and axonal guidance etc. The ectodermally derived neural tube forms the central nervous system, including the brain, the spinal cord, motor neurons and the neural pituitary in the developing embryo. Neural crest cells are derived from the embryonic dorsal-most part of the neural tube. Following epithelial to mesenchymal transition the neural crest cells migrate to their destined places in the developing embryo to generate the peripheral nervous system (PNS), facial cartilage and bone, dentine of teeth, melanocytes in the skin, connective and smooth muscle tissue of large arteries from the heart, neuroendocrine cells such as chromaffin cells of the adrenal medulla and thyroid parafollicular cells (C-cells) and enteric neural cells (Gilbert, 2003). The fate of the precursor neural crest cells is in large determined by the cues of the environment and soluble factors secreted at their potential targets. The eventual location of the neural crest precursor cell is largely determining the fate of the cell. After commitment to a neuronal fate, the neuron ceases to divide and differentiate through axonal outgrowth to specific targets, and formation of synaptic connections, finally undergoing a period of programmed cell death.

Neurotrophic factors control several vitally important functions in the nervous system, both during development and in the mature brain. The most important and best studied roles of the neurotrophic factors are the control of neuronal number and stimulation of neurite growth, the functions through which they were initially discovered. In the development of almost every neuronal population, there is a period of programmed death, during which a significant portion of the initially generated neurons die (reviewed by Oppenheim, 1991). Thus, in most regions of the nervous system, the neurons are initially overproduced, but during the period of programmed death, the number is reduced to correspond to the actual requirement of a given tissue. 20-80% of the initial neurons die during the programmed death period, corresponding generally to the time of target field innervation. In fact, during the programmed death period, the target tissues control their own innervation density by regulating the number of surviving neurons, especially in the peripheral nervous system (sympathetic and sensory ganglia), but also in the motor neurons. The neurons are basically apoptotic during programmed death period and tend to commit suicide by default. Neurotrophic factors, produced from target tissues, neutralize the apoptotic program, thereby rescuing the neurons from death. By the current target-derived neurotrophic factor model (Barde, 1989), the target tissues produce neurotrophic factors in limiting amounts that are sufficient to support only a fraction of the neurons seeking to innervate the tissue, whereas the neurons remaining without neurotrophic support, die by default. Thus, by producing suitable levels of neurotrophic factors, the

tissues maintain alive only that number of neurons that is required for their innervation (reviewed by Davies, 2003; Huang and Reichardt, 2001). Neurotrophic factors secreted by target tissues, bind to their receptors on the nerve terminals, are endocytosed and carried to the somae (reviewed by Campenot and MacInnis, 2004), where they actively block the ongoing apoptotic program. The activated receptors trigger survival pathways and suppress various pro-apoptotic proteins (c-Jun, Bax, Bim, DP5 etc.) (reviewed by Kaplan and Miller, 2000; Putcha and Johnson, 2004). Different neurotrophic factors support survival of distinct, but partially overlapping populations of peripheral neurons, in addition to motor neurons (reviewed by Henderson et al., 1998). The importance of neurotrophic factors in the control of neuronal number is best proven in knockout mice of neurotrophins or their receptors, where the main phenotype was mostly the death of expected populations of peripheral ganglion neurons during the programmed death period (reviewed by Snider, 1994). Neurotrophic factors are indeed important in the induction and guidance of (at least) peripheral axons. In the central nervous system, the survival-promoting and neuritogenic functions of neurotrophic factors are not as obvious as in the peripheral ganglia, as evidenced by essentially mild brain phenotypes in the knockout mice (Snider, 1994). Thus, in the brain, neurotrophic factors appear to be involved in the differentiation and functional modulation of the neurons, rather than in survival. Indeed, neurotrophins have been shown to have both acute effects on synaptic transmission and plasticity, and long-term effects on synapse formation and function (reviewed by Lu, 2003) and have been shown to be important in the pathophysiology of mood disorders including major depressive disorder and bipolar disorder (reviewed by Hashimoto et al., 2004). Thus, neurotrophic factors seem to have a role in all main functions of the nervous system. Recent detailed *in vitro* and *in vivo* investigations have revealed important additional functions for neurotrophic factors in non-neural tissues and diseases (reviewed by Sariola, 2001).

Three families of polypeptides namely the neurotrophins, the neurokines and the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) are traditionally classified as neurotrophic factors because they were initially described as factors acting on neuronal survival and growth. However, other growth factors such as fibroblast growth factors (FGF), insulin-like growth factors (IGF), members of the transforming growth factor-β family (TGF-β), stem cell factor (SCF) and several other growth factors have in culture been shown to have neuronal survival promoting effects (Arumäe et al., 1997; Barde, 1989). Most neurotrophic factors are synthesized as precursor pre-pro proteins with a consensus secretory signal sequence which is cleaved in the endoplasmatic reticulum (ER) (Lessmann et al., 2003). Additional modifications occur in the Golgi where the pro-domain is cleaved. The neurotrophic factors are transported in vesicles to the cell membrane where they are released into the extracellular space. Most of the biologically active neurotrophic factors act as homodimers either by binding to single- or multicomponent receptors located on the

same cell (autocrine) or on neighbouring cells (paracrine mode of action). The signaling components are usually transmembrane tyrosine or serine/ threonine kinase receptors that autophosphorylate themselves upon growth factor binding and thus initiate intracellular signaling cascades leading to immediate, early and late responses in the cell.

The prototypic neurotrophic factor, nerve growth factor (NGF), was identified 50 years ago as a factor that induced neuronal growth (Levi-Montalcini, 1952). Brain-derived neurotrophic factor (BDNF) isolated from pig brain in the beginning of 1980´s was shown to be highly homologous to NGF. Shortly thereafter the family of neurotrophins was expanded to include two structurally and functionally similar members, neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5) (reviewed by Huang and Reichardt, 2001; Sofroniew et al., 2001). The neurotrophins share a common transmembrane low affinity receptor ($p75^{NTR}$) but use different members of the Trk (tropomyosin-related receptor kinase) family tyrosine kinase receptors for high affinity binding and signaling. NGF is the preferred ligand for TrkA, BDNF and NT-4 for TrkB and NT-3 for TrkC. The $p75^{NTR}$ receptor, belonging to the family of tumor necrosis factor receptors (TNFR), can induce death in both neuronal and non-neuronal cells in the presence of neurotrophin when its high affinity receptor Trk is absent (reviewed by Teng and Hempstead, 2004). Although proneurotrophins have been considered as inactive precursors, at least secreted proNGF has recently been reported to induce apoptosis by binding with high affinity to $p75^{NTR}$ and sortilin, a 95 kDa receptor for neurotensin, in sympathetic neurons and cells expressing both receptors (Nykjaer et al., 2004).

Neuropoietic cytokines such as ciliary neurotrophic factor (CNTF), interleukin-6 (IL-6) and leukemia inhibitory factor (LIF) are pleiotrophic cytokines which are produced by immune cells, Schwann cells, fibroblasts and sensory neurons. Together with neurotrophins they act as communicators between the immune and nervous system and are known to act in response to injury and stress (Otten et al., 2000; Sariola et al., 1994; Sleeman et al., 2000). All neurokines bind to a common transmembrane glycoprotein receptor gp130. In addition CNTF binds to a glycosyl-phospatidylinositol (GPI)-linked CNTFRα receptor and LIFRβ, LIF to LIF-R and IL-6 to IL-6-R (Heinrich et al., 2003). The ligand binding leads to activation of Janus kinases (JAK) and subsequent activation of STATs (signal transducer and activator of transcription).

The GDNF family ligands (GFLs) include four members: GDNF (Lin et al., 1993), neurturin (NRTN, Kotzbauer et al., 1996), persephin (PSPN, Milbrandt et al., 1998) and artemin (ARTN, Baloh et al., 1998b; Enovin, Masure et al., 1999; Neublastin, Rosenblad et al., 2000). They are small dimeric proteins secreted by the target tissue which promote the survival of central and peripheral neurons *in vitro* and/or *in vivo*. GDNF protects dopamine neurons in animal models of Parkinson´s disease and rescues motor neurons *in vivo* which makes it a valuable candidate for therapeutic use in several neurodegenerative diseases (reviewed by Airaksinen and Saarma, 2002). Outside the nervous system GDNF acts as a morphogen in kidney development. Each ligand binds to a preferred GDNF family

co-receptor (GFRα1-4) which is linked to the outer leaflet of the cell membrane with a GPI-anchor. Ligand binds to the co-receptor and triggers the transmembrane Ret tyrosine kinase receptor, dimerizing and trans-phosphorylating its intracellular tyrosine residues. GDNF preferentially binds to GFRα1, NRTN to GFRα2, ARTN to GFRα3 and PSPN to GFRα4. Activating mutations in the human *RET* receptor cause different types of cancers in the thyroid gland, whereas inactivating mutations cause Hirschsprungs´s disease which is characterized by the absence of neuronal ganglia in various parts of the colon (reviewed by Sariola and Saarma, 2003). The different actions and features of GFLs and their receptors will be discussed in detail below. Furthermore, the results and discussion chapter will deal with the characterization and roles of the mammalian GFRα4 in more detail (publications I, II and III).

1.2 Roles for neurotrophic factors outside the nervous system

The neurotrophins have been extensively studied as molecules important for the survival and differentiation of both central and peripheral neurons (reviewed by Fariñas, 1999; Huang and Reichardt, 2001). However, recent findings have pointed to a diversity of roles for the neurotrophins outside the nervous system. NGF (and to some extent NT-3 and BDNF) stimulate proliferation, antibody synthesis and differentiation of a broad spectrum of cell types in the immune system (reviewed by Vega et al., 2003). Gene ablation studies for Trk receptors and their ligands have revealed that TrkC and NT-3 are important for cardiac outflow tract development (Donovan et al., 1996; Srivastava and Olson, 1996). Furthermore, both TrkA- and TrkB-deficient mice showed structurally abnormal thymus with lower density of thymocytes and increased apotosis leading to depletion of T- and B-cells, suggesting that both NGF and BDNF are important for the survival of immune cells and in thymic organogenesis (García-Suárez et al., 2000; García-Suárez et al., 2002). Levels of NGF are increased during inflammation, allergies and diseases of the immune system secondary to an initial rise in anti-inflammatory cytokines (reviewed by Levi-Montalcini et al., 1996). In agreement with this, NGF can suppress inflammation in the brain in an animal model for human multiple sclerosis (Villoslada and Genain, 2004). Neurotrophins may thus function in the maintenance of immune cells as well as in repressing the inflammatory response. BDNF-deficiency in mice has been shown to result in cardiac vascular hemorrhage leading to early postnatal death (Donovan et al., 2000). Furthermore, BDNF has been shown to support the survival of cardiac endothelial cells endogenously expressing TrkB and increase capillary density if overexpressed. NGF has been shown to indirectly stimulate the release of vascular endothelial growth factors from endothelial cells and to stimulate wound healing after experimental limb ischemia (Emanueli et al., 2003). The complete absence of $p75^{NTR}$ results in defects of blood vessel formation (von Schack et al., 2001), which further suggests an angiogenic role for neurotrophins.

Expression of neurotrophins and their receptors in germ cells and Sertoli cells

of the testis has suggested a role in germ cell development (Ayer-LeLièvre et al., 1988; Park et al., 2001). According to this, increased germ cell apoptosis and developmentally delayed gonad morphology was detected from embryonic and postnatal testicles of TrkA and TrkC homozygous knockout mice compared to wild-type mice (Cupp et al., 2002). Neurotrophic factors are also involved in hair follicle cycle as NGF/TrkA and NT-3/TrkC signaling appear to stimulate hair follicle development (anagen), whereas NT-4, BDNF and $p75^{NTR}$ are involved in the induction of apototic hair follicle regression (catagen) (Botchkarev et al., 2004).

Gene knockout studies of gp130, the common signaling receptor for the IL-6 family of cytokines and IL-6 itself, have revealed that gp130/IL-6 signaling is important for protective response of hepatocytes in liver injury (Streetz et al., 2003; Wuestefeld et al., 2003). Specific inactivation of gp130 in germ cells resulted in a major defect in ovulation whereas only a slight decrease in the number of germ cells (Molyneaux et al., 2003). The IL-6 family members are potent inhibitors of stem cell differentiation. LIF is commonly used in maintaining the undifferentiated state of embryonic stem cells in culture (Williams et al., 1988). Furthermore, LIF has been shown to be critical for blastocyst implantation (Robb et al., 2002). In addition, LIF has been found to induce differentiation of kidney mesenchyme to epithelia (Barasch et al., 1999) and is also suggested to be involved in regeneration of renal epithelium after acute injury (Yoshino et al., 2003). LIF and CNTF have *in vitro* shown to enhance the survival of Sertoli cells and gonocytes, which suggests that these factors may play a role in the initial steps of spermatogenesis (De Miguel et al., 1996).

GDNF family members may also contribute to the control of the follicle hair cycle. *Gfra1*, *Gfra2* and their ligands are highly expressed in the hair follicle during the anagen (active growth)-catagen (growth regression) transition (Botchkareva et al., 2000). Furthermore, NRTN and GDNF administered to mouse skin organ cultures retard hair follicle regression. In line with the previous results, Ret immunostaining has been shown in mouse hair follicles after birth (Kato et al., 2001). Interestingly, promotion of hair growth occurred in a transgenic mouse line overexpressing Ret in the skin (Kato et al., 2001), suggesting potential roles for GDNF/RET in human hair growth promotion.

GDNF and its receptors GFRα1 and Ret are crucial for ureteric branching in kidney development as respective gene knockout mice show relatively similar phenotypes, all lacking kidneys (See chapter 1.6.1). Furthermore, GDNF expressed by Sertoli cells in the testis is implicated in sperm differentiation and will be discussed in the result and discussion part of this thesis (publication IV).

1.3 Glial cell line-derived neurotrophic factor Family Ligands (GFLs) are distant members of the TGF-β family

1.3.1 GDNF

GDNF was biochemically purified in 1993 from a rat glioma cell line B49 growth medium on the basis of its ability to promote the survival of embryonic

dopamine neurons in culture (Lin et al., 1993; Lin et al., 1994). The full-length GDNF cDNA (633 bp) codes for a 211 amino acid precursor polypeptide with an amino-terminal signal sequence and a pro-sequence which is cleaved from the mature domain by a furin-like endoproteinase at a RXXR cleavage site (Lin et al., 1993) (Fig. 1B; Table 1). However, little is known about the processing and secretion of GDNF by neurons and non-neuronal cells *in vivo*. The mature protein consists of 134 amino acids (Mr 18-22kDa) and contains two putative glycosylation sites (Lin et al., 1993). GDNF contains seven cysteines in the same relative spacing as members of the TGF-β family (Chang et al., 2002; Eigenbrot and Gerber, 1997) although mature GDNF shares less than 20% sequence homology on amino acid level with any of the other TGF-β family members (Lin et al., 1993). According to the crystal structure the GDNF monomer forms two finger-like (Finger 1 and Finger 2) structures by pairs of anti-parallel β-strands with a α-helix at the opposite end (Chen et al., 2000; Eigenbrot and Gerber, 1997; Eketjäll et al., 1999) (Fig. 1A). A compact cystine knot structure, also common for other TGF-β superfamily members is produced by six cysteines forming three intermolecular cystinyl bonds (Eigenbrot and Gerber, 1997; Ibáñez, 1998). The biologically active GDNF dimer is formed by a covalent disulfide bond between the unpaired cysteines in monomers, which positions the monomers in a head-to-tail orientation with the knot structure in the middle of the dimer (Eigenbrot and Gerber, 1997; Eketjäll et al., 1999).

GDNF mRNA is expressed in many areas of the central and peripheral nervous system during embryonic development (Henderson et al., 1994; Nosrat et al., 1996; Golden et al., 1999) and after birth (Golden et al., 1998; Holm et al., 2002; Nosrat et al., 1996; Trupp et al., 1996). *GDNF* mRNA is also expressed by developing skeletal muscles (Arce et al., 1998; Chen et al., 2003; Henderson et al., 1994; Nosrat et al., 1996) and muscle layers of the gut wall (Golden et al., 1999; Nosrat et al., 1996; Suvanto et al., 1996). High levels of *GDNF* mRNA is expressed in the developing kidney mesenchyme surrounding the tips of ureteric buds (Nosrat et al., 1996; Sainio et al., 1997; Suvanto et al., 1996), in developing mouse whisker follicles and testis (Suvanto et al., 1996; Trupp et al., 1995). Expression of *GDNF* mRNA has also been detected in the mesenchyme of developing limb buds, tongue, teeth and cartilage and in adult ovary (Golden et al., 1999; Luukko et al., 1997; Nosrat et al., 1996; Trupp et al., 1995), but the exact role of GDNF in these organs is unclear.

GDNF supports *in vitro* the survival of midbrain dopamine neurons (Lin et al., 1993; Tomac et al., 1995), motoneurons (Henderson et al., 1994; Oppenheim et al., 1995), central noradrenergic neurons (Arenas et al., 1995), basal forebrain cholinergic neurons (Williams et al., 1996), peripheral sensory, parasympathetic, and sympathetic neurons (Arce et al., 1998; Buj-Bello et al., 1995; Forgie et al., 1999; Mount et al., 1995; Oppenheim et al., 1995; Trupp et al., 1995) and enteric neurons (Heuckeroth et al., 1998).

In vivo exogenous GDNF has been shown to prevent axotomized embryonic or injured adult motoneurons from atrophy and death and to induce sprouting

(Henderson et al., 1994; Hottinger et al., 2000; Li et al., 1995), which suggest that GDNF could be a potential therapeutic agent for the treatment of adult-onset motoneuron diseases, such as amyotrophic lateral sclerosis (ALS), a progressively paralysing disease affecting motoneurons. Animal models of Parkinson´s disease (PD) have shown that GDNF delivered to the striatum or substantia nigra can prevent further cell loss and also to restore function in dysfunctional or atrophic neurons in the degenerating nigrostriatal system (Björklund et al., 2000; Åkerud et al., 1999). In a recent clinical trial where GDNF was delivered directly to the putamen of five human PD patients, improvement in motorfunction with no side effects was reported (Gill et al., 2003), suggesting further GDNF as a potent candidate drug for treatment of PD. In rodents, an increase in *GDNF* mRNA and its receptors have been observed in the forebrain, hippocampus and in the striatum after experimentally induced global and focal forebrain ischemia and after kindling-stimulated epilepsy (Arvidsson et al., 2001; Kokaia et al., 1999). Direct delivery of GDNF before ischemic insult has shown to block the elevation of extracellular nitric oxide and reduce the infarction size (Wang et al., 1997). However, high doses of GDNF may increase ischemic cell death due to a yet unknown mechanism (Arvidsson et al., 2003). In animal models of experimentally induced neuropathic pain arising from injury in peripheral nerves, infusion of GDNF has been shown to normalize abnormal pain behaviour and restore the changes in neurochemical markers observed in neuropathic pain (reviewed by Sah et al., 2003). However, the broad expression range of the receptors for GDNF may elicit complicated side effects by activating cells normally silent.

1.3.2 Neurturin (NRTN)

NRTN was isolated from conditioned medium of Chinese hamster ovary cells on the basis of its ability to promote the survival of neonatal rat sympathetic neurons (Kotzbauer et al., 1996). Mature NRTN shows 42% sequence similarity with GDNF and has a predicted Mr of 12.5 kDa (Kotzbauer et al., 1996). Like GDNF, NRTN is synthesized as a prepro protein with seven cysteines in the same relative spacing as GDNF (Fig. 1B; Table 1). Since seven cysteines are also conserved in NRTN, it is likely that the general fold of NRTN is very similar to that of GDNF. However, data about the three dimensional structure of NRTN is lacking.

NRTN mRNA is detected in the developing brain, in dental epithelium and in developing lacrimal gland (Golden et al., 1999; Luukko et al., 1998; Rossi et al., 1999); Widenfalk et al., 1997). In addition, high *NRTN* mRNA levels are detected before birth in blood vessels, skin, testis, circular muscle layer of the gut, cutaneous glands, submandibular gland, and parotid gland (Golden et al., 1999; Widenfalk et al., 1997; Åkerud et al., 1999). Postnatally, NRTN expression remains high in skin, stomach, intestine, prostate, testis and urethra (Golden et al., 1999; Viglietto et al., 2000). *In vitro* NRTN supports the survival of populations of sensory and sympathetic neurons (Forgie et al., 1999; Kotzbauer et al., 1996), spinal motor neurons (Garcès et al., 2001), dopaminergic neurons

(Horger et al., 1998; Kotzbauer et al., 1996; Åkerud et al., 1999), enteric neuronal precursor cells (Heuckeroth et al., 1998) and basal forebrain cholinergic neurons (Golden et al., 2003).

In vivo, exogenous NRTN has been shown to prevent the death of dopamine neurons in mouse models of Parkinson´s disease (Horger et al., 1998; Åkerud et al., 1999) but in contrast to GDNF, it does not induce sprouting, neuritogenesis or hypertrophy. However, the mechanism for NRTN action is elusive, as GFRα2 expression has not been detected in dopamine neurons.

1.3.3 Persephin (PSPN)

PSPN was identified in 1998 by PCR, and subsequent rapid amplification of cDNA ends (RACE), using degenerate primers corresponding to very similar sequences in GDNF and NRTN (Milbrandt et al., 1998). Rat, mouse and human PSPN is a 156-amino acid long prepro protein with a predicted 21-residue signal sequence, a 39-residue pro region, a RXXR consensus cleavage site and a predicted 96-amino acid mature PSPN protein of Mr 10-12 kDa. PSPN shows about 40% sequence identity to GDNF and 50% to NRTN, with seven cysteines in the same characteristic spacing as other members of the TGF-β family (Milbrandt et al., 1998) (Fig. 1B; Table 1).

The expression of *PSPN* mRNA is very low in many rat embryonic and adult peripheral tissues as well as in many parts of the CNS and some sensory ganglia (Jaszai et al., 1998; Milbrandt et al., 1998). Because of its low expression, *PSPN* mRNA has been detected only by

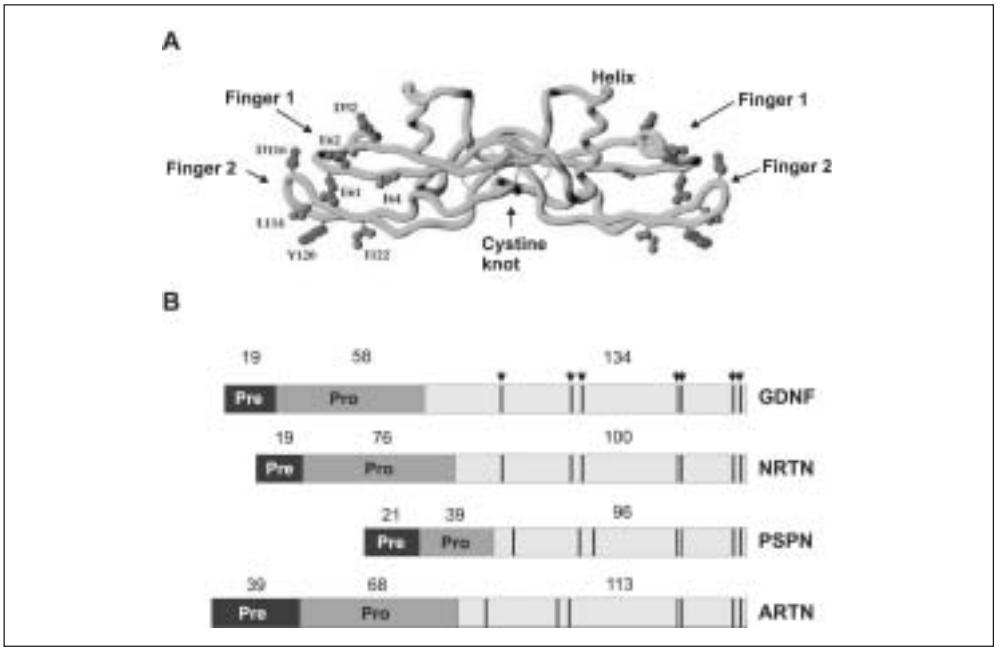

Figure 1. (A) Alpha-carbon chain feature of the GDNF homodimer. Adapted from (Eketjäll et al., 1999). **(B) Schematic organization of the prepro GFLs.** According to (Airaksinen et al., 1999). The relative lengths (number of amino acids) of pre-, pro- and mature domains of GFLs are shown. Conserved cysteines in the mature domains are marked by black lines and arrows.

reverse transcription PCR (RT-PCR). Curiously, the cDNA clones obtained from mouse, rat and human cDNA libraries contained an 88-bp intronic sequence, which was *in vitro* removed by splicing in mammalian cell lines transfected with the cDNA containing the short intron, to generate a transcript coding for the prepro PSPN (Milbrandt et al., 1998). In contrast, the intronic, unspliced transcript generates a premature stop codon when translated which does not give rise to a functional protein. As shown for GDNF and NRTN, PSPN can promote the survival of cultured rat embryonic midbrain dopamine neurons and rat embryonic motor neurons (Milbrandt et al., 1998; Åkerud et al., 2002). PSPN also promotes neurite outgrowth from chicken embryonic oculomotor neurons *in vitro* (Chen et al., 2003), but not postnatal rat motor neurons (Bilak et al., 1999). Recently, PSPN was also reported to promote the survival of embryonic rat basal forebrain cholinergic neurons (Golden et al., 2003). However, in contrast to GDNF and NRTN, PSPN does not promote the survival of cultured rat peripheral sympathetic, sensory or enteric neurons (Milbrandt et al., 1998). *In vivo*, PSPN signaling may protect the brain from insults as PSPN administered intraventricularly prior to experimentally induced focal cerebral ischemia, is shown to reduce the infarction size in mice and rats (Tomac et al., 2002). Furthermore, PSPN expressed by a neural stem cell line grafted to the striatal area in mouse was shown to rescue dopamine neurons from degeneration in a mouse model of Parkinson´s disease (Åkerud et al., 2002).

1.3.4 Artemin (ARTN)

ARTN (also named Enovin or Neublastin) the fourth member of the GDNF family, was independently identified by three groups using database homology search (Baloh et al., 1998b; Masure et al., 1999; Rosenblad et al., 2000). Full-length *ARTN* cDNA was obtained by RACE PCR from mouse and human cDNA libraries. Sequence data revealed that ARTN is a member of the GDNF family ligands (Fig. 1B; Table 1). Mature ARTN has the highest sequence identity to NRTN (51%) and PSPN (53%) and the lowest to GDNF (39%) (Masure et al., 1999). In rat and mouse embryos, *ARTN* mRNA expression is localized in close proximity to migration routes for sympathetic neuroblasts, in the wall of the dorsal aorta near the site of the organization of the primitive sympathetic chain and later its expression is localized to blood vessels and arteries along which sympathetic neurons migrate and differentiate (Enomoto et al., 2001; Honma et al., 2002). *Artn* mRNA has not been detected in the CNS. *In vitro,* ARTN is found to support the survival of a subset of sensory and sympathetic neurons (Baloh et al., 1998b; Enomoto et al., 2001). Recent studies have shown that ARTN can reverse experimental neuropathic pain, which suggests ARTN application as a treatment for different types of neuropathic pain (Gardell et al., 2003).

1.4 GDNF family receptor α´s (GFRα´s)

GFRα1, the preferential binding receptor for GDNF, initially termed GDNFR-α, was cloned by expression cDNA library screening and characterized

independently by two groups (Jing et al., 1996; Treanor et al., 1996) (Table 1). This proved to be a novel class of cysteine rich proteins (31 Cys of 468 amino acids) with three N-glycosylation sites and a predicted *M*r of 47 kDa. GFRα1 contains a N-terminal hydrophobic area characteristic for a secretory signal sequence (von Heijne, 1986) and a C-terminal sequence with 23 hydrophobic amino acids. A group of three small amino acids (ASS) proceeding the stretch of hydrophobic amino acids indicated a possible GPI-binding/cleavage site (Udenfriend and Kodukula, 1995). The GPI-linkage was verified by treatment of GFRα1 expressing cells with phosphatidylinositol-specific phospholipase C (PI-PLC) which cleaves GPI-linked proteins from the cell surface (Jing et al., 1996; Treanor et al., 1996). The GFRα1 receptor was found to bind ^{125}I-GDNF with high affinity, and this binding was effectively displaced with an excess of unlabeled GDNF (Cik et al., 2000). Immunoprecipitation of Ret

Table 1. Chromosomal localization of *GFL*, *GFRA* and *RET* genes.

Gene	Human Localization Accession[1], bp[2]	Mouse Localization Accession, bp	Rat Localization Accession, bp	Chicken Localization Accession, bp
GDNF	5p13.1 NM_000514 633 bp	15A2 NM_010275 720 bp	2q16 NM_019139 717 bp	Unknown* AF176017 Partial cDNA
NRTN	19p13.3 NM_004558 591 bp	17D NM_008738 585 bp	9q11 NM_053399 671 bp	28*
PSPN	19p13.3 NM_004158 468 bp	17E1.1 NM_008954 468 bp	9q11 NM_013014 468 bp	Unidentified*
ARTN	1p34.1 NM_003976 660 bp	14D1 NM_009711 672 bp	5q36 AF_184919 675 bp	8*
GFRA1	10q25.3 NM_005264 1395 bp	19D3 NM_010279 1404 bp	1q55 NM_012959 1407 bp	6* NM_205102 1407 bp
GFRA2	8p21.3 NM_001495 1392 bp	14D1 NM_008115 1389 bp	15p11 NM_012750 1392 bp	Unknown* NM_205101,
GFRA3	5q31.2 NM_001496 1200 bp	18 NM_010280 1191 bp	18p12 XM_341593 1267 bp	13*
GFRA4	20p13 AJ291673, 807 bp (a) AJ291674, 897 bp (b)	2F3 AJ276870, 780 bp (GPI) AJ276871, 879 bp (TM)	3q36 NM_023967 822 bp	4* NM_204991 1293 bp
RET	10q11.21 NM_000323 long 3342 bp NM_020630 short 3216 bp	6F1 NM_009050 long 3345 bp AY_326937 short 3219 bp	4q42 AJ_299016 long 3348 bp AJ_299017 short 3222 bp	6?* NM_205190 3192 bp short

[1] Genbank Accession number, [2] coding region, * localization based on BLAST analysis of the draft chicken genome assembly (http://www.genome.gov/11510730).

protein tyrosine kinase after cross-linking of ^{125}I-labeled GDNF to GFRα1 revealed that GDNF-GFRα1 forms a complex with the Ret receptor. The pattern of bands in reducing and non-reducing gels suggested that a GDNF dimer binds to a GFRα1 dimer that forms a complex with RET. GDNF was also shown to activate Ret tyrosine kinase when bound to either soluble or GPI-linked GFRα1 receptor (Jing et al., 1997).

Gfra1 mRNA is expressed in many brain areas of the developing and adult mouse which often is complementary to the sites where GDNF is expressed (Golden et al., 1998; Golden et al., 1999; Nosrat et al., 1997; Trupp et al., 1997). High *Gfra1* mRNA levels are detected in some peripheral organs of the developing mouse and rat such as the ureteric buds and mesenchyme of the kidney, the ganglionic plexuses of the developing gastrointestinal tract and whisker follicles (Golden et al., 1999; Nosrat et al., 1997; Yu et al., 1998).

Homology database searching using GFRα1 as a query resulted in the simultaneous cloning of GFRα2 (TrnR2, Baloh et al., 1997; NTNR-α, Buj-Bello et al., 1997; GFRα-2, Jing et al., 1997; NTNR-α, Klein et al., 1997; RETL2, Sanicola et al., 1997; GDNFR-β, Suvanto et al., 1997; GDNFR-β, Wang et al., 1998; GDNFR-β, Widenfalk et al., 1997) (Table 1). GFRα2 contains 464 amino acids with a predicted molecular weight of approximately 51 kDa and shares a significant homology with GFRα1 (48%) as well as nearly identical spacing of 30 of its 31 cysteines. GFRα2 contains a signal peptide at the amino terminus, three potential glycosylation sites and a GPI cleavage/binding site (Udenfriend and Kodukula, 1995). On the basis of internal homologous cysteine-rich sequences in GFRα1 and GFRα2, a secondary structure analysis prediction suggested three putative globular cysteine-rich domain structures in the two receptors (D1-D3), linked with less conserved hinge domains (Airaksinen et al., 1999; Suvanto, 1997) (Fig. 2). This domain model was recently verified by crystallization of GFRα1 D3 which revealed a new protein fold (Leppänen et al., 2004). The D3 domain forms a bundle of five α-helices connected with three less well-conserved loops. Five disulfide bridges form a compact structure including five conserved hydrophobic phenylalanines which form the core of the bundle. D1 and D2 were predicted to have the same structure as D3 on the basis of α-helical structure and conserved cysteines with the same positions with respect to disulfide bridges (Leppänen et al., 2004). Binding data revealed that NRTN binds with high affinity (K_d 10 pM) to the GFRα2 receptor (Cik et al., 2000; Klein et al., 1997). NRTN like GDNF was found to stimulate Ret phosphorylation in cell lines co-expressing Ret and GFRα2 (Baloh et al., 1997; Jing et al., 1997; Klein et al., 1997; Suvanto et al., 1997).

Gfra2 mRNA is expressed in the embryonic as well as adult CNS and PNS neurons and glia such as parasympathetic and enteric neurons and glia, in some sensory neurons, in the developing and postnatal myenteric plexus of the gastrointestinal tract and many peripheral organs (Golden et al., 1998; Golden et al., 1999; Heuckeroth et al., 1999; Jing et al., 1997; Rossi et al., 1999; Rossi et al., 2003; Suvanto et al., 1997; Widenfalk et al., 1997).

GFRα3, the third member of the

GFRα family was cloned by PCR (Baloh et al., 1998a; Jing et al., 1997; Masure et al., 1998; Naveilhan et al., 1998; Nomoto et al., 1998; Trupp et al., 1998; Widenfalk et al., 1998; Worby et al., 1998) (Table 1). GFRα3 (Mr 39 kDa) is 34% identical to GFRα1 and 36% identical to GFRα2 (Fig. 2). The conserved 28 cysteines suggested a similar domain structure as for GFRα1 and GFRα2 but with a shorter, less conserved C-terminal. *Gfra3* mRNA is expressed in some embryonic and adult sensory ganglia and throughout the entire sympathetic nervous system but not in the brain (Baloh et al., 1998a; Honma et al., 2002; Trupp et al., 1998; Widenfalk et al., 1998; Worby et al., 1998).

Chicken GFRα4 was identified from an embryonic chicken brain cDNA library by low stringency hybridization to a GFRα1 probe. The amino acid sequence revealed a 431-amino acid long protein with 28 conserved cysteines, a N-terminal putative hydrophobic signal sequence, and a putative consensus GPI-linkage sequence in the C-terminus, characteristic for the other GFRα receptors (Thompson et al., 1998). Sequence alignment revealed that chicken GFRα4 shares higher identity with both mouse and chicken GFRα1 and GFRα2 (40%) than with mouse GFRα3 (27%). In displacement binding experiments with different ligands, only mouse PSPN, but not rat GDNF nor human NRTN, was capable of binding to chicken GFRα4 (Enokido et al., 1998). However, higher concentrations (K_d approximately 1 nM) of unlabelled PSPN were needed for displacement compared to GDNF binding to GFRα1 and NRTN to GFRα2 (Enokido et al., 1998). Furthermore, Ret was shown to be the signaling receptor for PSPN together with GFRα4 as sympathetic neurons co-expressing GFRα4 and Ret, but not GFRα1 or GFRα2, survived in the presence of PSPN (Enokido et al., 1998). Chicken *Gfra4* mRNA was found to be expressed in embryonic kidney, skeletal muscle, skin, intestine and lung and within the CNS in spinal cord, cerebellum, midbrain, medulla oblongata and pons (Thompson et al., 1998). The mammalian GFRα4 was characterized in this work and its features compared to the other GFRα-receptors will be described in detail in the results and discussion part (I and II) (Fig. 2) (Table 1).

1.5 RET receptor tyrosine kinase

RET was identified in 1985 as an oncogene resulting from a chromosomal translocation during transfection of human lymphoma DNA into a fibroblast cell line (REarranged during Transfection) (Iwamoto et al., 1993; Takahashi et al., 1985; Takahashi, 1988) (Table 1). The RET proto-oncogene encodes for a single span transmembane protein with an extracellular domain (607 amino acids) consisting of four cadherin-like repeats (CDL1-4) based on computer modeling (Anders et al., 2001), a cysteine rich domain (Iwamoto et al., 1993; Kuma et al., 1993; Takahashi, 1988; Takahashi et al., 1989), a hydrophobic transmembrane region, and a cytoplasmic intracellular part (414 or 457 aa) with a kinase domain (amino acids 724-1016) divided in human RET into two lobes by a hinge region (Scott, 2002) (Fig. 2). A Ca^{2+}-binding site is localized between cadherin-like domains 2 and 3 (Anders et al., 2001). Two RET polypeptides with different molecular masses are usually seen in western blots, one fully processed,

membrane-bound protein of approximately 170 kDa and one smaller, partially glycosylated protein of approximately 150 kDa band that is thought to retain in the endoplasmatic reticulum (Carlomagno et al., 1996; Cosma et al., 1998). The *RET* gene encodes for multiple alternative 5´and 3´end splice variants (Ivanchuk et al., 1997; Lorenzo et al., 1995; Myers et al., 1995; Tahira et al., 1990). Alternative splicing of the 3´ *RET* mRNA gives rise to at least three RET isoforms differing in their C terminus downstream of amino acid 1062. The short isoform (1072 aa) with a 9-amino acid unique C-terminus and the long isoform (1114 aa) with 51-amino acid unique C terminus, are highly conserved between species (Carter et al., 2001). The short isoform (RET9) is important for early kidney and enteric nervous system development (de Graaff et al., 2001; Lee et al., 2002), whereas the long isoform (RET51) is important for the metabolism and growth of mature sympathetic neurons (Tsui-Pierchala et al., 2002a). *RET* transcripts are expressed in cells and tissues derived from the neural crest, branchial arches and ureteric bud (Lorenzo et al., 1995) often overlapping with the expression of GFRα receptor mRNAs (Trupp et al., 1997). Expression of *RET* is high in dopamine neurons, in motor neurons, in a subset of trigeminal neurons, gastrointestinal tract and kidney (Nosrat et al., 1997). However, the expression of *RET* is low or absent in many developing and adult brain areas such as hippocampus and developing cerebellum (Golden et al., 1998; Golden et al., 1999; Trupp et al., 1997). Expression of *RET* mRNA is also detected in the developing and adult thyroid parafollicular C-cells (Belluardo et al., 1999; Golden et al., 1999). High levels of *RET* mRNAs have been observed in human tumors with a neural crest origin, such as medullary thyroid

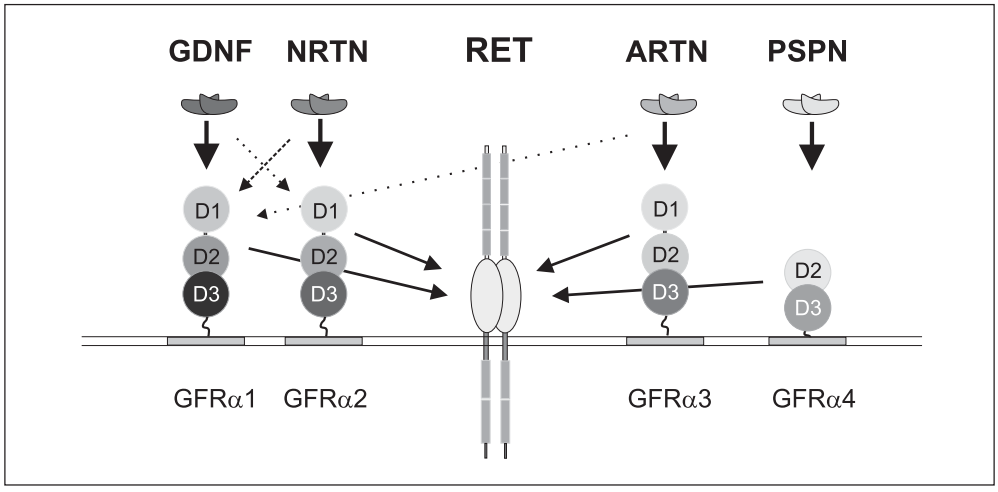

Figure 2. Interaction of GFLs with their cognate receptors. All GFLs activate the transmembrane tyrosine kinase RET through their preferred GFRα receptor (solid arrows). Weak cross-talk has been observed with NRTN and ARTN binding to GFRα1 and GDNF binding to GFRα2 (dotted arrows). The mammalian GFRα4 lacks the first Cys-rich domain D1 (I, II). Adapted from (Airaksinen and Saarma, 2002).

carcinoma and pheochromocytomas derived from the adrenal medullary chromaffin cells (Takaya et al., 1996). Several gain-of-function mutations in the *RET* gene result in human diseases including familial medullary thyroid carcinomas (FMTC), multiple endocrine neoplasia types 2A and 2B (MEN2A and MEN2B) and sporadic medullary and papillary thyroid carcinoma characterized by neoplasias of the thyroid gland (See chapter 1.9.2). Loss-of-function mutations in *RET* cause Hirschsprung´s disease (HSCR) characterized by megacolonic aganglionosis (See chapter 1.9.1).

1.6 Gene ablation studies reveal distinct activities for GFLs *in vivo*

1.6.1 GDNF, GFRα1 and RET are regulators in kidney morphogenesis and in the development of the enteric and parasympathetic nervous system

Many of the *in vivo* roles for GFLs, GFRα- and RET receptors and their interactions inside and outside the nervous system have been elucidated by gene ablation studies. Homozygous Ret-deficient mice (Schuchardt et al., 1994) as well as GDNF- (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996) and GFRα1-deficient mice (Cacalano et al., 1998; Enomoto et al., 1998) all display unilateral or bilateral renal agenesis or severe dysgenesis and lack of enteric neurons from the myenteric plexus in the small and large intestine (intestinal aganglionosis) (Table 2). However, the rest of the urogenital system such as gonads and the adrenal glands develop normally (Allmendinger et al., 2003). Consequently, these mice die 12-24 hours after birth possibly due to an inability to consume milk, a renal failure or/and a breathing defect.

Kidney development is characterized by a successive interaction of inductive signals between the metanephric blastema and the ureteric bud which branches from the Wolffian (nephric) duct into the metanephric blastema. The mesenchyme signals the proliferating bud both to divide and to elongate its collecting ducts (Suvanto, 1997). *Gdnf* mRNA is highly expressed in the condensing metanephric mesenchyme (Hellmich et al., 1996; Suvanto et al., 1996), whereas *Ret* and *Gfra1* mRNA are expressed by the tips of the ureteric bud (Sainio et al., 1997). *Gfra1* mRNA is also found in the nephrogenic mesenchyme (Sainio et al., 1997). GDNF has been shown to bind directly to tips of the ureteric buds and induce bud formation from the Wolffian duct from local sources in organ cultures (Sainio et al., 1997). In homozygous GDNF-, GFRα1- and RET-deficient mice, the ureteric bud is not developed and if it is, it never reaches the metanephric mesenchyme (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996; Schuchardt et al., 1994; Schuchardt et al., 1996), which suggests GDNF and its receptors to be critical for inducing the development of the ureteric bud. GDNF heterozygous mice display unilateral agenesis or bilateral dysgenesis, indicating that the level of GDNF produced by the nephrogenic mesenchyme is critical in the inductive process. Misexpression of *RET* mRNA throughout the bud also causes variable inhibition of ureteric bud growth and branching, probably due to reduction of

GDNF availability to the tips of the ureteric bud (Srinivas et al., 1999). However, signals other than GDNF are likely to count for the growth and elongation of the branches (Sariola and Saarma, 1999). Although both NRTN and PSPN *in vitro* can promote ureteric branching (Davies et al., 1999; Milbrandt et al., 1998), NRTN-, GFRα2- and PSPN-deficient mice show no obvious defect in kidney development (Heuckeroth et al., 1999; Rossi et al., 1999; Tomac et al., 2002).

The mammalian enteric nervous system (ENS) controls digestive activities such as peristaltic movements, endocrine and exocrine secretions. The development of the ENS starts with the migration of vagal and trunk (somites 1-7) neural crest cells (NC) from the neural tube ventrolaterally to invade the foregut mesenchyme. Previously, *in vitro* studies have shown that GDNF and NRTN promote the survival and proliferation of mammalian ENS progenitor cells and their differentiation into neurons and glia (Heuckeroth et al., 1998; Taraviras and Pachnis, 1999). GDNF has been shown to be important for guiding NC cells to their proper targets in the ENS and is expressed in the mesenchyme ahead of the invading NC cells that successively invade almost the entire length of the bowel in the developing mouse (Young et al., 2001; Natarajan et al., 2002). Homozygous GDNF-, GFRα1-and Ret-deficient mice all lack neural crest derived enteric neurons posterior to the stomach (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996; Schuchardt et al., 1994), a condition that resembles Hirschsprung´s disease (HSCR), or colonic aganglionosis in humans (Parisi and Kapur, 2000). In a recent study, knock-in mice were generated expressing monoisoformic forms of either RET9 or RET51 (de Graaff et al., 2001). The majority of mice homozygous for RET9 died as neonates with a slightly milder kidney- and ENS phenotype than Ret-deficient mice. The RET9 homozygous mice are characterized by distal colon aganglionosis which is analogous to the condition observed in HSCR (de Graaff et al., 2001). In contrast to RET9 mice, the RET51 homozygous mice are normal, suggesting that RET9 is sufficient for normal development of the kidneys and the ENS (de Graaff et al., 2001).

Peripheral neuronal deficits were also observed in GDNF-, GFRα1- and RET-deficient mice. Certain cranial (otic and sphenopalatine) parasympathetic ganglia are missing in newborn GDNF-, GFRα1- and Ret-mutant mice, indicating that GDNF signaling is important for early development of parasympathetic ganglia (Enomoto et al., 2000; Marcos and Pachnis, 1996; Rossi et al., 2000). Furthermore, there is a significant loss of a subset of motoneurons in homozygous GDNF- and GFRα1-deficient embryos (Cacalano et al., 1998; Moore et al., 1996; Oppenheim et al., 2000; Sánchez et al., 1996). Accordingly, the death of motoneurons is prevented by over-expression of GDNF in developing muscle or by exogenously injecting GDNF *in utero* (Oppenheim et al., 2000). Furthermore, transgenic mice over-expressing GDNF under a muscle-specific promoter show hyperinnervation of neuromuscular junctions (Nguyen et al., 1998), suggesting GDNF to be important for motor axon branching and synapse elimination. Even though *Gfra1* and to a lesser extent *GDNF* and *Ret*, are

highly expressed in the central nervous system of wild type mice, no obvious defects were found in the CNS of these mice at birth (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996). Although GDNF is known to be a potent survival factor for embryonic and mature midbrain dopamine neurons, no loss of dopamine neurons or projections to the striatum was observed in GDNF-, GFRα1- or Ret-deficient mice (Cacalano et al., 1998; Enomoto et al., 1998; Marcos and Pachnis, 1996; Moore et al., 1996; Sánchez et al., 1996). However, these mice die before maturation of the dopaminergic system. In rodents DA neurons undergo natural cell death in two phases the first peaking just after birth and the second 2 weeks after birth (Oo and Burke, 1997). It has recently been demonstrated that GDNF administred *in vivo* into the striatal target is able to suppress the natural apotosis during the first biphasic death event (Oo et al., 2003). In the same paradigm blocking antibodies to endogenous GDNF induced apotosis (Oo et al., 2003). Midbrain dopamine neuronal grafts transplanted from GDNF-null mouse fetuses, show poor survival compared to wild type or heterozygous tissue, when transplanted to wild-type adult mouse brain (Granholm et al., 2000), suggesting that GDNF is important for the survival of adult dopamine neurons. Moreover, GDNF is suggested to have many functions in the adult brain like in learning behavior, age related motor dysfunctions and in adaptation to drug abuse (Gerlai et al., 2001; Grondin et al., 2003; Messer et al., 2000). The best solution to *in vivo* study the effect of GDNF-, GFRα1- and Ret-deficiency in the young and adult mouse would be to generate conditional knock-out mice with deficiency in targeted tissues.

1.6.2 NRTN and GFRα2 regulate the development of parasympathetic and subsets of enteric neurons

Homozygous GFRα2- and NRTN-deficient mice are both viable and fertile, show no obvious defects in major organs and brain, but have a similar defect in the cholinergic innervation of some parasympathetic cranial ganglia and enteric myenteric plexus (Heuckeroth et al., 1999; Rossi et al., 1999) (Table 2). Both mice show a reduction in tear production caused by deficits in the cholinergic innervation of the lacrimal gland. Otic and sphenopalatine ganglia are present in GFRα2- and NRTN-deficient mice but reduced in neuronal size (Heuckeroth et al., 1999; Rossi et al., 1999). The sacral parasympathetic system is also affected as GFRα2-deficient mice have fewer parasympathetic axons innervating the penis (Laurikainen et al., 2000). It was shown recently that the innervation of the mucosa of reproductive organs by a subset of parasympathetic cholinergic neurons was reduced in GFRα2- and NRTN-deficient mice, whereas the smooth muscle layers and the urinary bladder were not affected (Wanigasekara et al., 2004). The reduction in parasympathetic neuronal number and size in many of the cranial and sacral parasympathetic ganglia indicates NRTN/GFRα2/Ret signaling to be important for parasympathetic target innervation and maintenance of cell size (Heuckeroth et al., 1999; Rossi et al., 1999; Rossi et al., 2000; Wanigasekara et al., 2004). Furthermore, a temporal

Table 2. **Phenotypes of mice lacking GFLs and their receptors.** Modified from (Airaksinen and Saarma, 2002).

Gene Knock-out	RET	GDNF/GFRα1	NRTN/GFRα2	ARTN/GFRα3	PSPN/GFRα4
Gross phenotype	P0 lethal	P0 lethal	Viable, fertile. Pseudoptosis growth retardation**	Viable, fertile Ptosis	Viable, fertile *Gfra4-/-*(III)
PNS: Sensory: PG	Breathing defect	<40%, breathing defect soma reduced			ND
DRG			Soma size reduced, loss of heat sensitivity	NS	
TG		loss in whisker follicles			
Autonomic: Sympathetic	SCG; migration defect	SCG; <35% in *Gdnf*-/- mice NS in *Gfra1*-/- mice	NS	SCG and other ganglia; defect in migration and axon growth	
Sympathetic cholinergic			Soma size,target innervation reduced		
Parasympathetic Cranial: SPG	Lack of ganglia	Lack of ganglia	NS		
OG	Lack of ganglia	Lack of ganglia	40% reduced no. of neurons		
SMG	Reduced number and soma size	Reduced number and soma size	42% reduced number of neurons	NS	
Enteric neurons	No neurons in bowel below stomach	No neurons in bowel below stomach	Moderate loss of fibers in small intestine	NS	
CNS: Spinal motorneurons	Loss in various nuclei	22-31% loss of neurons	No gross defects	No gross defects	No gross defects
Brain	Substantia nigra; ns	impaired learning, reduced loco-motor activity*	impaired behavioral flexibility and memory **	No gross defects	*Pspn-/-*: hypersensitive to cerebral ischemia
Other tissues	No kidneys, moderate thyroid C-cell loss(I)	No kidneys, testis degeneration in adult *Gdnf*+/- mice (IV)	ND	ND	*Gfra4-/-* mice; calcitonin levels reduced in young mice (III)

NS, not significantly different from wild-type; ND, not determined; PG, petrosal ganglion; DRG, dorsal root ganglion; TG, trigeminal ganglion; SCG, superior cervical ganglion; SPG, sphenopalatine ganglion; OG, otic ganglion; SMG, submandibular ganglion; **Gdnf*+/-, ** *Gfra2-/-* mice.

switch from GDNF- to NRTN dependence is suggested to occur in parasympathetic and enteric neurons late in embryonic development (Airaksinen and Saarma, 2002). A subpopulation of exicatory enteric neurons is lost in the small intestine of GFRα2- and NRTN-deficient mice and the motility of the gut shows impairment *in vitro* (Heuckeroth et al., 1999; Rossi et al., 1999). Interestingly, NRTN/GFRα2/Ret signaling has also proven to be important for adult cholinergic sympathetic neurons as three-week-old and adult GFRα2-deficient mice have a reduced number of cholinergic sympathetic neurons and soma size in footpad sweat glands in comparison to wild-types (Hiltunen and Airaksinen, 2004).

GFRα2-deficient mice experience growth retardation after birth compared to wild-type littermates, which is most pronounced at 4-6 weeks (Rossi et al., 1999; Rossi et al., 2003), while this phenotype is not observed in the NRTN-deficient mice (Heuckeroth et al., 1999). The differences between GFRα2-and NRTN-deficient mice in growth, even though fed with the same diet and bred almost in the same background, suggest that GFRα2/Ret signals yet through another ligand *in vivo* (Wanigasekara et al., 2004).

Although the morphology of the CNS in GFRα2-deficient mice appears normal, the GFRα2-deficient mice display a suppressed response to epileptic stimulus in a hippocampal kindling model (Nanobashvili et al., 2000), which further suggests functional roles for GFRα2 and NRTN in the brain.

1.6.3 ARTN and GFRα3 control sympathetic neuronal migration and initial axon growth

As substantial sympathetic defects were found in the homozygous RET knock-out mice (Durbec et al., 1996; Enomoto et al., 2001), it was logical to assume that GFRα3 and ARTN are important signaling mediators in the development of sympathetic ganglia. Profound deficits in the superior cervical ganglion (SCG) and other sympathetic ganglia are found in homozygous ARTN- and GFRα3-deficient mice (Andres et al., 2001; Honma et al., 2002; Nishino et al., 1999) (Table 2). These mice are viable and fertile and show no obvious defects in major organs, but 30% of the mice suffer from unilateral or bilateral ptosis caused by loss of sympathetic innervation from the SCG of the superior tarsus muscle (Nishino et al., 1999). The SCG on the ipsilateral side of the eye affected by ptosis is lacking or smaller in size and more caudally located. Newborn GFRα3- and ARTN-deficient mice exhibit smaller and aberrantly segmented sympathetic chain ganglia with a deficiency in axonal growth. It is well established that sympathetic axons follow blood vessels to reach their targets and it is now clear that ARTN expressed by smooth muscle cells in the wall of blood vessels probably acts as a chemoattractant for the developing sympathetic axons (Honma et al., 2002). It is thus suggested that ARTN is critical for guiding the migrating embryonic sympathetic neuroblasts to their targets and for guiding the axons to their innervating target organs, whereas other neurotrophic factors including NGF and

NT-3, are important for their postnatal survival and target innervation (Airaksinen and Saarma, 2002; Honma et al., 2002a). Compensatory mechanisms in the sympathetic nervous system must exist as most tissues eventually become at least to some part innervated by sympathetic neurons in the ARTN- and GFRα3-deficient mice.

1.6.4 PSPN is suggested to protect the brain from ischemic insult

PSPN-deficient mice are viable and fertile and exhibit no gross abnormalities in peripheral tissues or in the central nervous system (Tomac et al., 2002) (Table 2). Furthermore, extensive behavioral tests showed no significant abnormalities between PSPN-knockout mice and wild-type littermates. However, homozygous PSPN-deficient mice demonstrated an increased sensitivity to experimentally induced focal cerebral ischemia (Tomac et al., 2002). Furthermore, pretreatment of PSPN-deficient mice with low dose of PSPN (0.1 μg and 0.5 μg) before middle cerebral artery occlusion (MCAO) was shown to markedly reduce the infarction size and enhanced vertical locomotor activity compared to vechile-treated animals. Interestingly, a high dose of PSPN (10 μg) seemed toxic, as it increased the infarction volume. *In vitro,* low doses of PSPN were also able to inhibit cell death of cortical neurons after hypoxia and reoxygenation (Tomac et al., 2002). These results suggest PSPN signaling through mammalian GFRα4/Ret or yet another receptor. The *in vivo* effects of the mouse *Gfra4* gene ablation will be discussed in the results and discussion part of this thesis (III).

1.7 RET-dependent signaling

1.7.1 Assembly of the GFL/GFRα/Ret complex

GFLs are known to bind to a preferred GFRα co-receptor and activate the transmembrane receptor Ret (Baloh et al., 1997; Baloh et al., 1998b, Enokido et al., 1998; Jing et al., 1996; Klein et al., 1997; Treanor et al., 1996; Trupp et al., 1996). However, some cross-talk, at least *in vitro*, can occur between receptors and ligands (Fig. 2). NRTN and ARTN show weak interaction with GFRα1, and GDNF with GFRα2 (Baloh et al., 1997; Baloh et al., 1998a, Baloh et al., 2000; Klein et al., 1997; Sanicola et al., 1997; Suvanto et al., 1997; Wang et al., 1998). The physiological role for the cross-talk is however not understood. Since Ret cannot bind GDNF on its own, a model was first proposed that GDNF dimer binds GFRα1 monomer/dimer recruiting thereafter two Ret monomers into the complex leading to c-Ret dimerization and activation of the tyrosine kinase (Jing et al., 1996). However, it has been also suggested that GFRα-receptors and Ret are arranged in a preformed complex and that ligand binding stabilizes the complex (Eketjäll et al., 1999; Klein et al., 1997; Scott and Ibáñez, 2001; Treanor et al., 1996). The latter part of this hypothesis is supported by competition binding experiments, showing higher affinity of GDNF to GFRα1 in the presence of Ret (Cik et al., 2000; Leppänen et al., 2004). Clearly, further studies are needed to understand the molecular events of GDNF/GFRα1/Ret interaction.

Mutagenesis studies revealed 9 amino acid residues (of which 5 are conserved between other GFLs) on finger 1 and 2 of

GDNF that are critical for binding to GFRα1 (Eketjäll et al., 1999) (Fig. 1A). Studies with truncated or chimeric GFRα-receptors furthermore showed that the N-terminal cysteine-rich domain (D1) is dispensable for ligand binding to GFRα-receptors and Ret phosphorylation (Scott and Ibáñez, 2001). However, binding of GDNF is reduced in GFRα1 lacking D1, which could be caused by a loss of one of the two heparan sulphate binding consensus sequences or by loss of interaction with other receptors (Barnett et al., 2002; Scott and Ibáñez, 2001). Heparan sulphates which are borne by proteoglycans on the cell surface have been shown to be important for GDNF binding to GFRα1 in cell lines and suggested to concentrate the ligand in the vicinity of the receptor or to stabilize the ligand-receptor complex (Barnett et al., 2002; Rickard et al., 2003). Distinct ligand binding epitopes in domains 2 and 3 of GFR1α have been demonstrated to be important for ligand binding and Ret activation (Scott and Ibáñez, 2001). Furthermore, the highly conserved amino acid triplets ^{211}MLF and ^{224}RRR in D2 domain of GFRα1 show complementary properties to the receptor binding surface identified in GDNF (Eketjäll et al., 1999; Scott and Ibáñez, 2001). In agreement with previous results, a recent study based on crystal structure of D3 and homologous domain modeling of D2 of rat GFRα1, combined with site-directed mutagenesis studies, revealed that Phe213 of the MLF triplet, Arg224 and Arg225 of the RRR triplet and in addition Ile229 generate a putative binding surface for GDNF (Leppänen et al., 2004). The GFRα1 Arg224 and Arg225 as well as a hydrophobic residue at 229 are fully conserved between GFRα-receptors, which suggest that all GFRα-GFL complexes share these interaction sites (Leppänen et al., 2004). However, future studies are needed to identify the sequence and nature of interaction between the other GFL-GFRα complexes and Ret.

Ret activation requires both ligand and GFRα-receptor binding. A recent mutagenic analysis of the extracellular domain of human RET showed that three of the four N-terminal cadherin-like domains 1-3 (CLD1-3) are required for ligand binding, whereas the most important ligand determinants appeared to be in CLD1 (Kjaer and Ibáñez, 2003). Calcium binding to the extracellular Ret^{Ca2+}-binding site between CLD2 and CLD3 is crucial for GDNF- and NRTN-induced GFL/GFRα/RET oligomerization, for RET phosphorylation, to stabilize the signaling complex and for the transport of fully maturated Ret to the plasma membrane (Anders et al., 2001; Nozaki et al., 1998; van Weering et al., 1998).

According to the original model by Jing and co-workers where GFLs/GFRαs bind and activate Ret, both receptors should be expressed and interact on the same cell (Jing et al., 1996). This *in cis* model is supported by the overlapping expression of the Ret receptor and at least one of the GFRα-receptors in the same cell population (Golden et al., 1998; Golden et al., 1999; Rossi et al., 2000; Widenfalk et al., 1997; Widenfalk et al., 1999) (Fig. 3A). The ligand can act as a trophic factor or morphogen when the preferred ligand for the GFRα/Ret complex is expressed in cells complementary to those expressing GFRα and Ret, for example in the target organ for innervating neurons (Nosrat et

al., 1996; Rossi et al., 2000; Trupp et al., 1997; Widenfalk et al., 1997) or in the mesenchyme of the developing ureteric bud (Nosrat et al., 1996; Sainio et al., 1997; Suvanto et al., 1996). However, *Gfra1* and *Gfra2* mRNA levels are high in brain regions such as in the adult mouse cortex not found to express *Ret* (Golden et al., 1998; Trupp et al., 1997), indicating different Ret-independent signaling mechanisms. However, several *in vitro* experiments have shown that GFRα-receptors can be released from the cell surface by cleavage of the GPI-anchor and act as soluble receptors presenting the ligand to Ret located in the membrane of another cell *in trans* (Jing et al., 1996; Klein et al., 1997; Treanor et al., 1996) (Fig. 3B). GFRα1 has in fact been shown to be released into the conditioned medium after lesion of *ex vivo* cultured sciatic nerve, suggesting that GFRα1 could be released also *in vivo* at least as injury response (Paratcha et al., 2001). Furthermore, supernatants containing released GFRα1 from tissue explants have been shown to transactivate Ret in the presence of GDNF in sympathetic neurons (Ledda et al., 2002). Trans-activation of Ret also occurs in cell lines after GDNF treatment, even though GFRα1 is immobilized on beads, on surface or on other cells (Yu et al., 1998; Paratcha et al., 2001).

Most GPI-linked proteins are suggested to be localized to specialized detergent-resistant membrane fractions, called lipid rafts. These rafts are reported to be floating membrane platforms in the exoplasmic leaflet of the membrane

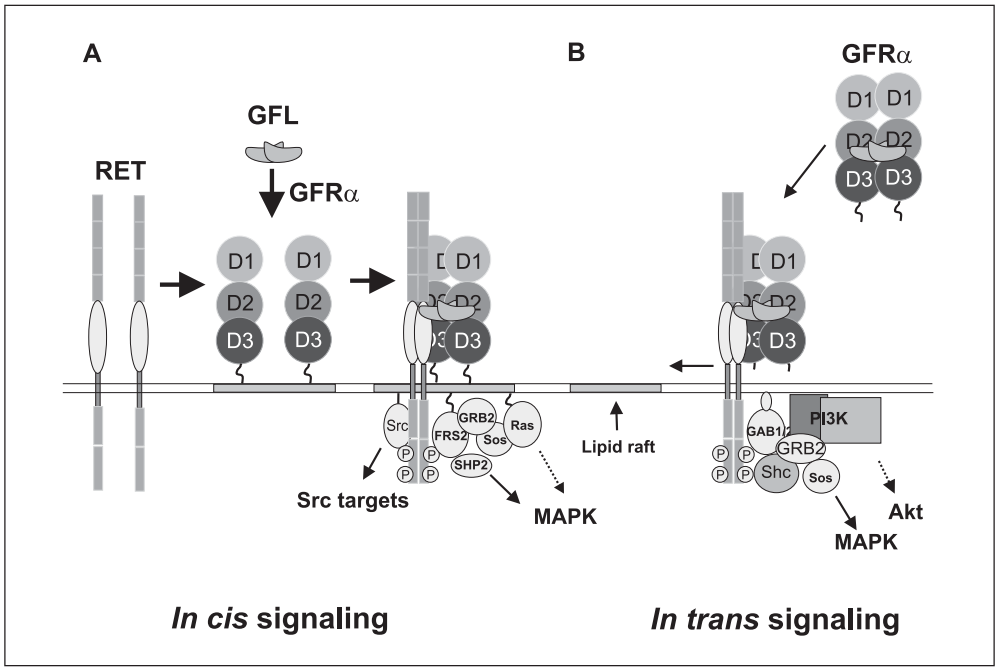

Figure 3. GFL signaling in lipid rafts (in cis) and outside lipid rafts (in trans). Inactive RET is located outside rafts but upon stimulation GFRα1 recruits RET by an unknown mechanism to the rafts. Activated RET in lipid rafts is preferentially associated with FRS2, whereas outside rafts RET is associated with Shc. Adapted from (Airaksinen and Saarma, 2002).

bilayer that are rich in cholesterol and sphingolipids (Simons and Ikonen, 1997). Many intracellular signaling molecules may be attached to these lipid rafts such as Src-family kinases (SFK) and small GTPases (eg. H-Ras), and have been implicated as signaling platforms for various transmembrane and GPI-linked proteins (Saarma, 2001; Simons and Toomre, 2000). However, the lipid raft concept is based on biochemical detergent extraction studies, and no direct visual evidence of their existence has yet been presented (Munro, 2003). Reports based on biochemical studies have shown that the GPI-linked GFRα1 is localized to lipid rafts and that GDNF bound to GFRα1 rapidly recruits Ret from the non-raft compartment of the cell membrane into the lipid rafts *in cis* (Tansey et al., 2000). Soluble GFRα1 bound to GDNF, is also able to recruit Ret to lipid rafts *in trans,* but in a more sustained manner which requires Ret activation (Paratcha et al., 2001) (Fig. 3). There is evidence that TGF-β acts in a synergistic way with GDNF to promote the survival of PNS and some CNS neurons (Krieglstein et al., 1998). TGF-β may cause clustering of GFRα1 receptors to lipid rafts and in that way aids in GDNF recognizing its receptor (Peterziel et al., 2002). However, the mechanism by which GDNF-signalling is assisted by TGF-β is still unknown.

1.7.2 RET tyrosine kinase activation

Upon ligand binding to GFRα-receptors and Ret, two transmembrane Ret molecules dimerize and trans-phosphorylate cytoplasmic tyrosines. The cytoplasmic domain of the long isoform of RET (RET51) contains 18 tyrosine residues of which 14 are known to be autophosphorylated, whereas 12 tyrosines out of 16 are identified as autophosphorylated in the short isoform of RET (RET9) (Encinas et al., 2004; Liu et al., 1996). These autophosphorylated tyrosines serve as docking sites for various proteins with phosphotyrosine binding (PTB) motifs or Src-homology 2 (SH2) domains which determine the pathways leading to survival, neurite outgrowth, differentiation etc. (Kurokawa et al., 2003). Several important cell signaling cascades are activated by RET transphosphorylation. Phosphorylated tyrosine 905 binds GRB7 (growth factor receptor- bound protein 7) and GRB10, pTyr1015 binds PLCγ (phospholipase Cγ), and pTyr1096 only present on RET51 isoform binds GRB2. The phosphorylated tyrosine 1062 present in both isoforms serves as a docking site for many effector proteins including Shc (SH2 domain protein C1), FRS2 (FGF receptor substrate), IRS1 (insulin reseptor substrate 1) and DOK4/5 (downstream of tyrosine kinase 4/5), and is a crucial docking site for signaling during embryogenesis and carcinogenesis (reviewed by Ichihara et al., 2004; Manié et al., 2001; Takahashi, 2001) (Fig 4). This tyrosine includes a PTB binding motif NXXpY (Kurokawa et al., 2003). Enigma, a protein consisting of a PDZ-domain and three LIM-domains, can bind to Tyr1062 in RET9 but poorly to RET51 regardless of its phosphorylation status (Borrello et al., 2002). Enigma may be involved in the clustering and recruitment of RET to the plasma membrane (Durick et al., 1996). GDNF induces intracellular signaling cascades in Ret which include RAS/mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-

kinase (PI3K)/AKT, p38 MAPK and c-Jun N-terminal kinase (JNK), pathways known to be activated also by other protein kinases. Activation by GDNF of both the MAPK and the PI3K/AKT pathways requires binding of Shc adaptor protein to the pTyr1062 of RET which further associates with GAB1/2 and GRB2 to activate the RAS/ERK or the PI3K/AKT pathways. The MAPK signaling pathway mediated by SHC binding to pTyr1062, results in activation of downstream targets GRB2+Sos, Ras, ERK1/2 and transcription factor cAMP responsive element (CREB), whereas FRS2 binding to pTyr1062 results in activation of SHP2 tyrosine phophatase, ERK and CREB (Hayashi et al., 2000). FRS2 is a lipid anchored docking protein proposed to activate the MAPK signaling cascade mainly after recruiting Ret to the lipid rafts by GDNF/GFRα1, whereas Shc may be associated to activated Ret outside the lipid raft in order to keep balance

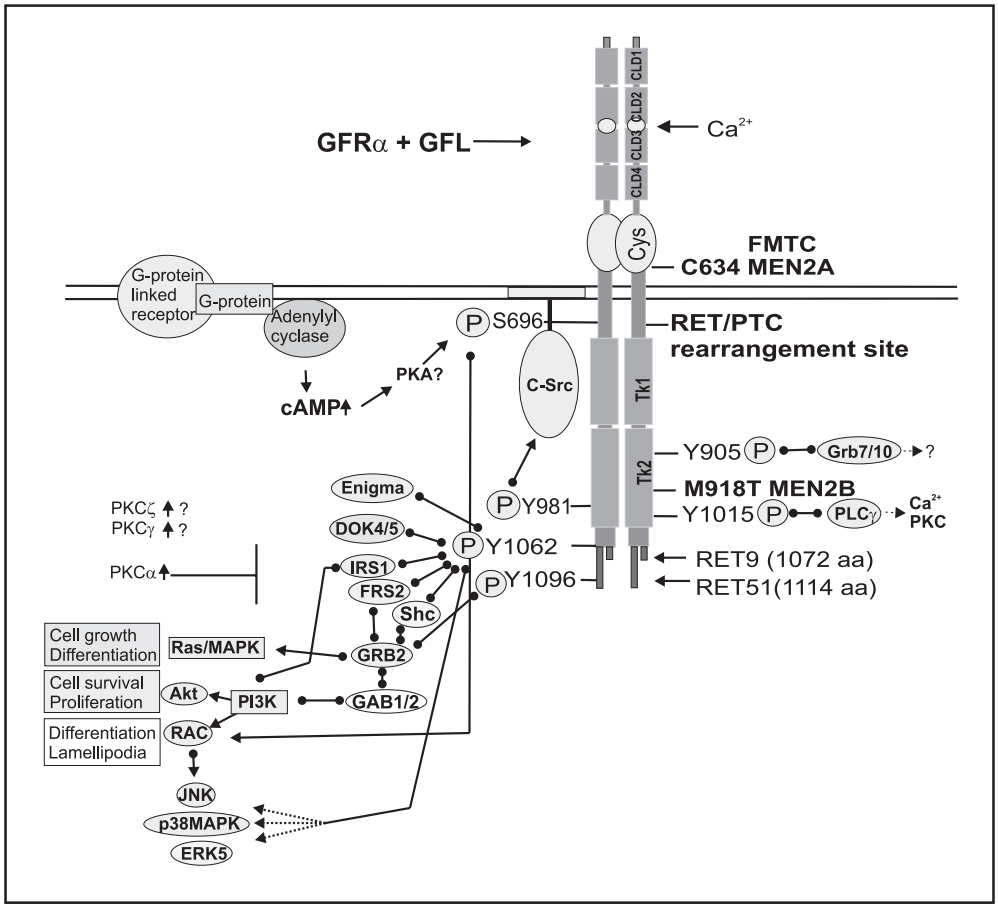

Figure 4. Intracellular pathways through activated RET and sites for activating mutations in RET. Transphosphorylation of intracellular tyrosines in RET generate binding sites for various adapter molecules which specify the down-stream signaling pathway and biological response.

between the two competing signaling pathways (Kurokawa et al., 2003; Paratcha and Ibáñez, 2002) (Fig. 3). The binding of FRS2 is suggested to be necessary for sustained MAPK activation and neuronal differentiation. The MAPK pathway is important for neurite outgrowth and survival, neuronal differentiation and ureteric epithelial branching morphogenesis (Califano et al., 2000; Fisher et al., 2001; De Vita et al., 2000).

Recruitment of Shc to pTyr1062 also results in the phosphorylation PI3K and downstream activators serine-threonine kinase Akt (protein kinase B) and focal adhesion kinase (FAK) activation which both activate transcription factor NFκB. pTyr1096 seems to activate only the PI3K/AKT pathway although it binds GRB2 (Bessett et al., 2000). The PI3K/AKT pathway has proven to be important in neurite lamellopodia formation, neuronal survival, neurite outgrowth and cell proliferation (Fukuda et al., 2002; Sariola and Saarma, 2003). Nerve growth cones are enriched in Src family kinases and the activation of Ret by ligand generates Src-SH2 docking site on Ret irrespective of the localization of the receptor (Tansey et al., 2000). Recently, the major binding site for c-Src was localized to pTyr981 in Ret (Encinas et al., 2004) (Fig. 4). C-Src has been shown to be essential for GDNF- but not NGF-induced maximal neuronal survival and differentiation signaling (Encinas et al., 2001), which implies a RET specific neuronal SFK response.

Lamellopodia formation in neurons by GDNF is controlled through at least two different signaling pathways, via tyrosine 1062 and serine 696 in Ret (Fukuda et al., 2002). The phosphorylation of Ser696 was shown to be mediated by protein kinase A (PKA) through elevated intracellular cAMP-levels regulated by unknown G-protein-linked receptors. This result suggests cross-talk with Ret and G-protein-linked receptors in the regulation of cytoskeletal structures. Furthermore, cross-talk between Ret and TrkA receptor has been observed, as NGF promoted Ret phosphorylation without GFL stimulation in sympathetic postnatal neurons (Tsui-Pierchala et al., 2002b). Among RET autophosporylated tyrosines and phosphorylated serines/threonines, additional important signaling and inhibiting pathways mediated by RET are likely to be discovered in the future.

1.8 RET-independent GFL/GFRα signaling

In many brain areas especially in the forebrain, cortex and inner ear, GFRα receptors are more widely expressed than Ret (Kokaia et al., 1999; Trupp et al., 1997; Ylikoski et al., 1998), which suggests that GFLs can signal independently of Ret in collaboration with other transmembrane receptors. GDNF treatment of a cell line lacking Ret but expressing GFRα1 resulted in Src family kinase (SFK)-dependent CREB phosphorylation, leading to a fast upregulation of transcription factor c-fos mRNA (Trupp et al., 1999). Additionally, GDNF was shown to induce SFK activation, PLCγ, CREB and MAPK phosphorylation in DRG neurons derived from homozygous Ret-deficient embryos and in Ret-negative cell lines (Poteryaev et al., 1999). However, the mechanisms of how intracellular SFKs are activated by GDNF binding to extracellular GFRα1 remained unclear. The finding that

exogenous GDNF is able to increase branching morphogenesis in kidney explants from $Ret^{-/-}$ mice led to the discovery of an alternative signaling receptor for GDNF, namely the receptor tyrosine kinase Met (Popsueva et al., 2003). Hepatocyte growth factor (HGF), the ligand for Met, induces scattering, tubule formation and chemotaxis of Met expressing dog epithelial MDCK cells. GDNF was shown to activate Met and induce tubulogenesis through GFRα1 and SFK in MDCK cells which lack endogenous Ret receptor (Popsueva et al., 2003). However, the activation of Met through SFK was not mediated by direct binding of GFRα1/GDNF to Met, and the role for GDNF-induced Met activation *in vivo* is still unresolved.

In a recent study, exogenous GDNF was able to induce Schwann cell migration and axonal growth of hippocampal and cortical neurons from Ret-deficient mice known to express GFRα1 and the neural cell adhesion molecule NCAM (Paratcha et al., 2003). GDNF was shown to bind NCAM with high affinity in the presence of GFRα1 and to rapidly activate cytoplasmic SFK, Fyn as well as focal adhesion kinase (FAK) in cells lacking Ret. This suggested that NCAM is an alternative binding and signaling receptor for GDNF and GFRα1. Furthermore, GFRα1 was able to associate with NCAM in the absence of GDNF and downregulate NCAM-mediated cell adhesion, indicating a ligand independent role for GFRα receptors. Interestingly, NCAM knock-out mice and heterozygous GDNF-deficient mice have impairments in spatial learning (Cremer et al., 2000; Gerlai et al., 2001). Moreover, mice lacking GFRα2 have impaired behavioral flexibility and recall in different memory and learning tasks (Võikar et al., 2004). These results indicate that GFLs and GFRαs can regulate neuronal synaptic plasticity through NCAM in the developing and postnatal brain. The survival effect of GDNF on dopaminergic neurons could be inhibited by using blocking antibodies to NCAM, which further supports the importance of NCAM in GDNF signaling (Chao et al., 2003). Furthermore, there may be interplay between Ret and NCAM signaling by GFLs in cells expressing both receptors or even between adjacent recepors expressed by different cells *in trans*.

1.9 RET in disease

1.9.1 Loss-of-function mutations in RET cause developmental enteric nervous system defects

Hirschsprung disease (HSCR) or congenital aganglionosis of variable segments of the large bowel in humans is a relatively common disorder (1/5000 births), leading to intestinal obstruction or chronic constipation usually already at birth (reviewed by Parisi and Kapur, 2000). Most HSCR cases are sporadic, with 10-15% being familial forms. Mutations in different genes, many of which are involved in the RET and endothelin signaling pathways, have been associated to HSCR. Mutations in the *RET* gene accounts for 50% of the familial cases and for 10-20% of the sporadic cases. These mutations constitute deletions, insertions, missense, nonsense, and frameshift mutations. On the basis of the localization of a *RET* mutation, these can be divided into two

main types types; those that impair the RET cell surface expression and those that impair the activation of the RET tyrosine kinase (reviewed by Iwashita et al., 2001; Manié et al., 2001; Takahashi, 2001). All these mutations are likely to affect the GDNF-mediated enteric precursor cell migration from the neural crest, and they are believed to be one causative means in HSCR. Furthermore, premature cell death of enteric neural crest cells in HSCR may be caused by a recently discovered death pathway mediated by binding of caspase-3 directly to the kinase domain of RET which mediates cell self-destruction in the absence of impaired ligand activation (Bordeaux et al., 2000). Germline mutations in the genes encoding for GFRα1-GFRα4 are not major contributors to HSCR (Borrego et al., 2003). Although mutations in the *GDNF* and *NRTN* genes have been found in only a minority of HSCR cases, only a few mutations affect the ligand binding to GFRα-receptor (Eketjäll and Ibáñez, 2002). However, they may in conjuction with other lesions contribute to the pathogenesis of HSCR.

1.9.2 Gain-of-function mutations in RET promote thyroid tumors

Ret mRNA is expressed already in neural crest cells of the developing nervous system (Pachnis et al., 1993). High expression of *RET* is also detected in many tumors of neural crest origin (Miya et al., 1992; Nakamura et al., 1994; Santoro et al., 1990). Germline missense mutations in the *RET* gene cause multiple endocrine neoplasia type 2A and 2B (MEN2A and MEN2B) syndromes and familial medullary thyroid carcinoma (FMTC) (Table 3). The MEN2A syndrome is characterized by multifocal and bilateral medullary thyroid carcinoma (MTC) of the parafollicular C-cells, pheochromocytoma (pheo, 50%) of the adrenal medulla and hyperparathyroidism (20%), all with a neural crest origin. MEN2A is the most common form of MEN2 syndromes (90%) with a clinical onset below 20 years of age. MEN2B accounts for approximately 5% of all MEN2 cases but is associated with a more aggressive disease progression and an earlier tumor onset (<10 years of age). MEN2B patients suffer from MTC, pheochromocytoma (50%), intestinal and mucosal ganglioneuromatosis, musculoskeletal abnormalities including marfanoid features, and a male reproductive defect (reviewed by Bachelot et al., 2002; Ponder, 1999). The only disease phenotype in FMTC is bilateral, multifocal MTC with a later onset than in MEN2A and MEN2B and with a good prognosis. Missense mutations involved in the *RET* gene of MEN2A and FMTC are mainly clustered to exons 10 and 11, coding for the extracellular cysteine rich juxtamembrane domain. Cysteine substitutions in FMTC families occur frequently in codons 609, 611, 618, 620, 630 and 634 (Ponder and Smith, 1996). FMTC mutations are also identified in the tyrosine kinase domain of RET at codons 768, 790, 791, 804 and 891. The most common mutation found among MEN2A families is Cys634 substituted by Arg (52%) or Tyr (26%), but mutations in cysteines 609, 611, 618, 620 and 630 are also known to cause MEN2A. MEN2B is mainly caused by a single substitution of a methionine residue at position 918 to a threonine (95%) in the tyrosine kinase

domain, but rare MEN2B cases with mutations in codons 883 and 922 have also been reported. Approximately 75% of the diagnosed MTCs are sporadic with no familial background and 30-70% of these are reported to contain a somatic RET mutation which most frequently is the M918T mutation also found in MEN2B families (reviewed by Eng and Mulligan, 1997).

Papillary thyroid carcinoma (PTC) is a sporadic and somatic tumor derived from follicular epithelial cells of the thyroid. PTC is frequently associated with chromosomal rearrangements joining together the 5´end of a heterologous gene to the cytoplasmic tyrosine kinase domain of RET, generating a chimeric oncogene designated RET/PTC (reviewed by Santoro et al., 1999; Takahashi, 2001). RET is normally silent in thyroid follicular cells but production of RET/PTC oncoproteins can be driven by the promoter of the fusion partner. The chimeric proteins do not usually contain a signal sequence for membrane transport. Thus the dimerized RET/PTC proteins remain in the cytoplasm, activating RET specific signaling pathways leading to tumorigenesis. To date, 9 different fusion partner genes have been found to be associated with the RET kinase domain in RET/PTC rearrangements in which the RET/PTC1 and RET/PTC3 are the most frequently found with activating genes H4 and ELE1 (Pierotti et al., 1996; Santoro et al., 1994). The RET/PTC rearrangements are found with a highly variable frequency among different countries and populations, suggesting that genetic background and environmental factors play a role in their birth. Additionally, RET/PTC is likely to develop in the thyroid gland as a direct consequence of radiation exposure and the ability of thyroid follicular cells to concentrate radioactive iodine (Santoro et al., 2000).

Table 3. Clinical features associated with MEN2 syndromes

MEN2 type	MEN2A	MEN2B	FMTC
Medullary thyroid cancer	70-80% early onset (age)	100% earliest onset (age)	100% late onset
Pheochromocytoma	50% C634any	50%	-
Parathyroid hyperplasia	20-30% C634R	-	-
Other phenotypes	-	Mucosal neuromas Musculoskeletal abnormalities Impotence Infertility	-
RET mutation	Extracellular C634 85%	Intracellular or TK2* M918T (95%) **	Cys-rich domain or TK1* C634other (not R 30%)

*TK1 and TK2; Tyrosine kinase domain

** Most somatic RET mutations in MTC (>70%) are of MEN2B (M918T) type

1.9.3 Oncogenic Ret signaling

The signaling mechanisms for MEN2A, MEN2B and RET/PTC oncoproteins have been extensively studied in murine fibroblasts over-expressing respective oncoproteins. These are well known tools used in cancer studies because of their capacity to form foci in culture (Asai et al., 1995; Santoro et al., 1994; Santoro et al., 1995). A cell line derived from a metastatic MTC tumor heterozygous for a RET MEN2A mutation, known as TT, and a rat pheochromocytoma cell line, PC12, are also widely used for RET *in vitro* oncogenesis studies (Borrello et al., 1995; Carlomagno et al., 1995). In contrast to the ligand-induced Ret, the MEN2 and RET/PTC proteins are constitutively active. Upon activation, the cysteines in the extracellular Cys-rich region form intermolecular disulfide bridges in wild type RET, whereas the unpaired cysteines in MEN2A RET are believed to form intramolecular covalent disulfide bonds. This leads to constitutive dimerization of two RET MEN2A molecules and thereby constitutive transphosphorylation of tyrosines in the kinase domain (Santoro et al., 1995). The activation is thought to resemble that of the ligand induced RET activation. The weaker transforming activity by FMTC and some MEN2A mutations is thought to depend on a misfolding of the oncoproteins which results in an impared cell surface expression (Chappuis-Flament et al., 1998; Takahashi et al., 1999). The mechanism of MEN2B RET activation differs from that of MEN2A, as MEN2B and intracellular FMTC proteins are constitutively active as monomers (Santoro et al., 1995). The tyrosine kinase domain of RET is highly conserved among other tyrosine kinases and especially those motifs that are involved in ATP-binding, catalytic activity and substrate binding (Hubbard and Till, 2000; Scott, 2002). The autophosphorylation of tyrosines in the activation loop induces drastic changes in the conformation of the activation loop (Kawamoto et al., 2004). The MEN2B M918T mutation is thought to change the substrate specificity of the activation loop in the RET kinase domain, as Src family and most others non-receptor tyrosine kinases also have a threonine residue at this position (Zhou et al., 1995; Santoro et al., 1995; Songyang et al., 1995). Accordingly, c-Src is phosphorylated and associated with RET oncoproteins (Melillo et al., 1999). Additionally, dominant-negative Src has been shown to reduce tumorigenic activation in a tumor caused by a homologous MEN2B mutation in the MET receptor tyrosine kinase (Nakaigawa et al., 2000). These studies suggest a critical role for Src in MEN2B mediated cell transformation. The distinct activities of MEN2A and MEN2B RET oncoproteins are supported by phosphopeptide mapping analysis, downstream target protein activation and differential display analysis of genes that are induced or suppressed by MEN2A and MEN2B proteins (Bocciardi et al., 1997; Liu et al., 1996; Murakami et al., 1999; Murakami et al., 2002; Salvatore et al., 2001; Watanabe et al., 2002). The more aggressive phenotype in MEN2B might be explained by the stronger activation of the JNK-pathway through the association of pY1062 with Dok1 and Nck1 (Murakami et al., 2002). Upregulation of the stanniocalcin I (*STC1*) gene in MTC of MEN2B patients suggest that stanniocalcin might be

involved in the skeletal phenotype in MEN2B (Watanabe et al., 2002). GDNF is further reported to enhance the phosphorylation status of the MEN2A and MEN2B oncoproteins (Bongarzone et al., 1998; Borrello et al., 1995; Carlomagno et al., 1998; Mograbi et al., 2001). Tyrosine 1062 activation and subsequent binding of Shc and FRS2 adaptor proteins have both *in vivo* and *in vitro* proven to be crucial for the activation of PI3K/Akt and MAPK pathways in MEN2A, MEN2B and RET/PTC oncogenic signaling (Asai et al., 1996; Hayashi et al., 2000; Iwashita et al., 2000; Kurokawa et al., 2001; Ludwig et al., 2001; Melillo et al., 2001; Salvatore et al., 2000; Segouffin-Cariou and Billaud, 2000; De Vita et al., 2000). In addition, a double substitution of Y900 and Y905 to phenylalanine in RET MEN2A completely abolished the kinase activity, whereas the kinase activity and transforming activity was impaired in the MEN2A mutants Y806F and Y809F (Kawamoto et al., 2004).

RET9 and RET51 isoforms appear to be equally co-expressed in MTC and pheochromocytomas (Myers et al., 1995), although the oncoproteins of RET isoforms have different transforming activities. *In vitro*, the RET51 MEN2B and FMTC oncoproteins have been reported to have a more potent transforming ability than RET9 and can induce a more robust neurite outgrowth in PC12 cells (Iwashita et al., 1999; Pasini et al., 1997; Rossel et al., 1997).

To date, the primary treatment of tumors developed in MEN2 syndromes and sporadic thyroid tumors (MTC and PTC) is restricted to surgical removal of the tumor or gland. Specific molecules known to downregulate RET activation have been suggested to function as potential therapeutic agents for these endocrine tumors. Grap-2, a novel RET-binding protein has been shown to downregulate NF-κB activation and specifically reduce the focus formation ability induced by oncogenic RET (Ludwig et al., 2003). PP1, a pyrazolo-pyrimidine inhibitor was shown to induce oncoprotein MEN2A and MEN2B degradation and therefore been proposed as an efficient drug in the treatment of MTC tumors (Carniti et al., 2003). Indolocarbazole derivatives, CEP-701 and CEP-751 are able to effectively block RET phosphorylation and cell growth in MTC cell culture and xenographs (Strock et al., 2003). Furthermore, a specific orally available anilinoquinazoline tyrosine kinase inhibitor can effectively block RET oncoprotein phosphorylation in cell lines and RET/PTC3-induced tumor formation in nude mice (Carlomagno et al., 2002). It may thus be a promising drug in the treatment of RET-induced tumors, especially medullary thyroid carcinoma which responds poorly to conventional chemotherapeutics.

1.9.4 Proposed mechanisms for the tissue-specific tumor formation in MEN2

One major unresolved question concerning the roles of RET MEN2A and MEN2B in the development of endocrine tumors is why tumor formation is restricted only to distinct tissues in the syndromes, although the mutated heterozygous RET is expressed in many other types of cells during development (Pachnis et al., 1993; Tsuzuki et al., 1995).

Several possible explanations have been proposed. RET MEN2 activation in cell lines derived from different origin has been shown to lead to distinct cellular responses suggesting that RET MEN2 may be capable of tumor formation only in distinct cells (Califano et al., 1996; D'Alessio et al., 1995; Rizzo et al., 1996). Takahashi and co-workers suggested that co-expression of GFRα-receptors would interfere with the dimerization of at least MEN2A proteins and therefore lead to reduced and controlled signaling of RET in tissues co-expressing GFRα and RET (Kawai et al., 2000). In agreement with this, GFRα1 was co-immunoprecipitated with MEN2A RET in the presence of GDNF in a cell line transfected with MEN2A RET and GFRα1 cDNAs (Mograbi et al., 2001). Leukocyte common antigen-related protein (LAR), a cell surface protein-tyrosine phosphatase, was shown *in vitro* if overexpressed to reduce the constitutive tyrosine autophosphorylation and kinase activity of RET MEN2A and MEN2B proteins by interfering with the dimerization of RET (Qiao et al., 2001). Thus, it seems likely that expression of certain cell surface proteins in affected cells can modify the clinical phenotypes of MEN2 syndromes by impairing the RET dimerization. Interestingly, the LAR gene is localized to a region in the short arm of chromosome 1 known to be frequently deleted in tumors of neuro-endocrine origin (Jirik et al., 1992; Mulligan et al., 1993).

Furthermore, similarly to wild type RET, MEN2A RET was shown to promote the survival of neuroendocrine cells in the presence of GDNF and GFRα1, but triggered apoptosis when GDNF/GFRα1 was omitted (Mograbi et al., 2001). GDNF has also been shown to inhibit the proliferation of an embryonic carcinoma cell line NT2/D1 with an accompanying increase in levels of cyclin-dependent kinase inhibitor p27kip1, leading to a growth arrest and subsequent neuronal differentiation (Baldassarre et al., 2002). This indicates that at least those neural crest-derived cell lineages that co-express RET MEN2A and GFRα1 are able to respond to endogenous GDNF stimulation by differentiation rather than proliferation and are therefore able to develop normally.

Endocrine cells affected in MEN2 syndromes might be more susceptible to tumor formation caused by inactivation of the tumor suppression system. The retinoblastoma susceptibility gene (Rb) product is a tumor suppressor inactivating E2F transcription factor that is known to be important for activating genes involved in cell-cycle progression (Harbour and Dean, 2000). Interestingly, Rb+/- mice developed MEN2-like syndromes with tumors of the thyroid C-cells, adrenal gland, intermediate lobe of the pituitary and parathyroid (Nikitin et al., 1999). Furthermore, some Rb +/- mice showed a missense mutation in the *RET* gene corresponding to human MEN2A/FMTC cysteine rich domain (Coxon et al., 1998). MTC-samples from the corresponding mice did not stain for Rb, which suggests a multistep progression in the formation of MTC. However, RB downregulation in human endocrine tumors has not yet been clearly demonstrated.

Recently, the constitutively phosphorylated tyrosine 1062 in oncogenic forms of RET/PTC has been shown to transmit not only mitogenic, but also proapototic signals in rat thyroid

epithelial cells by downregulation of the antiapototic protein Bcl-2 and upregulation of the apoptotic protein Bax through the Ras/ERK pathway (Castellone et al., 2003). Thus it might be possible that cells involved in MEN2 syndromes have aquired a lower threshold for malignancy than other cells through their tendency to escape the apoptotic pathway.

1.9.5 RET MEN2 mutations in transgenic mice models

Several transgenic mice models expressing the corresponding human MEN2A or MEN2B RET mutation under tissue-specific promoters have been created in order to address issues concerning the role of RET in the pathophysiology of MTC and sympathoadrenal tumors (Acton et al., 2000; Kawai et al., 2000; Michiels et al., 1997; Reynolds et al., 2001; Smith-Hicks et al., 2000; Sweetser et al., 1999). Transgenic mice expressing multiple copies of RET9 or RET51 MEN2A or MEN2B in thyroid C-cells developed C-cell hyperplasia and subsequent bilateral MTC accompanying high levels of serum calcitonin (CT) (Acton et al., 2000; Michiels et al., 1997). However, tumor phenotype was shown to differ between transgene copy lines. Since transgenic mice expressing the corresponding wild type allele for RET did not develop tumors, it was evident that overexpression of RET51 was not responsible for the tumors found in the RET mutant mice (Reynolds et al., 2001). Furthermore, tumors were not found in tissues expressing the transgenic MEN2 *RET* but co-expressing *Gfra1*, *Gfra2* or *Gfra3* (Kawai et al., 2000). In addition, in western blot analysis disulfide-linked MEN2A homodimers were detected in non-reducing conditions from MTC tissue, but not from other tissues expressing the MEN2A-transgene (Michiels et al., 1997), suggesting that *RET* transgene was not able to form trans-signaling dimers in other tissues.

In a knock-in MEN2B mouse model, the M919T RET mutation corresponding to the human M918T was introduced to the mouse genome using the Cre/loxP site-specific recombination (Smith-Hicks et al., 2000). The heterozygous Ret^{MEN2B} knock-in mice displayed bilateral C-cell and chromaffin cell hyperplasia resembling those features found in human disease. The homozygous mice displayed a more severe thyroid and adrenal phenotype (pheochromocytoma), ganglioneuromas of the adrenal medulla, enlargements of the adrenal associated sympathetic ganglia and male infertility due to unknown mechanism. However, thyroid C-cell hyperplasia never developed into MTC. Surprisingly, the Ret^{MEN2B} homozygous mice did not show any developmental abnormalities, suggesting that GFL signaling through Ret^{MEN2B} must be basically normal or then the Ret^{MEN2B} introduced change in substrate specificity does not impair normal development of kidneys or ENS. Importantly, Ret^{MEN2B} mice containing one Ret kinase-deficient allele did not show any of the characteristics found in Ret^{MEN2B} homozygous mice. This demonstrates that the ganglioneuromas and male infertility are due to a gene dosage effect rather than a loss-of-function effect (Smith-Hicks et al., 2000).

MTC tumor penetrance appears to differ significantly between different mouse background strains (Cranston and

Ponder, 2003). This important finding indicates that genetic background could modulate the tumor onset. As tumor phenotypic expression varies both within and between MEN2 families, it would be of importance to identify and elucidate the effects of modifier genes involved in this disease. Furthermore, the relevance of the latter finding must be taken into consideration when studying the effect of other oncogenes. Crossing MEN2-transgenic mice with mutant mice deficient in a RET signaling component, should reveal the contribution for the signaling molecule in transforming capacity. Furthermore, these mice will be important tools for testing pharmaceutical compounds which might inhibit RET oncogenic function.

1. 10 Endocrine organs relevant in RET signaling

The endocrine system is based on a number of glands which synthesize and secrete hormones into circulating blood to act on target tissues. Together with the nervous- and immune systems, the endocrine system regulates the internal physiological homeostasis in the body by making appropriate adaptive changes to internal and external stimuli. The major endocrine glands are the pituitary, the thyroid, the parathyroid, the adrenal, the pancreas (islets of Langerhans), ovary and testis. Hormones can be chemically divided into three classes: the amino acid-tyrosine-derived hormones, peptide and protein hormones and cholesterol-derived steroid or steroid-like hormones. Hormones are either released to the extracellular space to act on the same cell (autocrine hormones) or on neighboring cells (paracrine hormones) or released into the bloodstream. Amino acid and peptide/protein hormones act by binding to specific tyrosine kinase- or G-protein-coupled receptors on the target-cell surface, whereas steroids and steroid-like hormones are recognized by intracellular receptors.

1.10.1 The thyroid gland

The structures that contribute to the mature thyroid gland, the follicular cells and the parafollicular cells, are derived from different pharyngeal regions during embryonic development. The thyroid gland forms from cells of two different origins. The posterior migration of cells of the diverticulum from the ventral floor of the pharynx and the medial and ventral migration of the ultimobranchial bodies from the fourth pharyngeal pouch, fuse to form the mature thyroid gland (Fontaine, 1979; Pearse and Carvalheira, 1967; Pearse and Polak, 1971). The parathyroids derived from the third pharyngeal pouch, migrate lateral to the thyroid gland (Manley and Capecchi, 1995). The parafollicular C-cells are derived from the ultimobranchial bodies that are embryonic structures derived from the pharyngeal pouch and the vagal neural crest ectoderm. Most of the thyroid follicular cells are derived from the endodermal diverticulum whereas a minority of the follicular cells is derived from ultimobranchial bodies (Reynolds et al., 2001). The thyroid develops into two distinct lobes on either side of the trachea and larynx connected by a narrow isthmus of thyroid tissue at the midline. The parafollicular C-cells are interspersed in small groups between the follicles in the intermediate part of the thyroid lobes. Capillaries and lymphatic vessels

surround the follicles and postganglionic sympathetic nerve fibers from the superior and middle cervical ganglia control the blood flow through the thyroid gland.

The characteristic features of follicle cells are to concentrate iodine from the bloodstream and produce the hormones thyroxine (T_4) and its bioactive triiodothyronine (T_3) from thryroglobulin which stimulate cell metabolism and are essential for body growth. The parafollicular C-cells secrete calcitonin (CT) which is a peptide of 32 amino acids. Exogenous calcitonin hormone binds to specific CT receptors on bone osteoclasts and suppresses the activity of osteoclasts to mobilize calcium from bone and thus may have a stimulatory effect on bone formation (Lin et al., 1991; Nicholson et al., 1986). Secretion of calcitonin is stimulated by elevation in serum Ca^{2+} levels which at the same time suppress secretion of parathyroid hormone (PTH), the main hormone for calcium resorption. Specific calcium-sensing G protein-coupled receptors contribute to the calcitonin secretion in C-cells, whereas similar receptors in parathyroid cells contribute to the inhibition of parathyroid hormone secretion (Freichel et al., 1996). Although calcitonin is effectively used to treat osteoporosis, the normal physiological role of calcitonin in bone formation remains unclear (Civitelli et al., 1988). Surprisingly, mice lacking the CT/CGRPα-gene or its receptor show normal bone resorption but increased bone mass due to increased bone formation (Dacquin et al., 2004; Hoff et al., 2002), which suggests an unrecognized effect of CT/CGRP in bone formation postnatally.

The role for Ret, GFRα-receptors and GFLs in the development of the thyroid is unclear. *Ret* mRNA has been localized to the endoderm of branchial arches and thyroid C-cells probably express *Ret* mRNA already as neural crest progenitors in the ultimobranchial body (Pachnis et al., 1993). The expression of *Ret* is reduced postnatally in the thyroid (Golden et al., 1999) but is, however, detected in the C-cells of normal adult thyroid (Belluardo et al., 1999; Tsuzuki et al., 1995). *Gfra*1 and *Gfra2* mRNA has not been detected in thyroid C-cells but *Gfra*1 was present in thyroid follicular cells (Belluardo et al., 1999; Golden et al., 1999). High *RET* expression together with elevated calcitonin levels has been localized in thyroid C-cell hyperplasia and consequently in MTC (Golden et al., 1999; Santoro et al., 1990; Takaya et al., 1996). *GDNF* mRNA has not been detected in the thyroid gland (Belluardo et al., 1999). However, *NRTN* was present in some thyroid follicular cells (Belluardo et al., 1999). No expression data of *Gfra3* mRNA in thyroid is available, but *ARTN* mRNA has been detected by Northern blot from thyroid gland (Baloh et al., 1998b). Transcripts for *Ret*, *Gfra1*, *GDNF* and *NRTN* were not found in the parathyroids of the developing mouse (Golden et al., 1999). However, *Gfra2* was highly expressed in the developing mouse parathyroid from E12 to E18 (Golden et al., 1999).

1.10.2 The adrenal gland

The adrenal gland consists of two main parts, the medulla and the cortex. The medullary chromaffin cells originate from the trunk neural crest or sympathoadrenal (SA) cell lineage which also give rise to sympathetic neurons (reviewed by Anderson, 1993). During

embryonic development, the SA cells migrate to the dorsal aorta to form the sympathetic primordium. The cells differentiate to cathecholaminergic tyrosine hydroxylase positive neuronal progenitor cells and remigrate to their final destinations in response to different cues from the dorsal aorta. The cells that migrate to the adrenal medulla loose their neuronal traits and differentiate into chromaffin cells. The adrenal cortex originates from mesothelial cells located at the cranial end of the mesonephros. The cortex is divided into three zones on the basis of cell types which release aldosterone, glucocorticoids and androgens. The adrenal medulla comprises about 20% of the adrenal gland and is composed of catecholamine-producing chromaffin cells, ganglion cells, venules and capillaries. Two distinct sets of chromaffin cells are found in the medulla: those that secrete epinephrine (85%) and those that secrete norepinephrine synthesized from phenylalanine. The release of catecholamines in concert with glucocorticoids prepares the body for maximum use of energy through an increase in blood pressure, vasodilation, increased heart rate and rate of breathing. Each chromaffin cell is innervated by a cholinergic, pregangliotic sympathetic neuron which releases acethylcholine, which stimulates the chromaffin cells to release catecholamine.

Expression of *Ret* mRNA is observed in the SA cells aggregating at the dorsal aorta (Pachnis et al., 1993). While *Ret* continues to be expressed in the developing sympathetic neurons, its expression is very low in the developing mouse adrenal and in adult adrenal cortex (Schober et al., 2000). Nerve fibers in the adult adrenal cortex are reported to express *Gfra1* and *Gfra2* but not *Ret*, whereas ganglion cells in the adrenal medulla express *Gfra1* and *Ret* (Schober et al., 2000). However, chromaffin cells express high levels of *Gfra2* and its ligand *Nrtn* but no *Ret* (Golden et al., 1999; Schober et al., 2000; Widenfalk et al., 1997). *GDNF* mRNA is expressed from E16 onward by rat chromaffin cells, but its function in the adrenal medulla is still enigmatic. GDNF is however suggested to offer trophic support for sensory neurons innervating chromaffin cells (Krieglstein et al., 1996).

Pheochromocytomas (pheos) are benign tumors originating from adrenal chromaffin cells of which approximately 80% are sporadic and 20% familial. Sporadic pheos are rarely associated with activating mutations in RET although the wild-type *RET* gene is frequently expressed in pheos (Miya et al., 1992; Santoro et al., 1990; Takaya et al., 1996). Additionally, the level of RET phosphorylation in pheos is comparable to that of MEN2 constitutively activated RET (Le Hir et al., 2000). Moreover, levels of GDNF transcripts are several times higher in pheos than in normal adrenals but the *Gfra1* transcript remains low in normal adrenal and pheos (Le Hir et al., 2000). The contribution for GDNF in pheos is thus unclear.

1.10.3 The pituitary gland

The hypothalamopituitary axis plays a central role in the endocrine system. It organizes appropriate hormonal response from the brain in response to changes in the external environment by positive/ negative feedback. The pituitary has three functional components the anterior lobe

(adenohypophysis), consisting of glandular epithelial tissue and the intermediate lobe, which derive from evagination of the ectoderm of the oropharynx or the Rathke´s pouch (Amar and Weiss, 2003). The posterior lobe (the neurohypophysis), is derived from a ventral extension of the dienchephalon, the infundibulum. The adenohypophysis is rich in secretory cells which release different hormones regulated via the hypothalamic portal vessel system. The acidophilic somatotrophs secrete growth hormones (GH) and the mammotrophs prolactin (PRL) whereas the basophilic thyrotrophs secrete thyroid stimulating hormone (TSH), corticotrophs adrenocorticotrophin (ACTH), and the gonadotrophs luteinizing and follicle stimulating hormones (LH and FSH). The neurohypophysis secretes two hormones, vasopressin and oxytosin originally released from neurons whose cell bodies are located in the hypothalamus. The intermediate lobe is rudimentary in humans but produces several hormones whose significance is only now being established.

In the mouse embryonic pituitary, transcripts for *Ret*, *Gfra1*, *Gfra2* and their respective ligands have been detected between E12 to E16 (Golden et al., 1999). In the adult rat pituitary, *GDNF* has been detected in the intermediate lobe (Saland et al., 2000) but it is also expressed together with *Ret* and *Gfra1* in anterior and posterior lobes (Urbano et al., 2000). GDNF in the anterior lobe was detected by immunohistochemistry in corticotrophs and gonadotrophs, while *Ret* was mainly expressed by somatotrophs (Urbano et al., 2000). In human pituitary, most of the somatotrophs express both GDNF and RET (Japon et al., 2002), suggesting possible autocrine regulation of somatotroph cell growth or/and function. Furthermore, a strong immunostaining for RET, GDNF or/and GFRα1 was found in different types of pituitary tumors secreting GH or ACTH (Japon et al., 2002), implying that GDNF and RET could be involved in the pathogenesis of pituitary tumors.

1.10.4 The testis

The two primary functions of the testis are the production of male gametes by a process of spermatogenesis and synthesis of testosterone, a major male sex hormone. The first stage in mouse testis development is the initiation of the primary testis cord formation from genital ridges at approximately E12 (Tilmann and Capel, 1999). The gonadal sex is determined by the *SRY* gene located in the sex determining region of the Y-chromosome. Testis-determining factor (TDF) encoded by the *Sry* gene is responsible for the testicular development. In addition, proper signaling via insulin receptors has been shown to directly influence the expression of the *Sry* gene in mice, as XY mice deficient for all three insulin and insulin growth factor receptors develop ovaries and a complete female phenotype (Nef et al., 2003). The primordial germ cells (PGC) migrate from the allantois along the hindgut endoderm to the genital ridges where they are surrounded by the primary sex cords. If the embryo contains one X and one Y chromosome the sex cords continue to proliferate and fuse to form a network of internal sex cords, loose contact with the surface epithelium and become surrounded by a thick

extracellular matrix, the tunica albuginea. The primary testis cords are composed of germ cells and precursor Sertoli cells which are surrounded by smooth muscle cells. Under hormonal influence of TDF and subsequent testosterone, these cords differentiate into seminiferous tubules, rete testis and septa separating the seminiferous tubules. The PGCs differentiate into gonocytes which are centrally located within the seminiferous tubules. The gonocytes proliferate for a few days and are then arrested in the G_0/G_1 phase of the cell cycle until birth. Mesenchyme separating the seminiferous tubules gives rise to Leydig (interstitial) cells that produce testosterone which is important for the development of the primordium into a testis and for the growth and differentiation of the Wolffian ducts to male genital ducts. The Sertoli cells within the seminiferous tubules produce Müllerian-inhibiting factor (MIF) which inhibits cell division of the Müllerian ducts, thus inhibiting the development of female reproductive organs.

Within a week (6 days) after birth the gonocytes resume proliferation and start to migrate and establish contact with the basement membrane of the seminiferous tubules and give rise to adult type spermatogonia (Orth et al., 1997). By postnatal (P) day 18-20 the appearance of secondary spermatocytes and haploid spermatids signify the onset of spermatogenesis. At this time, the blood-testis barrier is formed by tight junctions created by Sertoli cells. These junctions divide the seminiferous tubules into two compartments: the basal compartment where the spermatogonia and the earliest primary spermatocytes reside and the adluminal compartment which contains the more advanced secondary spermatocytes and spermatids. An essential role for the testis-blood barrier is to isolate the spermatogenic cells from the adult immune system. The initiation and maintenance of spermatogenesis requires the action of FSH and LH (reviewed by Toppari and Huhtaniemi, 1999). LH induces Leydig cells to produce testosterone which in turn regulate spermatogenesis through Sertoli cells, whereas FSH binds directly to Sertoli cells and regulates spermatogenesis by controlling the proliferation of Sertoli cells and the secretion of different Sertoli cell substances like inhibin and activin, both members of the TGF-β family (reviewed by Cooke and Saunders, 2002). In mammalian adult testes, sperms are continuously produced from spermatogonial stem cells within seminiferous tubules by spermatogenesis. This process consists of three phases (reviewed by de Rooij, 2001). During the mitotic phase, stem cells divide to give rise both to undifferentiated stem cells (self-renewal) and differentiated spermatogonia that proliferate and differentiate. The first phase takes place in the basal compartment between the blood-testis barrier and the basement membrane. In the last mitotic division of spermatogonia, the type B spermatogonia divide by mitosis to form early primary spermatocytes. During meiosis of spermatocytes, the spermatocytes move through tight junctions from the basal compartment of the seminiferous tubules towards the adluminal side (Lui et al., 2003). Before the first meiotic division, the primary spermatocytes replicate their DNA to contain twice the number of chromosomes (4n) and undergo

homologous recombination. The first meiotic division reduces the number of chromosomes to 2n and the cells now become secondary spermatocytes. The secondary spermatocytes quickly enter the second reduction division to form the haploid spermatids. The final phase, the spermiogenesis consists of the morphological change of the haploid germ cells to the mature spermatids that are released to the lumen of the seminiferous tubules and transported through rete tetis to the epididymal duct.

Spermatogenesis continues via a cyclic process during adult life if male spermatogonial stem cells are capable of both self-renewal and differentiation (de Rooij, 2001). It is assumed that spermatogonial stem cells either renew themselves or produce two interconnected spermatogonia predestined to differentiate into spermatids (symmetrical division), or that one stem cell can divide asymmetrically into one stem cell and one spermatogonia destined to differentiate. To preserve the steady state kinetics, the ratio between cell renewal and differentiation has to be close to one (de Rooij, 2001). In a situation where germ cells are severely damaged by exposure of the testis to irradiation or toxic substances, the surviving stem cells initially prefer self-renewal (van Beek et al., 1990). The mechanism regulating the balance between self-renewal and differentiation has until our study remained largely unknown. In this study we show that GDNF determines the spermatogonial stem cell fate decision (IV).

Spermatogenesis is a complex process requiring not only hormonal regulation to transfer the intracellular stimuli but also a large number of intracellular mechanisms that are mediated by local cell-cell interactions. The functions of somatic Sertoli cells in spermatogenesis are mediated by numerous paracrine and autocrine local factors. Being in close contact with the developing germ cells through desmosome-gap junctions they nurture them and regulate their proliferation and differentiation (Parvinen et al., 1986; Russell et al., 1983). Sertoli cells can also interact physically and chemically through paracrine factors with the somatic Leydig cells and the peritubular myoid cells in the interstitial space. Recent evidence from transgenic or gene knockout mice suggests that distinct growth factors play crucial roles in different aspects of spermatogenesis. Reduction of circulating epidermal growth factor (EGF) through removal of salivary gland in rodents reduces spermatogenesis and sperm count (Tsutsumi et al., 1986). However, transgenic mice over-expressing EGF showed the same testis phenotype, suggesting that the proper levels of EGF expression is important for completion of spermatogenesis (Wong et al., 2000). Targeted mutagenesis of the gene coding for bone morphogenic protein 8b (BMP8b) causes male infertility, whereas the homozygous *Bmp8a* mice show a normal initiation of spermatogenesis but a degeneration of germ cells in adult homozygous males (Zhao et al., 1998). Analysis of mice with naturally occurring mutations in the genes encoding for stem cell factor (SCF) and its receptor c-Kit have implicated that these proteins are crucial for primordial germ cell migration and survival, and in spermatogonial differentiation, survival and adhesion (reviewed by Sette et al.,

2000). Several studies indicate that the CSF/c-Kit system is important for the continuing differentiation of early undifferentiated spermatogonia (reviewed by de Rooij, 2001). SCF is produced by Sertoli cells and its receptor c-Kit is temporally upregulated at 6-7 days postnatum (dpn) in early differentiating spermatogonia (Schrans-Stassen et al., 1999). FSH has been reported to increase SCF mRNA in Sertoli cells (Rossi et al., 1993). Recently, BMP4 (bone morphogenetic protein 4) which is produced by Sertoli cells very early in the postnatal life has been shown to regulate early spermatogonia differentiation and proliferation through its receptors in spermatogonia and to upregulate the expression of c-Kit (Pellegrini et al., 2003). Thus, FSH and many paracrine factors produced by Sertoli cells contribute to the complex regulation of spermatogenesis. However, little is known about the factors involved in spermatogonial stem cell renewal.

Several studies (IV) have shown that GDNF and its receptors are produced by distinct cell types in the testis. *GDNF* is expressed by the Sertoli cells and *Gfra1* and *Ret* by spermatogonia in prenatal and early postnatal testes (Hellmich et al., 1996; Hu et al., 1999; Golden et al., 1999; Trupp et al., 1995; Viglietto et al., 2000). GDNF has been shown to stimulate postnatal germ cell proliferation (Viglietto et al., 2000) and together with FSH stimulate the proliferation of Sertoli cells (Hu et al., 1999). FSH also stimulates GDNF expression in Sertoli cells and promotes spermatogonial proliferation (Tadokoro et al., 2002). Although *NRTN* and *Gfra2* are highly expressed in the postnatal testis (Baloh et al., 1997; Golden et al., 1999; Viglietto et al., 2000), no testis phenotype has been found in NRTN- and GFRα2-deficient mice. However, transgenic mice overexpressing NRTN in the testis show a transient disruption of spermatogenesis (Meng et al., 2001b).

2. AIMS OF THIS STUDY

The aims of this thesis were to characterize mammalian GFRα4 and to study its biological roles in mouse and human. Another aim was to generate transgenic GDNF-overexpressing mice and study the biological consequences for the GDNF gene dosage primarily in testis.

The specific aims were:

- To characterize the mammalian receptors for PSPN
- To reveal sites of *Gfra4*-expression in human and mouse
- To study the features of GFRα4 *in vitro*
- To study the *in vivo* roles for GFRα4 by creating GFRα4-deficient mice
- To produce mice over-expressing GDNF
- To study the GDNF gene dosage effect in spermatogenesis

3. MATERIALS AND METHODS

Most of the methods and materials used in this work have been described in detail in the original articles (Table 4).

3.1 Generation of the GFRα4-deficient mice

A mouse BAC (Bacterial Artificial Chromosome) clone 389B9, a gift from Dr. Gunn (Stanford, California) containing the full coding region of mouse *Gfra4* gene, was digested with *Xba*I and approximately 8 kb fragments were isolated from agarose gel and cloned into *Xba*I-opened CIP-treated pGEM11f+ vector. *Gfra4* positive clones were screened by colony hybridization. A chromosomal region covering exons 2-5 of *Gfra4* (1440 bp) was replaced by a 1.6 kb PGKneo cassette (Neomycin resistance gene under the phosphoglycerate kinase promoter). The targeting vector was linearized and electroporated into R1 embryonic stem

Table 4. List of methods used in this work (articles I-IV and unpublished results).

Method	Reference or reagent	Used in
RACE cloning	Marathon-Ready cDNA (Clontech), GeneRacer Kit (Invitrogen)	I, II
RNA isolation Human blood RNA Human total RNA Human thyroid tumors	Trizol reagent (Life Technologies) RNAwiz (Ambion) QIAamp (Qiagen) Human Total RNA Panels I-IV (Clontech) Ultraspec-II (Biotecx Laboratories, Inc.)	I, IV II, III II II II
Reverse transcription	SuperscriptII-III (Life Technologies)	I, II, III
PCR	GC-rich PCR-kit (Roche) DynazymeII (Finnzymes) Expand Long Template PCR system (Roche) Expand High Fidelity PCR system (Roche)	I, II, III I, II, III II I, II
PCR cloning	TOPO TA PCR cloning system (Invitrogen)	I, II
Sequencing	ALF, Pharmacia or ABI Prism 377XL, DMSO 5-10% (DNA sequencing facility, Institute of Biotechnology)	I, II, IV
Northern blot hybridization	Sambrook and Russell, 2001	I, IV
Southern blot hybridization	Sambrook and Russell, 2001	III, IV
Colony hybridization	Sambrook and Russell, 2001	III
In situ hybridization	Rossi et al., 1999; Kokaia et al., 1999	I, II, III, IV
RNase protection analysis	RPAII kit (Ambion), Timmusk et al., 1993	
Immunohistochemistry	See below	I, III, IV
Cell transfections	Fugene6 (Roche), Lipofectamine 2000 (Invitrogen)	II
^{125}I PSPN binding assays	Laurikainen et al., 2000	II
Chemical cross-linking	Suvanto et al., 1997	II
Ret phosphorylation assay	Suvanto et al., 1997	II
Immunoprecipitation	Protein A and G Sepharose (Amersham)	II, IV
Neural microinjection	Hamnér et al., 2001	II
Oocyte microinjection for transgene production	Hogan et al., 1994	IV
ES cell work, morula aggregation	Papaioannou and Johnson, 2000	III
Calcitonin measurement	Rat calcitonin IRMA kit (Immunotopics)	III
Cell proliferation assay	BrdU (Amersham)	IV
In situ cell death assay	ApoTag (Intergen)	IV

cells, selected with G418 and screened by southern blot analysis. Chimeras produced by morula aggregation (Papaioannou and Johnson, 2000) and the mutant *Gfra4* allele was transferred into both C57BL/6/6JOlaHsd and 129/SvHsd mouse backgrounds by backcrossing for at least five generations. Resulting offspring was genotyped from tail DNA by PCR using primers 5´-CGA TTC GCA GCG CAT CGC CTT C-3´, 5´-ATA CAA GCC TTT GAC AGC TTG C-3´ and 5´-TGG ACA AGA TGC CTA CTG ACG-3´. In most experiments, we used F1 hybrid wild-type and GFRα4-KO littermates obtained from inter-crosses of the congenic heterozygous parents. F1 heterozygous hybrid GFRα4-KO mice were crossed with Ret^{MEN2B} knock-in mice in a mixed (129/terSV, C57BL/6J, FVB/N) background (Smith-Hicks et al., 2000) to generate GFRα4/RET^{MEN2B}-heterozygous mice. For thyroid C-cell calcitonin antibody staining and C-cell counting double homozygous mutant mice for *Gfra4*-/- and Ret $^{MEN2B/MEN2B}$ and control mice were obtained from inter-crosses of the heterozygous mice.

3.2 Generation of the hGDNF transgenic mice

The full-length human cDNA for GDNF (GenBank Accession No. L15306) was cloned into the *Xba*I site of the eukaryotic expression vector pEFBos, directed to express the transgene under a testis specific human translation elongation factor 1 α (EF-1α) promoter (Furuchi et al., 1996; Mizushima and Nagata, 1990). The resulting construct was sequenced and expression of human GDNF protein was verified by expressing the transgene in COS7 cells followed by western blot analysis with GDNF antibodies. A *Pvu*I-*Hind*III fragment of the construct containing the EF-1α promoter, the h*GDNF* insert, and the polyadenylation site, was injected into the pronuclei of newly fertilized FVB mouse eggs to produce the transgenic mice. Southern blot analysis using the human GDNF as a probe was used to analyze the founder mice. The transgenic offspring were genotyped from tail DNA by PCR using human specific GDNF forward primer 5´-TGT CGT GGC TGT CTG CCT GGT GC-3´ and reverse primer 5´-AAG GCG ATG GGT CTG CAA CAT GCC-3´. Four founder lines with different transgene copy numbers were used in this study. Whereas the phenotypic effect can be different in each mouse backround strain, the GDNF-transgenic mice were backcrossed to a NMRI mouse strain.

3.3 *In situ* hybridization

Radioactive *in situ* hybridization was performed as described (Kokaia et al., 1999). Antisense and sense cRNA probes (Table 5) were synthesized using appropriate RNA polymerases and ^{35}S-labelled UTP. Hybridization was performed in 52°C overnight, slides were rinsed to high stringency and treated with RNaseA. Thereafter the slides were dehydrated, air dried and dipped in NTB-2 emulsion (Kodak), exposed for 1-4 weeks and developed. The sections were counterstained by haematoxylin and photographed with an Olympus AX70 Provis microscope (Olympus Optical Co., Japan) equipped with a SenSys CCD camera (Photometrics Ltd.). The images were processed using Image Pro Plus 4.0 (MediaCybernetics) or Adobe PhotoShop (4.0-6.0) software.

3.4 Immunohistochemistry

For thyroid and adrenal histology, anesthesized mice were perfused transcardially with 4% paraformaldehyde (PFA) in PBS or Bouin´s solution. Those that were perfused with Bouin´s solution were postfixed for 12 h and washed for 2-3 days with PBS before processing into paraffin, serially sectioned at 10 µm and stained with primary antibody against calcitonin (Santa Cruz). For immunofluorescence staining, sections were stained with Cy2-conjugated secondary antibody (Jackson Laboratories).

For histology on testes and epididymides, freshly dissected tissues were fixed in Bouin´s solution or 4% paraformaldehyde for 2-24 hours, depending on the size of the biopsy. Thereafter the tissue was dehydrated and processed in paraffin, cut at 5-7 µm and stained with haematoxylin/eosin. The deparaffinized sections were incubated over night with primary antibodies diluted according to Table 6. Following washes with PBS and incubation with secondary IgG antibody, standard biotin-streptavidin-peroxidase labeling was performed according to manufacturer´s instructions (Vector Laboratories, Inc., CA).

Table 5. List of probes used for *in situ*, northern blot, southern blot, colony hybridization and RNase protection assay

Probe	Size	Nucleotides	Vector	Accession	Used in
Mouse *Gfra4* 3´EST	497 bp	1-497	pT7T3	AA823200	I, III
Mouse *Gfra4* 5´exon1a	127 bp	1-5, 122 bp 5´UTR	pCRII	AJ276870	IV
Mouse *Gfra1*	777 bp	1-777	pT7T3	AF012811	I, III, IV
Rat *Gfra2*	2002 bp	1-2002	pBS	AF003825	III
Mouse *Gfra3*	1193 bp	95-1288	pCDNA3	NM_010280	I, III
Mouse Ret	646 bp	2534-3217	pBS	X67812	I, III
Mouse *Gfra4* RPA	460 bp	95-555	pCRII	AJ276870	I
Mouse *Pspn*	471 bp	1-471	pCDNA3	NM_008954	Unpubl.
Mouse *Gdnf*	328 bp	Exon 3	pCRII	U36449	IV
Human *GFRA1*	379 bp	491-870	pGEM-T	AF042080	II
Human *GFRA2*	279 bp	148-427	pGEM-T	U93703	II
Human *GFRA3*	629 bp	574-1203	pGEM-T	NM001496	II
Human *GFRA4* 5´probe	206 bp	1-150+56 bp 5´UTR	pCR2.1	AJ291673	II
Human *RET*	281 bp	833-1114	pGEM-T	X12949	II
Human *GDNF*	636 bp	1-636	pCRII	L15306	IV

Table 6. List of primary antibodies used in immunoprecipitation or immunohistochemistry

Antibody/Antigen	Host	Source/Reference	Dilution	Used in
Calcitonin	Goat/polyclonal	Santa Cruz, CA.	1:500	I
Calcitonin	Rabbit/polyclonal	Oncogene	1:500	III
Calcitonin	Rabbit/polyclonal	DAKO	1:500	Unpub.
RET C-19	Rabbit/polyclonal	Santa Cruz, CA.	1 µg/ml IP*	II
Agarose conjugated phosphotyrosine	Mouse/monoclonal	4G10, Upstate	30 µl/ml IP*	II
EE2	Rat/monoclonal	Koshimizu et al., 1995 Gift from Dr. Y. Nishimune	1:200	IV
GATA1	Rat/monoclonal	Santa Cruz, CA.	1:50	IV
c-Kit	Rabbit/polyclonal	Santa Cruz, CA.	1:200	IV
GDNF	Rabbit/polyclonal	Santa Cruz, CA.	1:500 1:1000 W**	IV
GDNF	Rabbit/polyclonal	R&D Systems	1µg/ml IP*	IV
c-Ret	Rabbit/polyclonal	IBL, Fujioka, Japan	1:500	IV

* Used in immunoprecipitation, ** used in western blotting.

4. RESULTS AND DISCUSSION

4.1 Mammalian *GFRA4* mRNAs encode for receptors lacking the first Cys-rich domain found in other GFRα receptors (I, II)

A putative mouse *Gfra4* locus was reported in an approximately 48 kb BAC genomic sequence near the mouse *mahogany* locus (Gunn et al., 1999). From this raw genomic sequence, we identified by BLAST analysis putative *Gfra4* exon sequences homologous to other *Gfra* receptor genes and identified three expressed sequence tags (ESTs) from the public database that partially matched the 3′ exons of the *Gfra4* locus. We performed *in situ* hybridization analysis of newborn and adult mouse sections with one of these ESTs (GenBank Accession No. AA823200). From this initial information of *Gfra4* tissue expression, RACE cloning and reverse transcriptase (RT)-PCR of *Gfra4* cDNA from various tissues were used to identify the 5′and 3′sequences and splice variants of *Gfra4* mRNA. The mouse *Gfra4* gene was found to contain 6 exons with 2 alternative exons (1a and 1b) encoding two GFRα4 alternative signal sequences (I). *Gfra4* transcripts with exon 1a were obtained from mouse thyroid but not from brain or testis. Exon 1a was predicted to encode for a prototypic strong signal sequence, whereas the exon 1b signal sequence is weak, suggesting that proteins encoded by signal 1b remain in the cytoplasm. Alternative splicing of exon 6 gave rise to transcripts encoding for putative GPI-linked or transmembrane Gfrα4 proteins. Expression of full-length *Gfra4* mRNA (780 bp) containing the exon 1a and the GPI-tail (GenBank Accession No. AJ276870) (Fig. 5A), was detected in the thyroid gland of embryonic, young and adult mice ((I) and unpublished results), in the juvenile (4 wk) and adult pituitary and in the juvenile adrenal gland (I). The full-length *Gfra4* encoding for the transmembrane GFRα4 protein (879 bp, GenBank Accession No. AJ276871) (Fig. 5A) was also detected in thyroid, in adrenal and in pituitary glands. Semi-quantitative RT-PCR analysis using primers that amplify the full-length *Gfra4* mRNAs suggests that the relative expression of the GPI-anchored and transmembrane forms is rather similar between newborn, juvenile and adult thyroid (unpublished results), in contrast to RT-PCR using primers recognizing only the 3′end of the transcript (I). Additionally, a full-length transcript containing the small 53 bp intron was found in thyroid, adrenal and pituitary glands. This transcript was predicted to encode for a small putative secreted protein, as intron inclusion resulted in frame-shift with a premature stop codon in exon 3 (GenBank Accession No. AJ276872) (Fig. 5A). However, as the premature stop codon is located more than 50 nucleotides upstream of the last exon-exon junction of the full-length *Gfra4* mRNA, the mRNA for the putative secreted isoform is likely to be degraded by nonsense mediated decay (NMD), a recently identified surveillance mechanism that selectively degrades nonsense mRNAs (Lewis et al., 2003; Singh and Lykke-Andersen, 2003).

All transcripts analyzed from testis and brain contained a small 53 bp intron between exon 2 and exon 3 but lacked the sequence encoding for a putative signal

peptide which suggests an involvement of tissue-specific regulatory elements in the *Gfra4* promoter.

Alternative splicing increases the diversity of proteins produced by a single gene. It is estimated that alternative splicing occurs in more than one third of all pre-mRNAs of human genes (reviewed by Lou and Gagel, 2001). The 3´donor splice site sequence in the short 53-bp intron between exons 2 and 3 of mouse *Gfra4* was found to be weak with a suboptimal 3´polypyrimidine tract and branchpoint sequence (Lou and Gagel, 1998). Weak splice sites are typically found at alternatively spliced sites. For example, the calcitonin/CGRP gene contains six exons and differential splicing of the pre-mRNA gives rise to either calcitonin (CT, exons 1-4) mRNA in thyroid C-cells or calcitonin gene-related peptide (CGRPα, exons 1-3 and 5-6) mRNA in neurons. In the CT/CGRP pre-mRNA, a weak pseudo 3´splice with a non-canonical branchpoint nucleotide and a short stretch of purine-interrupted polypyrimidine tract were found in the intron preceding exon 4 and a weak polyadenylation signal was found in the 3´UTR (Lou et al., 1995; Lou and Gagel, 1998). Normal RNA splicing factors do not recognize these weak sequences and

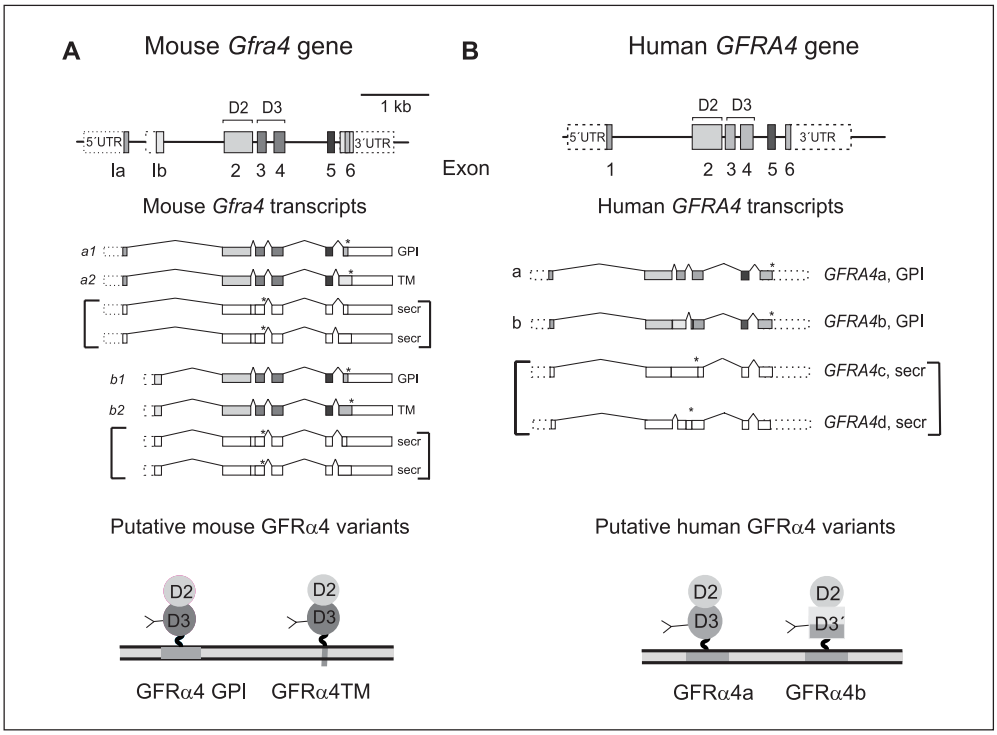

Figure 5. Structure of the mouse (A) and human (B) *GFRA4* genes, splice forms and GFRα4 protein variants. Mouse and human GFRA4 transcripts are alternatively spliced generating different GFRα4 variants, including putative GPI-linked and transmembrane (TM) variants. Asteriks denote stop codons. Transcripts in brackets contain premature stop codons and are probably subjected to nonsense-mediated mRNA decay. Putative N-glycosylation sites are indicated (Y).

additional factors are required to recognize these weak sites. Recently, exonic splice enhancer elements (ESE) have been identified in exon 4 of the CT-gene, that upon binding of two splicing proteins, SRp55 and human transformer β, increase CT splicing *in vitro* (Tran and Roesser, 2003; Tran et al., 2003). A splice regulator (CSR) isolated from rat brain has *in vitro* been demonstrated to bind specifically to the ESE element in brain and thus inhibit the CT-specific splicing in neurons (Coleman et al., 2003). Other sequence-specific splicing elements and factors are presumably used for alternative splicing of *Gfra4* pre-mRNA in endocrine cells versus brain, since the consensus sequence for ESE found in exon 4 of the *CT*-gene was not found in mouse or human *GFRA4* genes (unpublished data).

The GPI-linked and TM GFRα4 proteins have predicted molecular weights of 26 kDa and 30 kDa, respectively and contain one putative N-glycosylation site at position 184. The mammalian GFRα4 proteins are predicted to form a two domain (D2-D3) structure with 20 conserved cysteines in the same relative spacing as in other GFRα-receptors (Airaksinen et al., 1999). These domains contain highly conserved sequences homologous to the α-helix structures found in D3 of rat GFRα1, interrupted with less conserved loop regions found in GFRα1 (Leppänen et al., 2004) (Fig. 6). However, the mammalian *Gfra4* gene lacks the exons that encode for the first cysteine rich domain (D1) found in other GFRα receptors. Interestingly, isoforms lacking D1 are produced in many tissues by alternative

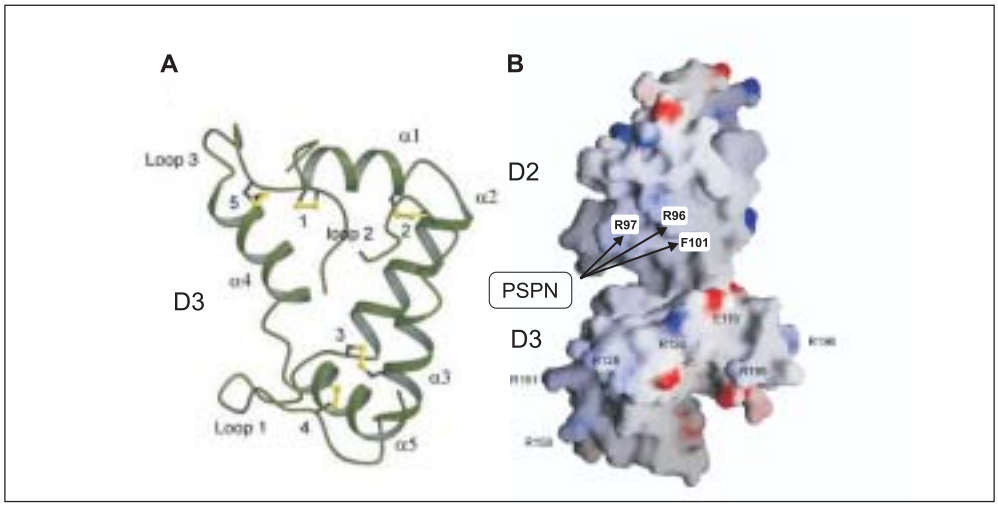

Figure 6. (A) Crystal structure of rat GFRα1 domain 3 shown as a ribbon diagram. α-helices are shown as coils, disulfide bridges (dsb-1-5) are numbered from 1-5. Modified from (Leppänen et al., 2004) with permission, from the EMBO journal, © 2004 European Molecular Biology Organization.
(B) Electrostatic potential surface representation of the proposed putative two-domain model for human GFRα4. Potential PSPN binding sites marked by arrows according to conserved binding sites for GDNF to GFRα1. By permission and courtesy of V.-M. Leppänen (2004).

splicing of *Gfra2* pre-mRNA (Dolatshad et al., 2002; Too, 2003; Wong and Too, 1998; Wong et al., 2002).

The amino acids in domain D2 of GFRα1 known to be responsible for GDNF binding (Phe213, Arg 224, Arg225 and Ile229) (Eketjäll et al., 1999; Leppänen et al., 2004) were also conserved in the corresponding domain (D2) of GFRα4 with a hydrophobic amino acid Phe at the position corresponding to Ile229 in Gfrα1 (Leppänen et al., 2004). Furthermore, the loop1 in D2 is longer in mouse GFRα4 (14 residues) compared to mouse GFRα1 (8 residues), GFRα2 (9 residues) and GFRα3 (4 residues), which may reflect allosteric positioning of D2 relative to D3 and thus ligand binding (Fig. 6). The region between D3 and the GPI-binding/ cleavage site is shorter in GFRα4 (9 residues) compared to mouse GFRα1 (87 residues) and GFRα2 (90 residues) and chicken GFRα4 (67 residues), which may reflect differencies in the interaction between GFRα4 versus GFRα1/GFRα2 to RET.

Mouse *Gfra4* gene sequence data was used to characterize the human *GFRA4* gene and transcripts (II). As in mouse, the human *GFRA4* gene contains 6 exons (Fig. 5B). A putative strong signal sequence showing high homology to mouse signal sequence 1a was found in human. However, no alternative signal sequence corresponding to exon 1b was found among human thyroid 5´RACE clones and no homologous sequence to mouse exon 1b is present in the *GFRA4* genomic sequence. Alternative splicing of *GFRA4* mRNA in human thyroid results in transcripts which encode for two GPI-linked and two putative soluble proteins. The short introns between *GFRA4* exon 2 and exon 3, and between exon 3 and exon 4 contain weak or pseudo splice sites like in mouse *Gfra4* gene. Splicing of all introns in the *GFRA4* pre-mRNA, gives rise to the GFRα4a protein (GenBank Accession No. AJ291673) (290 amino acids, Fig. 5B) with a predicted N-glycosylation site at position 178 and a stretch of hydrophobic amino acids in the C-terminus preceded by a hydrophilic linker region consistent with a GPI-linkage anchor (Udenfriend and Kodukula, 1995). The amino acid identity between mouse and human GFRα4a is 79%. Translation of *GFRA4*b mRNA in which the 79 bp intron is retained between exon 2 and exon 3, results in a protein that differs from GFRα4a in the N-terminal part of domain D3 (GenBank Accession No. AJ291674) (Fig. 5B). The globular domain structure of D3 is disrupted in GFRα4b by an additional proline- and glycine rich loop in the N-terminal part of domain D3, while two of the α-helical structures (α2 and α3) and two of the cysteine bridges (dsb-3 and dsb-4) are missing as compared to the determined GFRα1 D3 structure (Leppänen et al., 2004), making the D3 structure in GFRα4b different than D3 in GFRα4a. *GFRA4* transcripts, that retain both intron 2 and intron 3 (Accession No. AJ291675) or just intron 3 (unpublished data) code for putative soluble proteins with premature stop codons. As discussed with mouse *Gfra4*, these transcripts are susceptible to nonsense mediated RNA decay.

After completion of the study on mouse (I) and human GFRα4 (II), *Gfra4* cDNAs encoding for putative GPI-linked and soluble receptors were cloned from rat brain (Masure et al., 2000). The rat *Gfra4* gene was shown to contain 6 exons

as in mouse and human. The reported signal sequence in rat GFRα4 corresponded to the weak signal sequence encoded by exon 1b in mouse *Gfra4*. However, we identified by 5´RACE from rat thyroid a prototypic signal sequence for rat GFRα4 that shows high homology to the signal sequences encoded by the mouse *Gfra4* exon 1a (76%) and by human *GFRA4* exon 1 (67%). The rat GFRα4 showed 89% identity with the mouse GFRα4-GPI and 80% identity with the human GFRα4a.

4.2 The mammalian GFRα4 is the functional receptor for PSPN (II)

To study whether the mammalian GFRα4 receptor binds PSPN, a neuroblastoma cell line, Neuro-2a, expressing human GFRα4a and endogenous Ret was used in displacement binding experiments. Low concentrations of PSPN (~1 nM) effectively displaced ^{125}I- PSPN from the receptor, whereas not even high levels of GDNF (up to 300 nM), NRTN (up to 200 nM) or ARTN (up to 200 nM) were able to displace the bound ^{125}I-PSPN from the GFRα4-receptor. In the presence of Ret, PSPN binds to human GFRα4a with a dissociation constant (K_d) of ~100 pM, which is similar to the binding affinities reported for GFRα1, GFRα2 and GFRα3 and their cognate ligands (Baloh et al., 1998b; Klein et al., 1997; Trupp et al., 1998). The reported equilibrium dissociation constant for PSPN binding to rat GFRα4 (K_d ~6 nM) (Masure et al., 2000) is more than 50 times higher than the K_d for human GFRα4 that we determined. This probably reflects the nature of the rat GFRα4 fusion protein created by using a human signal sequence together with the rat coding sequence and an IgG-Fc domain. Furthermore, the reported binding affinity of mouse PSPN to chicken GFRα4 (K_d~1 nM) (Enokido et al., 1998) is also lower than found here, which might be due to the species difference in the PSPN and GFRα4 structure.

Cross-linking studies with ^{125}I-PSPN in cell lines expressing human GFRα4a revealed that PSPN could bind to human GFRα4a either alone or together with RET and that excess amounts of unlabelled PSPN reduce the levels of ^{125}I-PSPN bound to GFRα4/Ret. Furthermore, ^{125}I-PSPN cross-linked to GFRα4 and Ret in Neuro2a cells immunoprecipitated with Ret antibodies, suggesting interaction with PSPN, GFRα4 and Ret ((II), and Yang et al., 2004). These data are consistent with the model proposed by Jing and co-workers that the GFRα receptor first binds to the preferred ligand and that the ligand-GFRα receptor complex subsequently binds to Ret (Jing et al., 1996). However, these data do not rule out the possibility that a GFRα4 and Ret might be pre-associated before PSPN binding as suggested by an alternative model (Sanicola et al., 1997). Treatment of cells expressing GFRα4a, with phosphoinositide-specific phospholipase C (PI-PLC) after cross-linking reduced the levels of cross-linked complexes, confirming that the GFRα4a receptor is linked to the cell membrane by a GPI-anchor.

To determine whether PSPN could induce Ret autophosphorylation through GFRα4, hGFRα4/Neuro-2a cells were treated with different doses of PSPN and the levels of autophosporylated Ret were

shown by Ret staining after precipitation with phosphotyrosine antibody. In the presence of GFRα4, low levels of PSPN (0.1 ng/ml) rapidly (10 min) induced Ret autophosphorylation, whereas no Ret phosphorylation was detected in mock-transfected Ret-expressing cells. Moreover, we also demonstrated that PSPN can promote the survival of cultured mouse SCG neurons injected with expression vectors encoding for mouse *Gfra4-GPI* or human *GFRA4a* and *RET*. No survival promoting effect was seen when PSPN was added to either Ret or mock-injected neurons. Introduction of *Gfra4* alone was already sufficient to confer a PSPN promoting survival effect, probably due to endogenous expression of Ret.

Displacement binding and cross-linking studies with the mouse transmembrane GFRα4 isoform revealed a much weaker affinity for PSPN binding than for the GPI-anchored isoform (Yang et al., 2003). Cross-linking studies indicated that ^{125}I-PSPN did not bind to human GFRα4b in the presence or absence of Ret (unpublished data). This is consistent with the predicted disrupted secondary structure of the region corresponding to D3 in GFRα4b (See chapter 4.1). It remains unclear if GFRα4-TM or hGFRα4b can bind RET. Thus the functional roles for these putative alternative isoforms of mouse and human GFRα4 remain elusive. However, it is tempting to speculate that the mouse transmembrane GFRα4 and human GFRα4b isoforms may act as dominant negative forms of the receptors, or in fine tuning the function of the PSPN binding isoforms.

4.3 Tissue-specific splicing produces mRNA encoding for functional GFRα4 only in mouse and human endocrine tissues (I, II, III)

4.3.1 mRNA encoding for functional GFRα4 is not present in the normal mouse and human nervous system

In situ hybridization and northern blot analysis of *Gfra4* mRNA in embryonic and adult mouse showed relatively high levels of *Gfra4* mRNA expression in neurons of the adult and newborn mouse forebrain including cerebral cortex and hippocampus. Lower levels of *Gfra4* mRNA were detected in the brainstem including the ventral midbrain, where *Gfra4* appeared to be partially co-localized with *Ret*. *Gfra4* mRNA was also detected in the spinal cord where it did not co-localize with *Ret*. However, RT-PCR and RNase protection assay revealed that *Gfra4* transcripts did not contain exons coding for the signal sequences 1a or 1b. Consistent with this, *in situ* hybridization on brain sections from adult mouse with a probe specific for the GFRα4 signal sequence 1a, did not show any labeling above background (unpublished results). *Gfra4* transcripts with yet another alternative signal sequence might exist in brain, but may not have been retrieved by conventional cloning systems. Sequencing of GFRα4 cDNA clones required special conditions (high concentrations of DMSO and linearized products) presumably due to the GC-rich nature of the *Gfra4* mRNA (70%) and possible secondary structures. Even so, all the *Gfra4* transcripts from mouse brain

retained the intron (intron 2) that codes for a transcript with a premature stop codon, and which is likely to be degraded by nonsense mediated mRNA decay (See chapter 4.1). Unfortunately, lack of good antibodies to GFRα4 has so far prevented us from studying whether soluble GFRα4 is produced *in vivo*. In the human CNS, no *GFRA4* expression was detected from mRNA pools of either fetal or adult brain or in the adult spinal cord (II). Thus our results indicate that functional GPI-anchored GFRα4 is not produced (or produced in very low amounts) in the normal mouse and human CNS. This is supported by another study that failed to obtain the 5´end of human *GFRA4* from brain cDNA libraries (Zhou et al., 2001).

It has been reported that PSPN can *in vitro* support the survival of rat embryonic dopamine, motor and basal forebrain cholinergic neurons (Golden et al., 2003; Milbrandt et al., 1998; Åkerud et al., 2002) (Table 7), which suggests that a functional GFRα4 receptor exists in the embryonic rat brain or that PSPN is able to signal via a yet uncharacterized receptor. *In vivo*, exogenous PSPN has been reported to prevent the death of adult rat DA neurons following neurotoxic injury by 6-OHDA and to rescue rat P2 sciatic nerves from axotomy-induced neuronal cell death (Milbrandt et al., 1998). Moreover, PSPN produced by PSPN-overexpressing neural stem cells grafted to the striatum was able to protect adult mouse dopamine neurons from intrastriatally administered 6-OHDA induced cell death (Åkerud et al., 2002) (Table 7). Furthermore, exogenous and endogenous PSPN was reported to protect the mouse brain from experimentally induced ischemic insult (Tomac et al., 2002) (Table7). In these studies, it was assumed that PSPN induced neuronal protection and repair through GFRα4 and Ret, although expression of full-length GFRα4 was shown in none of these studies. GDNF and GDNF family receptor gene expression have been shown to be upregulated in neurons following insults to the nervous system, such as kindling-induced epileptogenesis and experimentally induced cerebral ischemia (Arvidsson et al., 2001; Kokaia et al., 1999). Although, *PSPN* mRNA levels were not affected after experimentally induced focal cerebral ischemia (Arvidsson et al., 2001) (Table 7), upregulation of *Gfra4* mRNA levels in brain insults has not been studied. Thus, although our results indicate that functional GFRα4 is not produced in intact normal brain, it remains possible that GFRα4 is upregulated *in vitro* and after brain lesion *in vivo*. Alternatively, PSPN may signal yet through other receptors. One alternative receptor could be NCAM, as PSPN has been shown to bind NCAM *in vitro* (Paratcha et al., 2003). This binding was further potentiated in the presence of chicken GFRα4.

In human and mouse CNS, *PSPN* expression was detected in cerebellum and in the spinal cord. In rat, low level of *Pspn* has been shown to be expressed throughout the CNS (Jaszai et al., 1998). The expression level of PSPN in the rat ventral midbrain and striatum has been reported to peak before birth and at P0, respectively and to be downregulated postnatally to reach low levels in adult (Åkerud et al., 2002).

Table 7. Reported effects of exogenous persephin *in vivo* and *in vitro*.

Model	Species	Age	*Gfra4* expression	Effect of exogenous PSPN	References
Cultured PNS and	Rat	E14	ND	No survival effect on peripheral neurons	Milbrandt et al. (1998)
CNS neurons	Rat	E14	ND	Survival effect on midbrain DA neurons and motor neurons (50 ng/ml)	
6-OHDA injections (striatum)	Rat	Adult	ND	*In vivo* protection (10 µg PSPN into SN)	
Sciatic nerve axotomy	Rat	P2	ND	Survival effect (140 µg/ml) following lesion	
Ureteric bud branching	Rat	E11	ND	Increase in number of ureteric buds (0.5-1.5 µg/ml)	
ChAT activity in motorneurons	Rat	P8	ND	No increase	Bilak et al. (1999)
Exitotoxic motor neuron death	Rat	P8	ND	High dose (100 ng/ml) added toxicity, low dose (0.1 ng/ml) protective against chronic glutamate toxicity	
Neurite outgrowth from spinal cord explants	Rat	P8	ND	No neurite outgrowth (100-200 ng/ml)	
Exitotoxic motor neuron death	Rat	P8	ND	Protective effect against chronic glutamate toxicity on motor neurons from 500 ng/ml No motor neurite outgrowth	Ho et al. (2000)
Focal cerebral ischemia induced by MCAO	Rat	Adult	ND	No upregulation of *PSPN* mRNA expression (*in situ* hybridization)	Arvidsson et al. (2001)
Focal cerebral ischemia induced by MCAO	Mouse Rat	Adult	ND	Neuroprotective activity by low dose of PSPN before ischemia (0.1 µg)	Tomac et al. (2002)
Cortical cell death induced by glutamate and hypoxia	Mouse	E17.5	ND	Low dose of PSPN reduced intracellular calcium influx (5 ng/ml) and hypoxia-induced cell death (10 pM)	
Cultured DA neurons	Rat	E14	ND	Promotes DA neurite outgrowth (30 ng/ml)	Åkerud et al. (2002)
6-OHDA (striatum)	Mouse	Adult	+, *in situ**	Protective effect on DA neurons (PSPN expressed by grafted neural stem cells)	
Basal forebrain neurons	Rat	E16	+, PCR (exons 4-5, 157 bp)	Promotes survival of basal cholinergic neurons (30-50 ng/ml)	Golden et al. (2003)

* *Gfra4* was expressed in the adult rat ventral tegmental area and in the substantia nigra pars compacta by *in situ* hybridization. The *Gfra4* cDNA used as probe was not described. PNS, peripheral nervous system; CNS, central nervous system; ND, not determined; 6-OHDA, 6-hydroxydopamine; SN, substantia nigra; DA, dopamine; MCAO, middle cerebral artery occlusion.

Peripheral ganglia studied including newborn and adult dorsal root ganglia, trigeminal ganglia, superior cervical ganglia, sympathetic chain ganglia and enteric ganglia, were positive for *Gfra4* mRNA by *in situ* hybridization. However, full-length *Gfra4* was not detected by RT-PCR from peripheral ganglia. *Gfra4* transcripts were detected by *in situ* hybridization in several other mouse tissues, including the condensing mesenchyme of developing limbs, postnatal testis and embryonic kidney (I). Nevertheless, in embryonic kidney, testis, liver and intestine, full-length transcripts encoding for membrane anchored GFRα4 were not produced, as assayed by RT-PCR (unpublished results). Of 26 adult human tissues examined only thyroid expressed *GFRA4,* and of the fetal tissues examined only fetal thyroid and adrenal gland showed expression of *GFRA4* (II, Table 8 and unpublished results), indicating a more restricted expression of human GFRα4 than rodent and chicken (where *Gfra4* is expressed in many tissues) (Masure et al., 2000; Thompson et al., 1998). Consistent with these findings, no human ESTs for *GFRA4* were found in public databases, in contrast to several mouse ESTs for *Gfra4* (I). The chicken and mammalian GFRα4 differ in domain structure and expression pattern which suggests that their biological functions are also different. The endogenous source of ligand for chicken GFRα4 is also unclear, since BLAST analysis of the draft chicken genome assembly failed to find a chicken homolog for mammalian PSPN (III). Furthermore, chicken GFRα4 shows only ~50% amino acid identity with the mammalian GFRα4. This indicates a less conserved role for PSPN/GFRα4 in vertebrate evolution compared to GDNF/GFRα1 and NRTN/GFRα2.

4.3.2 Full-length *GFRA4* mRNA is expressed in neuroendocrine cells of the pituitary, thyroid, and adrenal glands (I, II)

In the embryonic and adult mouse pituitary, *Gfra4* mRNA expression was located to the intermediate lobe, whereas *Ret* mRNA was expressed in the adjacent neurohypophysis, suggesting the possibility of Ret-independent signaling of GFRα4 in pituitary. Putative functional isoforms of GFRα4 proteins were produced in the mouse juvenile (4 wk) and adult pituitary with the transmembrane GFRα4 as the dominant form. However, *GFRA4* expression was not detected by RT-PCR in adult human pituitary probably due to the rudimentary nature of the pituitary intermediate lobe in human.

In the mouse adrenal gland, *Gfra4* and *Ret* mRNAs were highly expressed at P0 and P8 by a subpopulation of cells that seemed to be a subpopulation of chromaffin cells of the adrenal medulla. In adult adrenal medulla, *Gfra4* appeared to be evenly expressed in all cells, whereas *Ret* expression was only seen in a few cells in small clusters probably representing sympathetic ganglion cells, according to results by Schober et al., (2000) (Table 8). The majority of *Gfra4* transcripts obtained by RT-PCR from postnatal and adult mouse adrenal gland were shown to contain the short 53 bp intron between exon 2 and 3, encoding for the putative soluble protein. However, a minor fraction of *Gfra4* mRNAs in mouse adrenal gland encoded for the full-length GPI- or TM protein. In human, full-length *GFRA4* was detected in fetal (unpublished data) but not in adult adrenal gland (II). Expression of mRNA encoding for the functional correct

spliced PSPN was detected in embryonic and adult mouse adrenal gland and in adult human adrenal gland, suggesting a role for GFRα4/Ret signaling in adrenal chromaffin cell development (II, Table 8). Alternatively, if adrenal PSPN would be secreted to the circulating blood, it would be able to act on a broad range of cells/ tissues.

In mouse, *Gfra4* mRNA was identified together with *Ret* mRNA in the ultimobranchial body at E12, and at E16 at the beginning of fusion with the endothelial-derived diverticulum, and subsequent formation of the mature thyroid. At E18 *Gfra4* was co-localized with *Ret* in cells in the inner part of the thyroid lobe, which probably represents the parafollicular C-cells (Belluardo et al., 1999; Tsuzuki et al., 1995). In the adult human thyroid gland *GFRA4* and *RET* (consistent with previous reports, Belluardo et al., 1999; Tsuzuki et al., 1995) expression was detected by RT-PCR. As in mouse, *in situ* hybridization of human thyroid indicated co-

Table 8. mRNA expression of *RET, GFRAs* and *GFL*s in selected mammalian endocrine organs.

Tissue	*Ret*	*Gfra1*	*Gfra2*	*Gfra3*	*Gfra4*	*GDNF*	*NRTN*	*ARTN*	*PSPN*
Thyroid:								$+^{16}$	$-^{II}$
C-cells	$+^{I, II, 9, 10}$	$-^{III, 9}$	$-^{III}$	$-^{III}$	$+^{I, II, III}$	$-^{9}$	$-^{12}$	ND	ND
Follicular cells	$-^{I, II, 9, 10}$	$-^{III}$	$-^{III}$	$-^{III}$	$-^{I, II, III}$	$-^{9}$	$+^{12}$	ND	ND
Parathyroid	$-^{I}$	$-^{4}$	$+^{4}$	ND	$-^{I, III}$	ND	ND	ND	ND
Adrenal:				$+^{11}$				$+^{16}$	$+^{II,}$
Medulla:					$+^{I}$				
Ganglion cells	$+^{1}$	$+^{1}$	$-^{1}$	ND		$-^{2}$	$-^{12}$	ND	ND
Chromaffin cells	$-^{1}$	$-^{1}$	$+^{1, 5}$	ND	$+^{I}$	$+^{2}$	$+^{12}$	ND	ND
Cortex	$-^{1}$	$+^{1}$	$+^{1}$	ND	$-^{I}$	$-^{2}$	$+^{12}$	ND	ND
Pituitary:			ND	$+^{11}$				$+^{16}$	$-^{II}$
Anterior lobe	$+^{3}$	$+^{3}$		ND	$-^{I}$	$+^{3}$	$+^{12}$	ND	ND
Interm. lobe	$-^{I}$	ND		ND	$+^{I}$	$-^{6}$	$+^{12}$	ND	ND
Posterior lobe	$+^{I, 3}$	$+^{3}$		ND	$-^{I}$	$+^{3}$	$-^{12}$	ND	ND
Testis:				$+^{11, 13}$				$+^{15, 16}$	$+^{II, unpub.}$
Sertoli cells	$-^{IV, 8}$	$-^{IV, 8}$	$-^{7}$	ND	$-^{I, unpub.}$	$+^{IV, 7, 8}$	$+^{5, 7, 8}$	ND	ND
Spermatogonia	$+^{IV, 8}$	$+^{IV, 8}$	$-^{7}$	ND	$-^{I, unpub.}$	$-^{IV, 7, 8}$	$-^{7}$	ND	ND
Spermatocytes	$+^{14}$	$-^{IV}$	$+^{7}$	ND	$+^{I, npub.}$	$-^{IV, 7, 8}$	$-^{7}$	ND	ND
Spermatids	$-^{IV}$	$-^{IV}$	$+^{7}$	ND	$-^{I, unpub.}$	$-^{IV, 7, 8}$	$-^{7}$	ND	ND
Oviduct	$-^{4}$	$-^{4}$	$-^{4}$	ND	ND	$-^{4}$	$+^{5}$	ND	ND
Ovary	$-^{4}$	$-^{4}$	$-^{4}$	$+^{11, 13}$	ND	$+^{4}$	$+^{4}$	$+^{15, 16}$	ND

mRNA expression determined in this work is marked by +, whereas no expression is marked by – and a Roman numeral (I-IV) referring to article number. Expression determined by others is marked by a given number referred to as follows: [1]Schober et al., 2000 (rat), [2]Krieglstein et al., 1996 (rat), [3]Urbano et al., 2000 (rat), [4]Golden et al. 1999 (mouse), [5]Widenfalk et al., 1997 (mouse), [6]Saland et al., 2000 (rat), [7]Meng et al., 2001 (mouse), [8]Viglietto et al., 2000, [9]Belluardo et al., 1999 (rat), [10]Tsuzuki et al., 1995 (rat), [11]Baloh et al., 1998a (human), [12] Xian et al., 1999 (rat), [13]Masure et al., 1998 (human), [14]Creemers et al., 2002 (mouse), [15]Masure et al., 1999 (human), [16] Baloh et al., 1998b (rat). ND, not determined. Unpub., unpublished results.

localization of *GFRA4* and *RET* in thyroid C-cells. By RT-PCR *Gfra4* was expressed in the mouse thyroid by most developmental stages analyzed. Still, the overall expression level of *Gfra4* seemed lower in embryonic and adult than in neonatal and juvenile thyroid as measured by RT-PCR (unpublished data). In human, the two identified GPI-linked *GFRA4*a and *GFRA4b* transcripts were expressed in equal amounts in adult and fetal thyroid, as shown by RT-PCR (II and unpublished data). Strong positive signal for *Gfra4*, but not for *Ret*, was detected in the newborn and postnatal mouse parathyroid gland by *in situ* hybridization. However, *in situ* hybridization with an exon 1a-specific *Gfra4* probe that recognizes the mRNA encoding for the functional signal sequence in mouse GFRα4 (III, Table 8) did not reveal any signal above background in the parathyroid gland, suggesting that functional GFRα4 is not produced in the parathyroid gland. In conclusion, mRNAs encoding for functional PSPN, GFRα4, as well as other GFLs and their receptors are expressed in several distinct types of endocrine tissues and cells (Table 8). However, their roles in these tissues have not yet been determined (but see III and IV).

Except for the brain, adrenal gland and testis, *PSPN* expression was not detected in other mouse and human tissues, including thyroid at any developmental stages analyzed (I, II), raising questions about the source of ligand for the GFRα4/Ret signaling complex in thyroid. Members of the neurotrophin family, such as NGF and BDNF have been found in circulating blood (Aloe et al., 1986; Nakahashi et al., 2000). Unlike GDNF, that binds strongly to heparan sulfate proteoglycans (HSPG) in the extracellular matrix (Barnett et al., 2002; Hamilton et al., 2001), PSPN lacks the heparin-binding properties and does not bind to heparan sulphate (M. Bespalov and M. Saarma, personal communication). Thus it is possible that PSPN, if secreted from, for example, adrenal gland to the circulating blood, might be able to act in a hormone-like manner, binding to GFRα4/Ret on the surface of C-cells in the thyroid.

4.4 High expression of *GFRA4* and *RET* in medullary thyroid tumors suggests roles for GFRα4 in tumorigenesis (II)

As the RET proto-oncogene is known to play an important role in the oncogenesis of C-cell-derived tumors and *Gfra4* was shown to be co-expressed with *Ret* in developing C-cells, expression of *GFRA4* and *RET* mRNA was analyzed by RT-PCR from RNA derived from human thyroid tissue tumor samples. *GFRA4* mRNAs encoding the two GPI-linked isoforms, GFRα4a and GFRα4b were highly expressed in all samples derived from medullary thyroid tumors (MTC), whereas no expression was detected in any of the other primary thyroid tumors including papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC) and follicular thyroid adenoma (FTA). Consistent with previous reports (Nakamura et al., 1994; Santoro et al., 1990), *RET* was also highly expressed by the MTC tumors. *In situ* hybridization on sections from the same tumor samples, revealed that *GFRA4* and *RET* were highly and evenly expressed by virtually all the malignant cells in the MTC samples, indicating that the expression of

GFRA4 is C-cell specific. In line with a recent study that showed expression of *GFRA1* and *GFRA2* in some cells of MTC (Frisk et al., 2000) very low or undetectable levels of *GFRA1*, *GFRA2* and/or *GFRA3* were detected in a subset of tumor cells in some MTCs. However, low levels of *GFRA1-3* were also detected in PTCs, FTCs and FTAs, indicating that their expression is not specific to particular thyroid tumor cell type. Furthermore, low expression of *GFRA1*, *GFRA2* and *GFRA3* was also detected in the normal human thyroid. This strong expression of *GFRA4* (and not other GFRAs) in human C-cells, suggest that GFRα4 may be critical in the oncogenic action of RET MEN2 that leads to initial hyperplasia of C-cells and subsequent MTC. On the other hand, the absence of other GFRαs in C-cells may also be important. GFRα-receptors could interfere with the dimerization or/and constitutive activation of RET mutant receptors, and therefore inhibit tumor formation in tissues that are not involved in MEN2 syndromes but express RET (Kawai et al., 2000). Even GFRα4 might be able to suppress C-cell tumor formation by forming signaling complexes with RET, but it may not be able to do so as effectively as the other GFRα´s because of the limited amount of PSPN available, or because GFRα4 may be weaker than the other GFRα´s in its suppression of RET-mediated oncogenic activity. Thus it would be of importance to test whether GFRα4 is able to inhibit RET MEN2A dimerization as reported for GFRα1. Furthermore, these hypotheses could be tested by examining whether tumor development is altered when transgenic MEN2 mice are crossed with different GFRα-deficient mice. To test whether lack of GFRα4 could suppress C-cell hyperplasia, pheochromocytoma and ganglioneuromas of the adrenal medulla GFRα4-deficient mice (III) were crossed with Ret^{MEN2B} knock-in mice (Smith-Hicks et al., 2000). Preliminary data revealed that in the thyroid of 10-month-old double mutant mice as compared to MEN2B-mice, the number of C-cells is reduced (Fig. 7). This suggests a positive effect of GFRα4 in thyroid tumor formation or a reduced number of calcitonin expressing cells (See chapter 4.5). Unfortunately, C-cell hyperplasia did not develop in the 10-12-months-old $Ret^{MEN2B/MEN2B}$ mice in the mixed background strain, and thus we could not use this animal model to study the effect of GFRα4-deficiency in MTC.

To test the possibility that mutations in the *GFRA4* gene would be found in thyroid tumors, full-length *GFRA4*

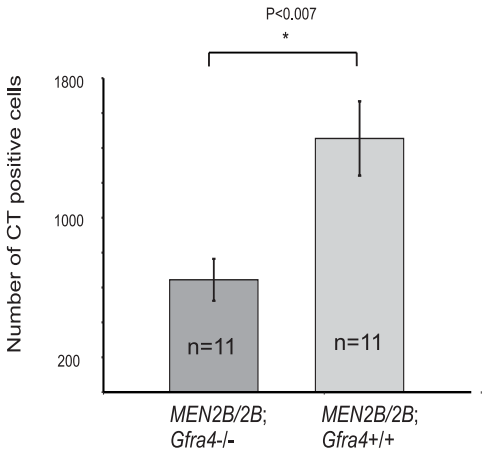

Figure 7. Number of C-cells in the thyroid of double Ret $^{MEN2B/2B}$; *Gfra4*-/- and Ret $^{MEN2B/2B}$; *Gfra4*+/+ littermate mice. Lack of GFRα4 appears to reduce the number of calcitonin positive (CT) C-cells in the thyroid of aged $Ret^{MEN2B/MEN2B}$ knock-in background.

cDNAs from eight MTC tumors, the TT cell line and normal thyroid were sequenced. No mutations except for some allelic polymorphisms were found in the coding region of the *GFRA4* gene (II).

4.5 Calcitonin synthesis by C-cells is reduced in newborn and juvenile GFRα4-deficient thyroid (III)

The GFRα4-deficient mice produced in this study are viable and show no obvious defects in growth and gross behavior (III). Basic histological analysis of CNS and pituitary did not reveal differences between wild type and GFRα4-deficient mice. No structural differences were found in the adrenal medulla, including tyrosine hydroxylase or PNMT-immunostaining of adrenal chromaffin cells or their cholinergic innervation (Hiltunen et al., 2001). However, signs of mild testis degeneration were observed in these mice, with significantly elevated numbers of TUNEL positive spermatogonia in the seminiferous tubules (Hiltunen et al., 2001). Yet, this defect does not have an obvious effect on male fertility. The mechanism for the testis phenotype remains unclear (See chapter 4.9).

As *Gfra4* is highly expressed in thyroid C-cells, and C-cells appear to be the only cells that clearly co-express functional GFRα4 and Ret, we focused our studies on the effect of GFRα4-deficiency in the development and function of C-cells. No differences in the number, distribution or morphology of the C-cells were observed between the genotypes in newborn or adult mice. To rule out the possibility that expression of other GFRα-receptors might compensate for the lack of GFRα4, *in situ* hybridization was used to detect RNA for *Gfra1*, *Gfra2* and *Gfra3* in the thyroid of 1-week-old wild-type and GFRα4-deficient thyroids. No expression of *Gfra1* and *Gfra3* was detected in wt or *Gfra4*-/- thyroid. However, *Gfra2* mRNA was detected in both wild-type and GFRα4-deficient thyroid glands (Table 8), but immunostaining with calcitonin and GFRα2-specific antibodies showed that *Gfra2* is not expressed in the C-cells (III).

Ret signaling is likely to be necessary for the early development of at least some C-cells, as Ret-deficient mice (E18) show a clear reduction in number of C-cells (37%) (I). *Gfra1* was shown to be co-expressed together with *Ret* in the ultimobrachial body, the structure from where C-cells are derived, but not in neonatal thyroid C-cells (III). Therefore it is tempting to speculate that Ret/GFRα1/GDNF signaling might be important for the migration of C-cells to the thyroid gland. To address this issue, detailed analysis of the developing thyroid gland of GFRα1 or GFRα1/GFRα4 double knockout mice should be carried out.

The physiological effect of GFRα4-deficiency was studied in juvenile compared to adult and newborn thyroid by measuring thyroid calcitonin levels using an immunoradiometric assay. The tissue calcitonin content was significantly reduced (by 80%) in newborn and (by ~45%) in juvenile but not in the adult GFRα4-deficient mice compared to wild type littermates. The reduced calcitonin production in young GFRα4-deficient mice, appear to correlate with the coexpression of functional GFRα4 and Ret in thyroid C-cells. Furthermore, the expression levels of functional GFRα4

and Ret seem to be higher in young mouse thyroid C-cells than in adult ((I) and unpublished results). Additional work is needed to quantify the relative expression of different GFRα4 isoforms and Ret in mouse C-cells at different developmental stages. In conclusion, these results suggest that GFRα4 is not necessary for the development of thyroid C-cells but is required in young, and not in adult mouse thyroid, for the synthesis of calcitonin. Thus, our results indicate a physiological role for GFRα4 in the production of calcitonin in the thyroid of juvenile mice. Recent observations have shown that GFLs via their cognate receptors may regulate neurotransmitter release at some synapses (Bourque and Trudeau, 2000; Pothos et al., 1998; Ribchester et al., 1998; Wang et al., 2001; Yang et al., 2001). Nevertheless, the present study is the first to show that GFRα4 regulates calcitonin production in C-cells. This result further suggests that other GFRα receptors may also regulate transmitter production in other cells.

Our results suggest that GFRα4, presumably via Ret signaling, helps to regulate calcitonin synthesis, but the mechanism remains to be studied. The mechanisms that regulate the signal transduction pathway leading to calcitonin production are only partially known. Elevated extracellular calcium $[Ca^{2+}]_e$ activates G protein-coupled calcium sensing receptors (CaR) on C-cells, resulting in an upregulated expression of the calcitonin and CaR genes (reviewed by Brown and MacLeod, 2001; Freichel et al., 1996) presumably through activation of the thyroid transcription factor 1 (TTF-1) (Suzuki et al., 1998). CaR-induced calcitonin secretion from cultured thyroid C-cells is triggered by the activation of PI3-Kβ and downstream effector protein kinase Cζ (PKCζ) via Gβγ (Liu et al., 2003). Recent studies have shown evidence for crosstalk between G protein-coupled receptors and tyrosine kinase receptors in the regulation of cell proliferation (Brown and MacLeod, 2001). Interestingly, in cells co-expressing the EGF receptor and CaR, dominant negative CaR inhibited EGF induced ERK-1 phosphorylation (McNeil et al., 1998). Furthermore, the levels of PKCs, including PKCζ, are upregulated upon EGF-stimulation in fibroblasts expressing a chimeric EGF-receptor consisting of a RET kinase domain (Andreozzi et al., 2003). Thus, one mechanism for GFRα4/Ret regulation of calcitonin synthesis could be synergistic activation of signaling cascades mediated by GFRα4/Ret and CaR. Whether PSPN is required for calcitonin production, awaits verification from PSPN-deficient mice (Tomac et al., 2002). Furthermore, it might be of importance to study whether enhanced Ret signaling in MTC caused by activating mutations in *RET* can increase the calcitonin production in C-cells.

The calcitonin production rate in osteoporotic women has been reported to be reduced (Reginster et al., 1989; Tiegs et al., 1985). Furthermore, calcitonin is successfully used to treat hypercalcemia and metabolic bone diseases such as osteoporosis and Paget´s disease reflecting high osteoclast activity (Civitelli et al., 1988; Zaidi et al., 2002). In humans, the functional GPI-linked GFRα4 receptor continues to be expressed in adult thyroid calcitonin producing C-cells (II), which suggests that GFRα4 signaling may control

calcitonin production in adult humans and thus be relevant in osteoporosis.

4.6 Expression of *GDNF*, *Gfra1* and *Ret* in wild type and GDNF-transgenic mouse testis

Transgenic mice expressing the human *GDNF*-gene were produced under the human translation elongation factor-1 α (eEF1A) promoter. The *eEF1A* gene is known to be highly expressed in various cell types (Wakabayashi-Ito and Nagata, 1994). Surprisingly, transgenes driven by the eEF1a promoter in the pEFBos construct are mainly expressed in the testes (Furuchi et al., 1996). Accordingly, the expression of h*GDNF* transgene was detected only in the testes of the transgenic mice analyzed by northern blotting. Four independent transgenic founders were analyzed with the transgene copy number ranging from 3-20 analyzed by southern hybridization. The males were sterile independent of strain background, and defects were not found in other organs than in testis. Transgenic mice showed significant reduction of the testicular weight at 4 and 8 weeks of age. Northern blot analysis showed high endogenous expression of *GDNF*, *Ret* and *Gfra1* mRNA in neonatal wild type mouse testes, but the levels were down-regulated after the first postnatal weeks. *In situ* hybridization has revealed that *GDNF* is expressed by Sertoli cells (Trupp et al., 1995; Viglietto et al., 2000) (Table 8). Consistent with these results, GDNF protein was detected in Sertoli cells by immunohistochemistry (X. Meng, unpublished results). Messenger RNAs for *Gfra1* and *Ret* were shown to be expressed by a subset of spermatogonial cells that might include spermatogonial stem cells (IV). However, Ret protein detected by immunohistochemistry has been reported also in early spermatocytes (Creemers et al., 2002) (Table 8). In hGDNF overexpressing transgenic testis, elevated levels of human *GDNF* mRNA and protein were detected in postnatal testis. *Gfra1* and *Ret* mRNA levels were high in spermatogonia from birth to adulthood in transgenic testis. Endogenous *Gdnf* mRNA level was also elevated in the transgenic testis that may be due to the alteration of the ratio of cell population (Sertoli/germ cell).

Sertoli cells are known to release soluble factors regulating germ cell differentiation (Skinner, 1991). The distribution of GDNF and its receptors in wild type mouse testis thus suggests that GDNF can regulate spermatogonial cell proliferation and differentiation by binding to GFRα1 and Ret in a paracrine way. However, in the transgenic GDNF-overexpressing mouse testis, the expression of GDNF was targeted in spermatogonia thus acting in an autocrine manner on the regulation of spermatogonial proliferation and differentiation.

4.7 High doses of GDNF lead to spermatogonial accumulation and testicular tumors in hGDNF-transgenic mice (IV)

The testicular morphology of hGDNF-transgenic mice was normal at birth but began to display large cell clusters in the seminiferous tubules after 2-3 weeks. The large cell clusters expressed spermatogonial and germ line cell markers, but not markers for Leydig or Sertoli cells, suggesting that the cells of these clusters are derived from

spermatogonia. Furthermore, they did not express c-Kit which is a marker for differentiating spermatogonia (Schrans-Stassen et al., 1999), suggesting that the cells within the clusters are undifferentiated spermatogonia including stem cells. In the hGDNF-overexpressing mice the normal segmental nature of cell proliferation was disrupted, showing some mitotic figures in all the clusters, but the overall proliferation rate of spermatogonia was not enhanced. In addition, these cells were unable to respond properly to differentiation signals but underwent apotosis instead of differentiation when treated with retinoic acid, which is known to be important for spermatogonial differentiation (Akmal et al., 1997). These results suggested that the majority of cells in the clusters mainly represent stem cells of spermatogenesis and their differentiation pathway was inhibited by high dose of GDNF. Furthermore, this idea is supported by a recent germ cell transplantation experiment where GDNF-overexpressing spermatogonia were transplanted to testes devoid of endogenous spermatogonia due to prior irradiation (Creemers et al., 2002). The transplanted spermatogonia formed similar clusters to those in the young GDNF-overexpressing mice, verifying that the undifferentiated state is unchanged because of the high expression of GDNF in the host clusters. More recent supportive data showed that mouse testes overexpressing GDNF only in Sertoli cells (transfected by electroporation) developed spermatogonial clusters similar to those found in our GDNF-overexpressing mice. When these clusters were transplanted to progeny cell-deficient mouse testes, proliferation and regeneration of normal spermatogenesis was continued in the absence of high GDNF levels (Yomogida et al., 2003). This study verifies that the transplanted cells possess the normal stem cell property. Furthermore, FSH was shown to induce the expression of GDNF in Sertoli cells cultures (Tadokoro et al., 2002) and to stimulate rat Sertoli cell proliferation *in vitro* (Hu et al., 1999), suggesting a hormonal control of spermatogenesis conducted via the GDNF/GFRα1/Ret system.

The undifferentiated spermatogonia in GDNF overexpressing transgenic mice (IV) were able to proliferate, but not differentiate, which eventually lead to germ cell degeneration and sterility. Apotosis in cell clusters was increased with the peak at week 4. At 10 weeks of age only remnants of clusters were visible and a rim of spermatogonia remained at the base of atrophic seminiferous tubules. No sperm was visible in seminiferous tubules or the epididymis, and the somatic cells of the seminiferous tubules were normal as compared to wild type testes.

Bilateral (56%) and unilateral testicular tumors frequently developed in mice over 1 year (89%) from the dormant spermatogonia which spread into the interstitial tissue but did not distantly metastasize. Interestingly, morphological and histological analysis of the GDNF-transgenic mouse tumors revealed many of the characteristics found in human classical seminomas, a common testicular tumor in young men (Meng et al., 2001a). They consist of homogenous round cells with large nuclei, express germ cell markers and are positive for placental alkaline phosphatase. In contrast, spermatocytic seminomas are more heterogenous; often showing gigantic cells derived from spermatocytes or B

spermatogonia and usually not positive for alkaline phosphatase staining (Chaganti and Houldsworth, 2000). The GDNF transgenic mouse can thus serve as the first experimental animal model for this tumor type. However, although murine and human seminomatous tumors are similar in many aspects, some differences exist which have to be taken into consideration when using this model. The tumors appear in old transgenic mice in contrast to tumors in young men, they show no lymphocyte infiltrates that are common in human seminomas and they do not stain for c-Kit which is frequently detected in human classical seminomas (Devouassoux-Shisheboran et al., 2003; Meng et al., 2001a).

High expression of transgenic *GDNF*, and endogenous *Gfra1* and *Ret* was detected from tumors along with high Ret phosphorylation (Meng et al., 2001a). Expression of GDNF and its receptors has also been detected in human seminomas in contrast to non-seminomas (Viglietto et al., 2000), which indicates that GDNF signaling is involved in the pathogenesis of classic seminomas. This issue could be addressed *in vitro* by measuring the proliferative effect of GDNF on a recently

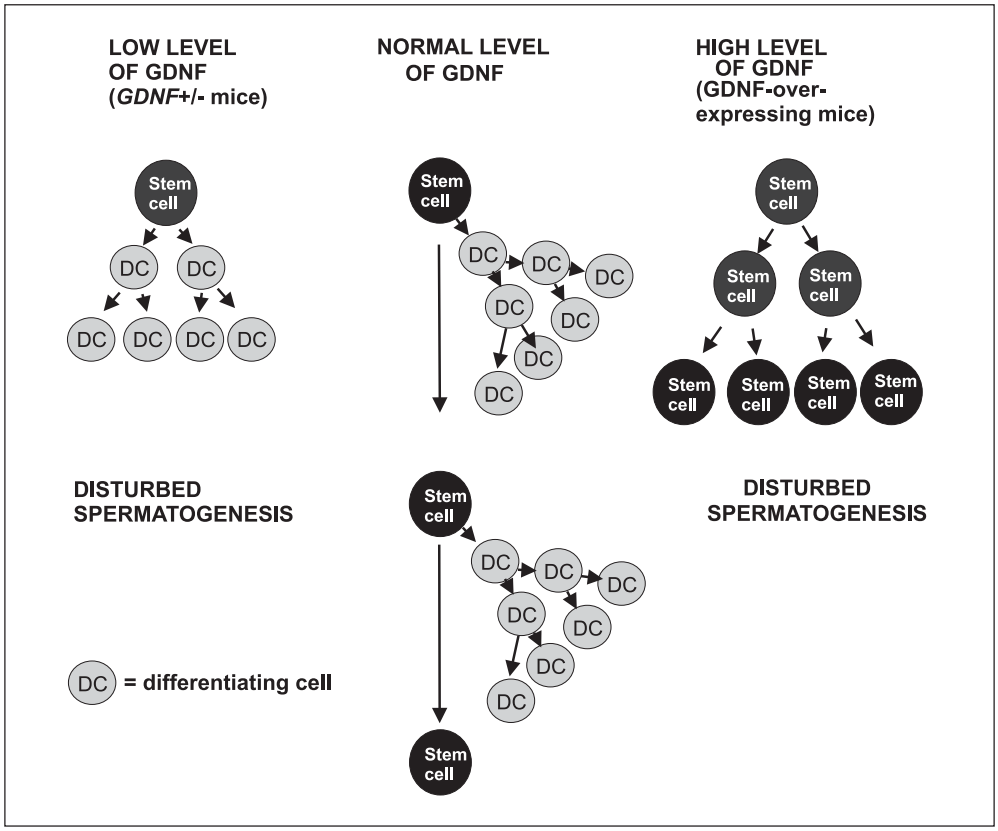

Figure 8. Control of spermatogonial stem cell differentiation and renewal by GDNF dosage. Appropriate level of GDNF controls the balance between differentiation and proliferation of spermatogenic stem cells. Low dosage results in depletion of stem cells, whereas high dosage results in depletion of differentiating spermatogonia.

established rat spermatogonial stem cell line (van Pelt et al., 2002) or by overexpressing GDNF in mutant mice with progeny-deficient testes (Tadokoro et al., 2002).

4.8 Low doses of GDNF result in depletion of stem cells in heterozygous *GDNF*+/- mice (IV)

While mice lacking GDNF die during the first postnatal day, most *GDNF*+/- mice survive to adulthood but show developmental renal and ENS abnormalities probably due to haploinsufficiency (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). In order to reveal the role for decreased GDNF expression in mouse testis, histological analysis of testes from heterozygous *GDNF*+/- mice (Pichel et al., 1996) was performed. The *GDNF*+/- mice survived to adulthood and were fertile though spermatogenesis was disturbed. Histological analysis of 5 and 8 week-old *GDNF*+/- testes revealed that atrophy was detected in about half of the seminiferous tubules but spermatids could be observed. However, sperm cells were observed in the epididymal ducts and in well preserved seminiferous tubules. In older *GDNF*+/- mice, spermatogonia were depleted and cell proliferation was reduced resulting in Sertoli cell-only tubular segments. In conclusion, the spermatogonia differentiate in excess in *GDNF*+/- mice, leading to depletion of undifferentiated germ cells.

In summary, by using GDNF-overexpression and loss-of-function approaches we have shown *in vivo* that GDNF dosage determines the cell fate decision of spermatogonial stem cell renewal and differentiation in mouse. High dosage inhibits stem cell differentiation, leading to degeneration of tubular germ cells, whereas low dosage leads to progressing stem cell depletion (Sertoli-cell-only) due to the prevailing differentiation exceeding proliferation of stem cells (Fig. 8).

4.9 Does NRTN or PSPN have a role in testis?

Apart from GDNF, NRTN is also expressed by Sertoli cells and can stimulate proliferation in *Ret* expressing spermatogonia (Viglietto et al., 2000). However, no testis phenotype has been reported in NRTN- or GFRα2-deficient mice (Heuckeroth et al., 1999; Rossi et al., 1999). Yet, transgenic mice overexpressing NRTN in testes, although fertile, show a transient degeneration of spermatocytes at 3-5 weeks of age in a fraction of mice (Meng et al., 2001b), which indicates that a proper concentration of NRTN is needed for differentiation of spermatocytes. *Gfra2* mRNA is highly expressed by spermatids in wild type mouse testis, whereas *Nrtn* expression is upregulated in testes after puberty. The *Gfra2* transcript in postnatal testis is smaller in size (1-2 kb) compared to *Gfra2* in somatic tissues (~4 kb) (Baloh et al., 1997; Meng et al., 2001b). RT-PCR and RACE-cloning revealed that the full-length *Gfra2* is expressed in embryonic and newborn testis, whereas only truncated *Gfra2* transcripts are expressed in adult mouse testis (unpublished results). These transcripts lack sequences corresponding to exons 1-4 of the *Gfra2* gene which encode for the N-terminal part of GFRα2 (signal sequence and

domains D1 and D2). These results suggest that the functional GFRα2 receptor is not produced in pubertal and adult spermatocytes. The role for NRTN produced by Sertoli cells thus remains elusive, but may signal through GFRα1, as the expression of *Gfra1* and *Ret* mRNA is downregulated but not absent from the postnatal mouse testis (IV).

PSPN was shown to be expressed in human and mouse adult testis by RT-PCR (I, II). The cellular localization of *PSPN* expression in testis could not be determined because the expression levels were beyond detection by *in situ* hybridization. In adult mouse testis, moderate levels of *Gfra4* transcripts were found in pachytene spermatocytes (unpublished data, Table 8). Northern blot analysis also revealed a clear *Gfra4* transcript from testis that was smaller in size (1.2 kb) than the mRNA retrieved from thyroid (1.4 kb) (I). RT-PCR showed aberrant splicing of the *Gfra4* transcript and 5´RACE using adult testis cDNA resulted in short clones containing only the transcript encoding for D3 of GFRα4 without signal sequence. Despite several attempts, transcripts encoding for full-length GFRα4 containing a signal sequence were never achieved by RACE cloning.

Many genes normally expressed in somatic cells appear to be overexpressed in mammalian pachytene spermatocytes and round spermatids (reviewed by Kleene, 2001). These transcripts differ in size and structure from the somatic transcripts and often use TATA-independent spermatogenic cell-specific transcription start sites (SCS) located upstream or downstream the somatic start site and alternative splicing as well as upstream polyadenylation sites. Some of these spermatogenic cell-altered transcripts (SCATs) are at least partially translationally repressed or encode for severely truncated proteins that cannot perform the same functions as the proteins encoded by the same gene in somatic cells. The role for this leaky non-specific process in spermatocytes is unknown, but at least some of these truncated proteins appear to be functional. For example, the CSC isoform of c-Kit in round spermatids lacks its extracellular and transmembrane domains but it may still participate in the activation of development of fertilized oocytes (Sette et al., 1997). Thus, truncated mRNAs of *Gfra2* and *Gfra4* in testis probably represent SCATs and, if anything, they might have a distinct function in testis or in the fertilization process. It would be necessary to confirm whether truncated GFRα2- and GFRα4-proteins indeed exist in spermatogenic cells.

5. CONCLUSIONS

- Mouse and human GFRα4 receptor is smaller in size lacking the first Cys-rich domain found in other GFRα-receptors, including chicken GFRα4. PSPN, but not other GFLs, can bind with high affinity to the mammalian GFRα4 receptor and autophosphorylate Ret tyrosine kinase. In the presence of GFRα4 and RET, PSPN, but not other GFLs, can induce survival of cultured rat sympathetic neurons. Thus GFRα4 is the functional PSPN receptor.

- Mammalian *GFRA4* mRNA expression is restricted to endocrine cells, such as thyroid C-cells and adrenal chromaffin cells. Tissue specific splicing of *Gfra4* pre-mRNA produces functional GFRα4 in young mouse thyroid gland. High expression of *GFRA4* together with RET in human medullary thyroid carcinoma suggest a role for GFRα4 in tumor formation.

- Decreased calcitonin synthesis in young, but not adult GFRα4-deficient thyroid, indicate a novel role for GFRα signaling in controlling transmitter synthesis. This also suggests that GFRα4/Ret-signaling regulates calcium homeostasis and may therefore be relevant in human osteoporosis.

- In testis, the ratio between stem cell renewal and differentiation is regulated by GDNF dosage as spermatogonial stem cells are exhausted (Sertoli-cell-only) in GDNF-deficient mice, while overproduction of GDNF leads to clustering of spermatogonial stem cells. These mouse lines could serve as useful tools for studying the pathogenesis of male infertility and testicular cancers. The importance of GDNF signaling in spermatogenesis suggests that specific molecules in the GDNF/GFRα1/Ret signaling pathway could serve as potential targets for designing male contraceptives.

6. ACKNOWLEDGEMENTS

The present study was carried out at the Institute of Biotechnology, University of Helsinki, during ~1998-2004.

I wish to express my sincere gratitude to Professor Mart Saarma, Director of the Institute, my supervisor and tutor, for encouraging me to carry out this work with his endless support and enthusiasm and his vast scientific knowledge. I am grateful for his advice in various situations and for providing me an excellent working athmosphere.

I warmly thank Docent Matti Airaksinen for his excellent scientific guidance and supervision. His sharp interpretations and helpful discussions have been of fundamental importance throughout my study.

Professor Dan Lindholm and Professor Jorma Palvimo are acknowledged for giving their time to pre-examine and improve the manuscript of this thesis.

The whole personnel of the Institute of Biotechnology are acknowledged for the stimulating and friendly environment and for the help in practical issues whenever needed.

I am grateful to Dr. Urmas Arumäe for his initial guidance in the world of primary neuronal cultures, his help in improving this manuscript and his friendly support during the years in the Neuro lab.

Professor Leevi Kääriäinen is acknowledged for pushing me to write this thesis and for guiding me in the practical issues concerning dead-lines for faculty meetings.

I especially thank Dr. Tõnis Timmusk without whom the cloning of *Gfra4* would ever have been accomplished and for his friendship and support.

I want to thank Dr. Xiaojuan Meng for sharing the early life of GDNF-overexpressing mice and for her help in improving the testis part of this manuscript.

All of my co-authors are acknowledged for fruitful collaboration.

Many thanks go to Päivi Hiltunen, Jari Rossi and Kaija Berg from Mattis lab, for sharing the ups and downs of the GFRα4 knockout mice.

Jukka Hiltunen, my former colleague, is acknowledged for his kind support during the early years in Marts lab. and recently for his help in patenting matters.

My gratitude extends to the people at the computer helpdesk and especially Tinde for his excellent assistance in preparing the manuscript into a printable format.

I am truly thankful to Matthew Phillips for improving the language in this manuscript.

I wish to thank members from the Kidney group, especially Kirmo, Marjo, Tiina, Mervi, Kirsi and Hannu, for the brief but enjoyable time we shared in C-noppa.

Thanks BIKO (especially Miika and Henri) for the great fun at the Sähly court.

I warmly thank all members of the Neuro group, both present and previous for their help, and friendship, and for numerous memorable occasions: Päivi L., Pia, Judith, Svetlana, Maria L., Maili, Marjo, Yu, Ulla, Johanna, Anastasia, Hong, Heidi, Kristiina, Miika, Yang, Maxim, Misha, Claudio, Johan, Antti, Veli-Matti, Janne, Juha, Pavel, Tero, Petro, Ülo and Maxim M.

I am truly grateful to Satu, Eila, Maria v. N, Mari and Maarit for their skilful assistance in the lab, and for sharing many good laughs and jokes.

I highly appreciate the support and affection from all of my friends and relatives near and far!

I am privileged to have such a wonderful family! Special thanks go to my mother Cirre, my father Ronnie, Heicci, Anja, my brother Pate, Ebba and Tor-Anders and all of the Lindahl clan for their big support, help and love. I could not have managed without my sisters Lotta and Miccus, including families. Their endless support, trust and true friendship have been of utmost importance to me.

My deepest gratitude goes to my husband Thomas and our children Anna and Filip for sharing the most precious moments in life. You are the essence of my life!

Helsinki, August 2004

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