Structure and Function of GDNF Receptor Alpha Splice Variants

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Academic dissertation

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To my family

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Abbreviations:

aa	amino acid
AKT	serine-threonine protein kinase
ARTN	artemin
BDNF	brain-derived neurotrophic factor
cDNA	complementary deoxyribonucleic acid
CGN	cerebellar granule neurons
CNS	central nervous system
CNTF	ciliary neurotrophic factor
D	domain
3D	3 dimension
DRG	dosal root ganglion
E	embryonic day
EGFP	enhanced green fluorescent protein
ENS	enteric nervous system
GAS1	the growth arrest-specific gene 1
GDNF	glial cell line-derived neurotrophic factor
GFLs	glial cell line-derived neurotrophic factor family ligands
GFRα	GDNF family receptor alpha
GPI	glycosylphosphatidylinositol
GRAL	GDNF receptor-alpha-like genes
HSCR	Hirschsprung´s disease
Kd	equilibrium dissociation constant
kDa	kilodalton
MAPK	mitogen-activated protein kinase
MEN2	the inherited cancer syndrome multiple endocrine neoplasia type 2
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydopryridine
mRNA	messenger ribonucleic acid
MTC	medullary thyroid carcinoma
NCAM	neural cell adhesion molecule
NGF	nerve growth factor
NRTN	neurturin
6-OHDA	6-hydroxydopamine
P	postnatal day
PD	Parkinson´s disease
PI-3K	phosphatidylinositol-3 kinase
PI-PLC	phosphoinositide specific phospholipase
PNS	peripheral nervous system
PSPN	persephin
RET	rearranged during transfection
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription polymerase chain reaction
SCG	superior cervical ganglion
Sol	soluble
TH	tyrosine hydroxylase
TK	tyrosine kinase
TM	transmembrane

List of publications and manuscripts:

I. **Yang J**, Lindahl M, Lindholm P, Virtanen H, Coffey E, Runeberg- Roos P, Saarma M. (2004). PSPN/GFRalpha4 has a significantly weaker capacity than GDNF/GFRalpha1 to recruit RET to rafts, but promotes neuronal survival and neurite outgrowth. *FEBS Lett. 569(1-3):267-271.*

II. **Yang J**, Runeberg-Roos P, Leppänen V-M, Saarma M. (2006).The mouse soluble GFRα4 receptor activates RET independently of its ligand persephin. *Oncogene* (In press).

III. Virtanen H, **Yang J**, Bespalov MM, Hiltunen JO, Leppanen VM, Kalkkinen N, Goldman A, Saarma M, Runeberg-Roos P. (2005). The first cysteine-rich domain of the receptor GFRα1 stabilizes the binding of GDNF. *Biochem J. 387(Pt 3):817-824.*

IV. **Yang J**, Lindahl M, Saarma M. A novel transmembrane GFRα4 receptor silences persephin-mediated RET signalling, neuronal differentiation and survival. *Manuscript.*

Summary

The growth factors of the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs), consisting of GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN), are involved in the development, differentiation and maintenance of many types of neurons. They also have important functions outside the nervous system in the development of kidney, testis and thyroid gland. Each of these GFLs preferentially binds to one of the glycosylphosphatidylinositol (GPI)-anchored GDNF family receptors α (GFRα). GDNF binds to GFRα1, NRTN to GFRα2, ARTN to GFRα3 and PSPN to GFRα4. The GFLs in the complex with their cognate GFRα receptors all bind to and signal through the receptor tyrosine kinase RET. Alternative splicing of the mouse GFRα4 gene yields three splice isoforms. These had been discribed as putative GPI-anchored, transmembrane and soluble forms. My goal was to characterise the function of the different forms of mouse GFRα4.

I firstly found that the putative GPI-anchored GFRα4 (GFRα4-GPI) is glycosylated, membrane-bound, GPI-anchored and interacts with PSPN and RET. We also showed that mouse GFRα4-GPI mediates PSPN-induced phosphorylation of RET, promotes PSPN-dependent neuronal differentiation of the rat pheochromocytoma cell line PC6-3 and PSPN-dependent survival of cerebellar granule neurons (CGN). However, although this receptor can mediate PSPN-signalling and activate RET, GFRα4-GPI does not recruit RET into lipid rafts. The recruitment of RET into lipid rafts has previously been thought to be a crucial event for GDNF- and GFL-mediated signaling via RET.

I secondly demonstrated that the putative transmembrane GFRα4 (GFRα4-TM) is indeed a real transmembrane GFRα4 protein. Although it has a weak binding capacity for PSPN, it can not mediate PSPN-dependent phosphorylation of RET, neuronal differentiation or survival. These data show that GFRα4-TM is inactive as a receptor for PSPN. Surprisingly, GFRα4-TM can negatively regulate PSPN-mediated signaling via GFRα4-GPI. GFRα4-TM interacts with GFRα4-GPI and blocks PSPN-induced phosphorylation of RET, neuronal differentiation as well as survival. Taken together, our data show that GFRα4-TM may act as a dominant negative inhibitor of PSPN-mediated signaling.

The most exciting part of my work was the finding that the putative soluble GFRα4 (GFRα4-sol) can form homodimers and function as an agonist of the RET receptor. In the absence of PSPN, GFRα4-sol can promote the phosphorylation of RET, trigger the activation of the PI-3K/AKT pathway, induce neuronal differentiation and support the survival of CGN. Our findings are in line with a recent publication showing the GFRα4-sol might contribute to the inherited cancer syndrome multiple endocrine neoplasia type 2. Our data provide an explanation to how GFRα4-sol may cause or modify the disease.

Mammalian GFRα4 receptors all lack the first Cys-rich domain which is present in other GFL co-receptors. In the final part of my work I have studied the function of this particular domain. I created a truncated GFRα1 construct lacking the first Cys-rich domain. Using binding assays in both cellular and cell-free systems, phosphorylation assays with RET, as well as neurite outgrowth assays, we found that the first Cys-rich domain contributes to an optimal function of GFRα1, by stabilizing the interaction between GDNF and GFRα1.

1. Review of the literature

1.1. General introduction to neurotrophic factors

In 1951 Levi-Montalcini and Hamburger discovered the first neurotrophic factor, the nerve growth factor (NGF). NGF was able to support the survival of sensory and sympathetic neurons of chick embryos (Levi-Montalcini and Hamburger, 1951). Later several families of neurotrophic factors have been discovered, such as the neurotrophin family and the glial cell line-derived neurotrophic factor (GDNF) family. In addition some members of the cytokine family (also called neurokines), the insulin-like growth factor (IGF) family, the fibroblast growth factor (FGF) family, the other members of the transforming growth factor beta (TGF-β) superfamily, and the hepatocyte growth factor have neurotrophic activity (Mitsumoto and Tsuzaka, 1999). All neurotrophic factors have in common that first, they support the survival of a subset of neurons during naturally occuring programmed cell death (PCD) period; second, they are synthesized in and secreted from the target tissues (Barde, 1988; Mitsumoto and Tsuzaka, 1999). The growth factors that are classified as neurotrophic factors are similar in many ways, even sometimes with overlapping actions, but are eventually not identical. Here I will give a short introduction of the neurotrophin family and the cytokine family, then focus in more detail on the GDNF family.

1.1.1. Neurotrophin family

The neurotrophin family consists of NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3, -4/5, -6, -7 (NT-3, NT-4, NT-6, and NT-7) (Gotz *et al.*, 1994; Mitsumoto and Tsuzaka, 1999; von Boyen *et al.*, 2002). NT-6 and NT-7 are only in fish species. Neurotrophins are first synthesized as precursors (pre-pro-form). Pre-region is cleaved in the endoplasmic reticulum (ER) during secretion. The pro-form of the immature proteins is then proteolytically cleaved either in the Golgi by furin or in the secretory granule by pro-protein covertases into active, mature proteins (neurotrophic factors) (Seidah *et al.*, 1996).

1.1.1.1. Structure of neurotrophins

Neurotrophins contain a cysteine “knot” which is formed by three disulfide bonds, and neurotrophins exist exclusively as dimers. The core structure of neurotrophin is formed by two pairs of intertwined two-strand β sheets which are assembled by three disulfide bonds. This core structure is conserved in all members of the neurotrophin family (Butte *et al.*, 1998; Butte, 2001).

1.1.1.2. Receptors of neurotrophins

Neurotrophin receptors consist of two types of receptors, the Trk tyrosine kinase receptor family and neurotrophin receptor p75 (p75NR). The Trk receptor family includes three high-affinity receptors, Trk A, Trk B, and Trk C, each of them specifically bind a set of neurotrophin; Trk A binds NGF, NT-6 or NT-7; Trk B binds BDNF or NT-4/5; and Trk C binds NT-3. The p75 receptor, also called a low-affinity receptor, belongs to the tumor necrosis factor receptor (TNFR) family that signal through the intracellular death domain to activate the apoptotic machinery. Recent studies have demonstrated that the pro-form of BDNF can interact with p75NR with a high affinity and affect the neuronal fate (Lee *et al.*, 2001; Egan *et al.*, 2003; Nykjaer *et al.*, 2004; Teng

et al., 2005). These two neurotrophin receptors can either consort or inhibit each other´s actions to mediate the effects of neurotrophins (Chao, 1992; Lindsay *et al.*, 1994; Kaplan and Miller, 2000).

1.1.1.3. Splicing of neurotrophins and their receptors

Most of the eukaryotic genes are mosaics and consist of protein encoding sequences (exons) and intervening sequences (introns). The transcripts of this kind of split genes (including introns as well as exons) are called pre-messenger RNAs (mRNA). During protein synthesis in the cells, mRNAs containing a contiguous stretch of codons are translated into proteins. Noncoding sequences from introns can not at this stage be identified and skipped, meaning that the introns in the pre-mRNA must be removed before the initiation of the translation process. The removal process of introns from pre-mRNA is called splicing.

Alternative splicing gives rise to variants of neurtrophins and their receptors. For instance, alternative splicing and different initiation from two promoters, result in four different NGF-encoding mRNA transcripts, A-D (Selby *et al.*, 1987). Alternative splicing and multiple promoters generate eight unique BDNF transcripts (Timmusk *et al.*, 1993) that might be translated into eight different BDNF forms. Alternative splice variants of BDNF have been shown to be differently regulated (Timmusk *et al.*, 1993) and to be responsive for behavioral learning (Rattiner *et al.*, 2004). The *nt-3* gene consists of two small upstream exons (exons IA and IB) and a larger downstream exon (exon II). The mature NT-3 proteins are encoded by two different transcripts (A and B) which are formed by alternatively splicing of exon IA or exon IB to the common exon II (Leingartner and Lindholm, 1994). A third transcript of NT-3 was also identified in rat. This transcript is derived from the splicing of exon 1A to exon 1B as well as to exon II (Kendall *et al.*, 2000).

Like the neurotrophins, their cognate receptors are also translated from alternatively spliced mRNAs. For example, *trkA* has two splice forms, *trkA* I and *trkA*II. TrkA II proteins contain an insert of six amino acids. Both forms have an equal ability to bind the ligand, but their expression pattern is different (Barker *et al.*, 1993). Although the binding ability of these two receptor variants to their ligands is similar, TrkA II has significantly higher activation by NT-3 than TrkA I (Clary and Reichardt, 1994). Very recently a novel alternative *trkA* splice variant, *trkA* III, was identified in undifferentiated early neural progenitors, human neuroblastomas (NBs), and a subset of other neural crest-derived tumors (Tacconelli *et al.*, 2004). The mammalian *trkB*, by alternatively splicing, gives at least eight different transcripts and results in two different receptor classes. The first class consists of the full-length TrkB receptor ($TrkB^{TK+}$) and the second class consists of truncated TrkB receptors ($TrkB^{TK-}$). $TrkB^{TK-}$ has the same extracellular and transmembrane domains as $TrkB^{TK+}$, but contains only a short cytoplasmic domain of 23-amino acids, lacking the entire catalytic kinase region (Klein *et al.*, 1989; Klein *et al.*, 1990; Middlemas *et al.*, 1991). These two classes of the TrkB receptors have been shown to have different effects on dendritic arborization and neuronal morphology (Haapasalo *et al.*, 1999; Yacoubian and Lo, 2000). However, *in vivo* and *in vitro* studies indicate that the $TrkB^{TK-}$ receptors negatively influence neuronal survival by interfering with the function of the $TrkB^{TK+}$ receptors (Haapasalo *et al.*, 2001;

Luikart *et al.*, 2003). In chicken there are two splicing forms of *trkB*, a full-length receptor and a truncated form which is lacking 11 amino acids in the extracellular domain. These two forms have different response to BDNF and NT-4/NT-5 (Boeshore *et al.*, 1999). The *trkC* gene encodes multiple NT-3 receptor isoforms with distinct biological properties and substrate specificities (Lamballe *et al.*, 1993), including receptors with inserts or deletions in the catalytic domain (Tsoulfas *et al.*, 1993; Valenzuela *et al.*, 1993). The TrkC isoforms with inserts (TrkC14 and TrkC25) appear to be inactive as receptors for NT-3 (Tsoulfas *et al.*, 1996), and the truncated TrkC receptors have pleiotropic modulatory functions *in vivo* (Palko *et al.*, 1999). Two isoforms of p75 have been identified, a full length transmembrane and a truncated form lacking the second, third and fourth extracellular cysteine-rich repeats (von Schack *et al.*, 2001). The truncated p75 does not bind neurotrophins, and is expressed at markedly reduced levels compared to the full-length p75 in human and birds. The biological functions of the truncated form are unclear. Gene-targeted animals lacking both isoforms exhibit more profound losses in sensory neurons and Schwann cells, and an additional defect in the development of the great vessels, compared to animals lacking only the full-length p75 (von Schack *et al.*, 2001; Hempstead, 2002).

1.1.2. Cytokine family

The cytokine family members are small ubiquitous pleiotropic molecules that are synthesized and released in response to a variety of stimuli (Kishimoto *et al.*, 1992). This family consists of numerous members including ciliary neurotrophic factor (CNTF), interleukin 6 (IL-6), lukemia inhibitory factor (LIF), and several other growth factors. This family is sometimes also called CNTF-family of neurotrophic factors. They have a multitude of actions throughout the body, including actions on the central and peripheral nervous systems (CNS and PNS). It has been shown that CNTF acts as a trophic factor for many types of neurons, including parasympathetic, sympathetic, sensory, and motor neurons *in vitro*. In addition it was considered as the most potent trophic factor for motor neurons (Arakawa *et al.*, 1990). However, later *in vivo* studies have shown that it is not required for embryonic development of motor neurons, but it is required for maintaining the number of motor neurons in adult mice (Masu *et al.*, 1993). IL-6 has been reported to affect neuronal survival (embryonic rat sensory neurons, cultured hippocampal neurons, rat striatal cholinergic neurons, and dosal root ganglion (DRG) neurons), neuronal differentiation (sensory neurons and motorneurons), nerve regeneration (DRG), and synaptic plasticity in the hippocampus (Gadient and Otten, 1997; Tancredi *et al.*, 2000; De Jongh *et al.*, 2003). LIF has been shown to promote the proliferation of olfactory sensory neuron precursors *in vivo* and *in vitro* (Kim *et al.*, 2005).

1.1.2.1. Structure of cytokines

CNTF is not a cysteine knot growth factor, it is rather a four-helix bundle protein belonging to the IL-6 family of hematopoietic cytokines. The primary structure of CNTF shows the four helices, named A-D (Butte, 2001). Two helices, B and C, contribute to the dimer interface, whilst the other two show pronounced kinks. Analysis of the electrostatic surface of CNTF identified residues within these kinked helices that may contact the CNTF receptor-α. The A and C helices contribute to the interaction with gp130,

the common receptor for the CNTF family of neurotrophic factors (McDonald *et al.*, 1995).

1.1.2.2. Receptors of cytokines and their splice variants

The receptor of IL-6 (IL-6-R) has two splice forms, the membrane-bound and soluble forms. The biological functions of these two isoforms are basically the same (Nowell *et al.*, 2003; McLoughlin *et al.*, 2004). The receptor of CNTF has two variants, glycosylphosphatidylinositol (GPI)-anchored and soluble forms. The GPI-anchored form is expressed in neuronal and skeletal muscle cells (Davis *et al.*, 1991; Ip *et al.*, 1993). The soluble form serves as a cofactor in potentiating CNTF actions on cells (Davis *et al.*, 1993). Several isoforms of the common gp130 receptor of cytokines have been identified. There are at least three different splice variants of soluble gp130, in addition to the membrane-associated gp130. The size of these three variants is 50 kDa, 90 kDa and 110 kDa, respectively, and these may be involved in regulating IL-6 trans-signaling. Also, the purified recombinant soluble gp130 (90 kDa and 110 kDa) showed an inhibitory effect on the biological function of IL-6 via gp130 (Narazaki *et al.*, 1993; Zhang *et al.*, 1998; Richards *et al.*, 2006).

The members of the cytokine family signal via multicomponent receptor complexes. All cytokine receptors have a single transmembrane domain and an intracellular signaling domain, except the CNTF receptor α (CNTFRα), which is anchored to the membrane by a GPI-anchor. The common receptor for cytokines is gp130. In order to signal, cytokines need to bind their own cognate receptors and then induce either the homodimerization of gp130 receptors, or the heterodimerization of gp130 and LIF receptor (LIFR). There are two theories on how the signal complexes are formed. One is based on immunoprecipitation and gel filtration, and predicts that the complex is hexameric with a stoichiometric ratio of 2:2:1:1 (cytokines: cognate receptors: gp130:LIFR/gp130). The other theory is based on molecular modeling, and predicts that the complex is tetrameric with a stoichiometric ratio of 1:1:1:1. IL-6 is an example of the former type of ligands whereas LIF, oncostatin M (OSM), CNTF and cardiotrophin 1 (CT-1) are the examples of the latter type (Sanchez-Cuenca *et al.*, 1999; Butte, 2001; Vergara and Ramirez, 2004).

1.1.2.3. Splicing variants of cytokines

Some of the cytokines have been reported to have splicing isoforms. IL-6 and IL-6-alt arise through alternative splicing. Both form signaling complexes with p80 and gp130, although IL-6-alt is lacking the second exon. This second exon encodes amino acid residues which are known to be important for the gp130-mediated signal transduction pathway. Therefore IL-6-alt has less activity comparing to IL-6, but does not act as an inhibitor of IL-6 (Kestler *et al.*, 1995; Kestler *et al.*, 1999). Several splice variants of LIF have also been described in human tissues. By alternative use of the first exons (D, M, and T), three variants are formed (LIF-D,-M, and -T). The LIF-D is a biologically active, secreted protein and can signal via the LIF receptor. The LIF-M is a secreted and intracellular protein, and may have biological activity when it is in the extracellular matrix; the LIF-T is an intracellular protein which might function as a transcription factor (Hisaka *et al.*, 2004).

1.2. Glial cell-derived neurotrophic factors and their receptors

There are four different types of GDNF family ligands (GFLs), GDNF (Lin *et al.*, 1993), neurturin (NRTN) (Kotzbauer *et al.*, 1996), artemin (ARTN) (Baloh *et al.*, 1998b), and persephin (PSPN) (Milbrandt *et al.*, 1998). ARTN is also called neublastin (NBN) (Rosenblad *et al.*, 2000) or enovin (EVN) (Masure *et al.*, 1999). These four factors have their own preferential receptors, respectively, so called GDNF family receptor alphas (GFRαs), which are GPI-linked cell surface proteins. There are four GFRαs, GFRα1, GFRα2, GFRα3, and GFRα4, and a common receptor tyrosine kinase called RET. I will introduce the growth factors and their signaling receptors in the following chapters.

1.2.1. GFLs and their splice variants

1.2.1.1. GDNF

GDNF was first cloned and purified as a potent neurotrophic factor that enhances the survival of midbrain dopaminergic neurons. The full-length GDNF consists of 211 amino acids and harbours both a signal sequence and a pro-region. The pro-region is cleaved from the mature domain possibly at a RXXR cleavage site (Lin *et al.*, 1993), but the biological activity of the pro-GFLs are still uncharacterised. In this context it is interesting to note that the pro-forms of NGF and BDNF have some biological functions naturely inducing cell death (Lee *et al.*, 2001; Egan *et al.*, 2003). Mature GDNF consists of 134 amino acids with a predicted molecular weight of ~15 kDa. Biochemical analysis has shown that mature GDNF is a glycosylated protein and forms covalent disulfide-bridged homodimers (Lin *et al.*, 1993). GDNF is a growth factor which contains a cysteine "knot", and the monomer of the three-dimensional structure is characterized by two long fingers formed by pairs of anti-parallel β-strands connected by a loop and a helical portion in the opposite site (Fig. 1) (Eigenbrot and Gerber, 1997). The monomers are associated in a head-to-tail orientation to form the dimer. Because of the anti-parallel arrangement, the structure of GDNF has a left-right symmetry, which may indicate that there is a symmetrical binding site for a dimerized receptor. The structure-function analysis showed that the first 39 amino acids in the N-terminus of GDNF are not required for its biological activities in motor neurons (Chen *et al.*, 2000). It should be noted that

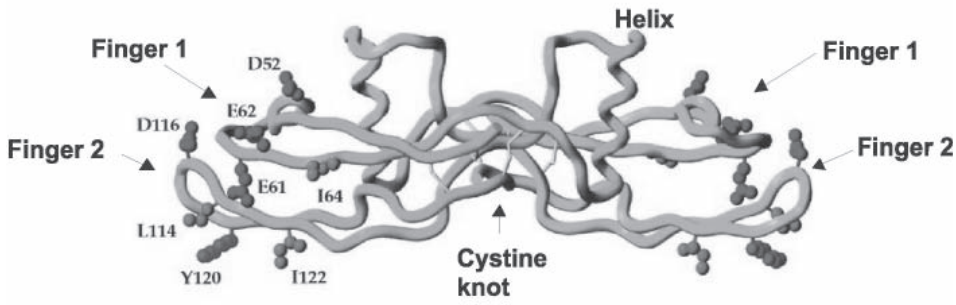

Fig. 1. The 3D structure of GDNF. Note that 38 amino acids at the N-terminus of GDNF are not visible on this structure. GDNF is a homodimer. Indicated amino acids on the finger 1 and 2 are important for receptor binding (Adapted from Eketjäll *et al.*, 1999).

the 3D structure of the N-terminal region is still missing. Most likely this region is structurally very dynamic and therefore not visible in the crystal structure. The C-terminus is critical for the stability and biological activity of GDNF, the α-helix, finger 1 and finger 2 are involved in the binding of GDNF to the GFRα1 receptor (Chen *et al.*, 2000). More precisely, Eketjäll *et al.* have identified a set of eight residues, four negatively charged (Asp52, Glu61, Glu62, Asp116) and four hydrophobic (Ile64, Leu114, Tyr120, Ile122), that are involved in the binding of GDNF to the GFRα1 receptor. Any individual mutation in these residues results in a major effect on the binding affinity of GDNF to GFRα1, indicating that each of these amino acids is critical for the binding (Eketjäll *et al.*, 1999).

GDNF is expressed in many tissues and in many cell types throughout the body, including neurons (Schaar *et al.*, 1993; Stromberg *et al.*, 1993; Springer *et al.*, 1994; Trupp *et al.*, 1995; Suvanto *et al.*, 1996; Golden *et al.*, 1999). It is widely expressed in the CNS and PNS, and has multiple neuronal targets in the nervous system. The effects of GDNF on nervous systems are summarized in Table 1 (modified from Bohn, 2004).

Outside the nervous system, GDNF has a crucial role in kidney development and spermatogenesis. $GDNF^{-/-}$ mice displayed complete renal agenesis due to a lack of the induction of the ureteric bud formation which are early steps in kidney development (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez, *et al.*, 1996). *In vitro* assays show that exogenous GDNF stimulates both the branching and proliferation of embryonic kidneys (Vega *et al.*, 1996; Sainio *et al.*, 1997). It was also showed that GDNF can stimulate the chemotaxis in kidney organ culture (Tang *et al.*, 1998). During spermatogenesis, GDNF regulates the cell fate decision of undifferentiated spermatogonia. $GDNF^{+/-}$ mice displayed a disturbed spermatogenesis with degenerated tubules. However, mice overexpressing GDNF show an accumulation of spermatogonia stem cells and are unable to respond properly to differentiation signals, later these mice develope nonmetastatic testicular tumors (Meng *et al.*, 2000; Meng *et al.*, 2001).

1.2.1.2. NRTN and ARTN

1.2.1.2.1. NRTN

NRTN is a GFL which binds preferentially to GFRα2 receptor and signals via RET. NTRN was identified on the basis of its ability to support the survival of sympathetic neurons in culture. Mouse NRTN is synthesized as a pre-pro-form consisting of 195 amino acids, where 19 amino acids form the signal sequence and 76 amino acids form the pro-region (Fig. 2). After cleavage of the pro-protein, a 100 residues long mature NRTN is formed. Human mature NRTN is 91% identical to mature mouse NRTN and both proteins have a predicted molecular weight of about 11.5 kDa (Kotzbauer *et al.*, 1996). As all other GFLs, NRTN is also biologically active as a homodimer.

The distribution of NRTN has been described in detail for developing and adult mice and rats (Golden *et al.*, 1999; Xian *et al.*, 1999). In addition to the sympathetic neurons, NTRN also maintains the enteric neurons in the gut (Heuckeroth *et al.*, 1998; Rossi *et al.*, 1999), neurons in the DRG, trigeminal and nodose ganglion (Kotzbauer *et al.*, 1996; Rossi *et al.*, 1999), CNS dopaminergic neurons in the ventral midbrain (Horger *et al.*, 1998) and the spinal motor neurons (Klein *et al.*, 1997).

Outside of the nervous system, NRTN can induce the branching of the ureteric bud in an *in vitro* assay (Milbrandt *et al.*, 1998). The expression of NRTN in and around bone suggests that it might be involved in the bone development. In the prostate the high-level of NRTN may influence the growth of prostate epithelium and it is expressed at high-levels in the epithelium and mucosa of the oviduct (Golden *et al.*, 1999). Recently, NRTN, as well as PSPN have been found to promote the survival of rat basal forebrain neurons *in vitro* including both cholinergic neurons and a population of non-cholinergic neurons (Golden *et al.*, 2003).

1.2.1.2.2. ARTN

ARTN, mentioned already earlier is called enovin and neublastin (Masure *et al.*, 1999; Rosenblad *et al.*, 2000), is more similar to NRTN and PSPN (about 45% identity), than to GDNF (about 36% identity). Like GDNF and NRTN, ARTN is also synthesized as a pre-pro-form (Fig. 2). Human mature ARTN consists of 113 amino acids with a molecule weight of around 12.5 kDa. ARTN is a trophic factor for sensory and sympathetic neurons *in vitro* (Baloh *et al.*, 1998b), and enhances the neuronal generation, growth and neurite outgrowth of sympathetic neurons *in vitro* (Andres *et al.*, 2001; Enomoto *et al.*, 2001). In the CNS, this factor is expressed at a very low level. ARTN supports at least the survival of dopaminergic neurons in the midbrain *in vitro* (Baloh *et al.*, 1998b). The physiological functions of ARTN were demonstrated by characterizing ARTN-deficient mice, where the migration and axonal projections of sympathetic neuroblasts were disrupted. However, only small effects on cell survival or proliferation were observed, which is in contrast with the *in vitro* data (Honma *et al.*, 2002). Furthermore, the activity of ARTN is mediated by heparin sulfate proteoglycans (HSPGs). The crystal structure reveals that the arginine residues in the pre-helix and amino-terminal regions of ARTN are involved in ARTN binding HSPGs, and the substitution of these residues can reduce the ability of ARTN to activate RET (Silvian *et al.*, 2006). ARTN is also expressed in smooth muscle cells of the vessels during development of the vasculature, and it may thereby act as a guidance factor for sympathetic

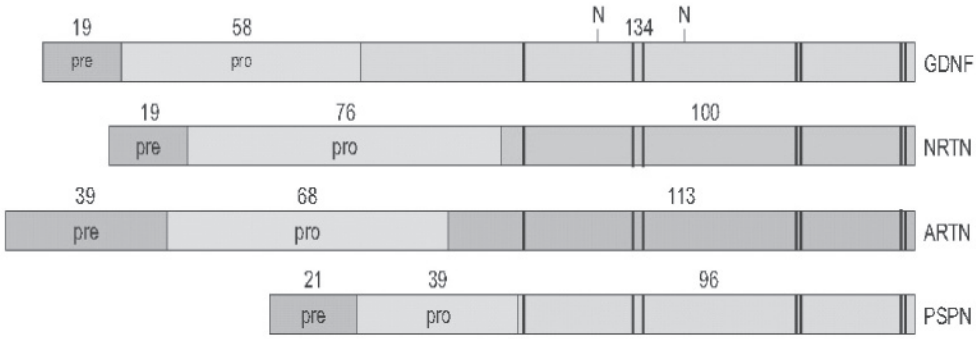

Fig. 2. Schematic structure of GFLs showing the relative lengths (in amino acid number). The conserved seven cysteines in the mature regions are marked (black lines), and "N" stands for the glycosylation sites (kindly provided by Professor Saarma).

Table 1. GDNF has multiple neuronal targets in CNS and PNS

Neuronal class	Target neurons	Effects of GDNF	Literatures
CNS: motoneurons	Spinal motoneurons	Increased survival of spinal motoneurons at all levels of spinal cord *in vitro* and *in vivo*, and the regeneration of motoneneurons following axotomy; increased activity of choline acetyltransferase (ChAT) in dissociated culture, and the sciatic nerve regeneration in mice; decreased programmed cell death during development; protection against ethanol challenge.	(Escandon *et al*., 1994; Zurn *et al*., 1994; Li *et al*., 1995; Munson and McMahon, 1997; Naveilhan; *et al*., 1997; Barrow Heaton *et al*., 1999; Boyd and Gordon, 2003)
	Rat facial nucleus	Increased survival of facial neurons in neonatal rat after axotomy and decrease in ChAT after axotomy in adult rat	(Yan *et al*., 1995; Matheson *et al*., 1997)
	Corticospinal neurons	Increased survival following axotomy at level of internal capsule and also, increased size and function after spinal cord lesions	(Giehl *et al*., 1998; Tang *et al*., 2004)
	GDNF transgenic mouse	Hyperinnervation of neuromuscular junctions by overexpression of GDNF in muscle; delayed synapse elimination; decreased programmed cell death in trigeminal, facial and hypoglossal nuclei; and also decreased lesion-induced death.	(Nguyen *et al*., 1998; Oppenheim *et al*., 2000; Zwick *et al*., 2001; Zhao *et al*., 2004)
CNS: Retina	Retinal ganglion cells	Increased survival after optic nerve lesion; and also increased survival of rod photoreceptors.	(Klocker *et al*., 1997; Bennett *et al*., 1998; Koeberle and Ball, 1998; Frasson *et al*., 1999)
CNS: sensory neurons	Chick vestibular ganglion	Increased neurite outgrowth at E12–16	(Hashino *et al*., 2001)
CNS: dopamine neurons	Dopamine neurons	Increased survival of dopamine neurons *in vitro*; *in vivo*, GDNF increased survival in rat or mouse brain after 6-OHDA or methamphetamine, also the survival and function with GDNF gene delivery before or after 6-OHDA; increased survival in vivo in MPTP treated or aged monkey brain with GDNF protein or GDNF gene delivery. Increased survival and decreased apoptosis of human embryonic dopamine neurons in vitro.	(Lin *et al*., 1993; Hoffer *et al*., 1994; Beck *et al*., 1995; Kearns and Gash, 1995; Sauer *et al*., 1995; Tomac *et al*., 1995; Clarkson *et al*., 1995; Cass, 1996; Hou *et al*., 1996; Gash *et al*., 1996; Choi-Lundberg *et al*., 1997; Kordower *et al*., 2000; Kozlowski *et al*., 2000;)
CNS: striatal neurons		Increased survival of NeuN, calbindin, and GABA/substance P immunoreactive neurons after quinolinic acid or kainic acid lesion; increased neuronal survival of transient ischemia; and in aged and MPTP-treated monkey, increased number of tyrosine-hydroxylase immunoreactive neurons in striatum	(Perez-Navarro *et al*., 1996; Hermann *et al*., 2001; Alberch *et al*., 2002; Palfi *et al*., 2002; Kells *et al*., 2004)
CNS: noradrenergic neurons	6-OHDA lesion GDNF mull mouse	Increased survival of noradrenergic neurons in locus coeruleus Disrupted the postnatal development of locus coeruleus noradrenergic neurons in the brainstem-hippocampal cografts	(Arenas *et al*., 1995; Quintero *et al*., 2004)

Table 1. continuing

CNS: Purkinje cells	Dissociated rat cerebellum in culture and in shaker rat	Increased survival of Purkinje cells	(Mount *et al*., 1995; Tolbert *et al*., 2001; Tolbert and Clark, 2003)
CNS: hippocampus	Kainic acid in rat Global ischemia in gerbils	Decreased free radical damage to CA3 and CA4 pyramidal neurons Decreased apoptosis of CA1 pyramidal neurons	(Shirakura *et al*., 2003; Cheng *et al*., 2004)
PNS: sensory neurons	DRG lesioned and in culture GDNF knockout mouse	Increased survival of sensory neurons Decreased number of sensory neurons in DRG and nodose ganglion	(Buj-Bello *et al*., 1995; Moore *et al*., 1996)
PNS: sympathetic neurons and parasympathetic neurons	GDNF knockout mouse Chick neurons in culture	Decreased number of sympathetic neurons, loss of several parasympathetic neurons. Increased survival of sympathetic and parasympathetic neurons; increased neurite outgrowth	(Buj-Bello *et al*., 1995; Ebendal *et al*., 1995; Moore *et al*., 1996; Rossi *et al*., 2000)
PNS: enteric neurons	GDNF knockout mouse	Enteric neurons lacking	(Moore *et al*., 1996)
Auditory system	Guinea pig Guinea pig	Hair cells protected from cisplatin induced ototoxicity Increased survival of cochlear neurons after noise-induced lesions of inner hair cells	(Ylikoski *et al*., 1998; Kuang *et al*., 1999)

fibers to project toward their final target tissues *in vivo*. This finding indicates that ARTN is an important guidance factor for sympathetic fibers to follow blood vessels as they project toward their final target tissues (Honma *et al.*, 2002).

1.2.1.3. PSPN

Pspn was cloned based on the sequence homology to *gdnf* and *nrtn*, and the mature PSPN is about 40% identical to mature GDNF and NRTN. The rat and mouse *pspn* genes contain an intron in the sequence encoding the pro-region of PSNP, and removal of this intron leads to the formation of an open reading frame encoding a pre-pro-protein of 156 amino acids, with a predicted 21 residues long signal peptide (Fig. 2). The mature PSPN is a 96 amino acid long protein with a molecular weight of about 10~12 kDa (Milbrandt *et al.*, 1998).

Human *PSPN* also has an intron present in the pro-domain. After splicing, a 156 amino acid protein with 80 % identity to mouse and rat PSPN can be translated. Human PSPN shows 38% identity to human NRTN (50% in the mature region), and 30% identity to human GDNF (40% in the mature region). The pro-region is cleaved from the mature protein at the RXXR site five residues upstream of the first conserved cysteine (Milbrandt *et al.*, 1998).

Pspn mRNA is expressed at very low levels and was detected in all tissues examined in embryos and adult animals. Two species of PSPN encoding mRNA (unspliced and spliced mRNA) were detected using RT-PCR. The larger, more abundant species correspond to the unspliced mRNA (Milbrandt *et al.*, 1998; Lindfors *et al.*, 2006). The levels of both *pspn* mRNA species were stable from E10 through E18. Comparison of the expression levels in adult tissues and their embryonic counterparts showed that PSPN encoding mRNA appears to be present at relatively similar levels in most tissues examined, but with slightly higher levels in embryonic tissues (Milbrandt *et al.*, 1998).

PSPN supports the survival of rat motoneurons in culture and *in vivo* after sciatic nerve axotomy. Surprisingly, unlike GDNF and NRTN, PSPN does not maintain any of the peripheral neurons examined, including those from the superior cervical, dorsal root, nodose, trigeminal, and enteric ganglia (Milbrandt *et al.*, 1998). The recent findings of its effects on the rat basal forebrain neurons including both cholinergic neurons and a population of non-cholinergic neurons shed light on the possibility that PSPN may be effective as a neuroprotective agent for basal forebrain cholinergic neurons (BFCNs) *in vitro* and *in vivo* (Golden *et al.*, 2003). The potential neurobiological function of PSPN *in vivo* was also investigated and it was demonstrated that mice lacking PSPN have markedly increased cerebral infarction after focal ischemia (Tomac *et al.*, 2002). Moreover, PSPN prevented by ~70% the loss of dopaminergic neurons and also by 51-66% the behavioral abnormalities in a rodent Parkinson´s disease model (Åkerud *et al.*, 2002).

PSPN can support the survival of rat dopamine neurons and spinal cord motor neurons both *in vitro* and *in vivo* (Milbrandt *et al.*, 1998; Bilak *et al.*, 1999). The potential clinical application of PSPN was recently reported. Engineered neural stem cells (NSCs), expressing PSPN were used in a PD model. In this study PSPN appeared to enhance the dopamine-dependent behavioral parameters in unlesioned mice, prevented the loss of dopamine neurons and the behavioral

impairment of mice exposed to 6-OHDA. These results suggest that PSPN has a clinical potential in the treatment of PD (Åkerud *et al.*, 2002). Besides the potential in the treatment of PD, PSPN also has a potential to bring a new strategy to stroke treatment. Mice lacking PSPN are hypersensitive to cerebral ischemia showing an increase of about 300 % in infarction volume as compared with the controls (Tomac *et al.*, 2002). The glutamate-induced Ca^{2+} influx was thought to be a major component of ischemic neuronal cell death (Zipfel *et al.*, 1999; Lee *et al.*, 1999; Zipfel *et al.*, 2000). *In vitro* experiments demonstrated that PSPN can reduce hypoxia-induced cortical neuronal death. Furthermore, *in vivo* administration of recombinant PSPN before ischemia can attenuate the neuronal cell death. Taken together, these events indicate that PSPN may have an important control function in the context of stroke and glutamate-mediated neurotoxicity (Tomac *et al.*, 2002).

1.2.2. GFL family receptors and their splice variants

1.2.2.1. GFRαs

After the discovery of GDNF, one of the main targets was the search for a functional receptor for GDNF signaling. Under this effort, Jing *et al.* in 1996 found a novel membrane-associated protein which can bind GDNF, GDNFR-α (later named GFRα1).

Recently a GDNF receptor-alpha-like gene, *gral* has been identified from mouse. Sequence analysis showed that the *gral* is a distant homolog of the GFRα family, and its amino acid sequence is 20, 26, 30 and 30 % identical to that of GFRα1,-2, -3, and -4, respectively. There are two splice variants of *gral*, *gral*-A (2080 bp) and *gral*-B (1833 bp). *Gral*-A and *gral*-B encode proteins of 393 and 238 amino acid residues respectively, and the GRAL-A is a putative transmembrane protein, whereas the GRAL-B is a putative soluble protein. GRAL transcripts have been detected primarily in the CNS in adult mouse, it is highly expressed in the cerebrocortex and hippocampus at birth, thereafter decline in the developing mouse brain (Li *et al.*, 2005). The function of GRAL remains unclear.

GAS1 (the growth arrest-specific gene 1) protein has been recently demonstrated as an alternative receptor for GFLs. GAS1 protein shows high structural similarity to GFRαs. It can bind RET, therefore modify the downstream signaling. The functional similarities between GFRαs and GAS1 may be in their functions in embryogenesis, differentiation and glia maintenance (Schueler-Furman *et al.*, 2006; Cabrera *et al.*, 2006).

1.2.2.1.1. GFRα1

Based on the conserved cysteine residues, it was predicted that the GFRα1 receptor has a three-domain structure, D1, D2, and D3 (Suvanto, 1997; Airaksinen *et al.*, 1999). This three-domain structure seems to be common to all GFRα receptors except for the mammalian GFRα4 receptors, which lack the first domain, D1. Rat GFRα1 consists of 468 amino acids, there are three potential N-linked glycosylation sites and a predicted molecular weight of about 47 kDa. Structural and biochemical analysis show that this protein is actually linked to the cell surface by a GPI-linkage (Jing *et al.*, 1996). It has also been shown that GFRα1 may act as the alternative receptor for NRTN to activate RET, but NRTN binding to GFRα1 is much weaker than to GFRα2 (Creedon *et al.*, 1997).

Alternative splice isoforms of *gfrα1* have been identified and named *gfrα1*a (includes exon 5) and *gfrα1*b (excludes exon 5) (Sanicola *et al.*, 1997; Dey *et al.*, 1998; Shefelbine *et al.*, 1998). GFRα1a is expressed predominantly in the whole brain, while GFRα1b was found in peripheral tissues. This indicates that these two isoforms may have distinct functions (Yoong *et al.*, 2005). Indeed, *in vitro* studies show that the two isoforms have different binding affinities for GDNF and NRTN. Moreover, the GFRα1b isoform, which has a higher affinity for both GDNF and NRTN, is also mediating more efficiently the RET autophosphorylation upon ligand stimulation (Charlet-Berguerand *et al.*, 2004). These results suggest that the regulation of GFRα1 by alternative splicing may have a function in neuronal differentiation.

GFRα1 is expressed in both neuronal and non-neuronal tissues. Those neurons include the enteric, parasympathetic, sympathetic, sensory, motor neurons, and dopamine neurons in the ventral midbrain (Golden *et al.*, 1999). GFRα1 is also expressed in non-neuronal cells, such as in the cells of the urogenital system, the respiratory system, spermtogonial stem cells (Golden *et al.*, 1999). GFRα1 has also recently been found to be expressed in the developing rat carotid body (Leitner *et al.*, 2005).

Recently structural studies resulted in a first model of the GFRα1 fragment that binds GDNF. The crystal structure of rat GFRα1 domain 3 (residues 239–346) and a model of the homologous domain 2 (residues 150–238), combined with site-directed mutagenesis, led to the identifaion of the residues Phe213, Arg224, Arg225 and Ile229 as part of a putative GDNF-binding surface. Furthermore, the crystal structure of GFRα1 domain 3 revealed a new protein fold with all-α fold with five disulfide bridges (Leppänen *et al.*, 2004).

1.2.2.1.2. GFRα2

Gfrα2 was identified in 1997 from human, rat, mouse and chicken tissues based on the homology to *gfrα1* (Baloh *et al.*, 1997; Buj-Bello *et al.*, 1997; Klein *et al.*, 1997; Suvanto *et al.*, 1997). This protein consists of 464 amino acids with 48% identity to GFRα1 and is anchored to the cell surface by GPI-linkage. Like the other family members it has an N-terminal signal peptide for secretion and three putative glycosylation sites.

Gfrα2 has three isoforms, named *gfrα2*a, *gfrα2*b and *gfrα2*c. These three variants have the same 5´-UTR (Wong and Too, 1998). GFRα2a is expressed in the SCG neurons and DRG. In the adult brain it acts preferentially as a receptor for NRTN. GFRα2a consists of 463 amino acids with N-terminal signal sequence and C-terminal hydrophobic domain. The amino acid sequence of GFRα2b is the same as GFRα2a except for the 105 amino acid deletion at the N-terminal region (14-119 a.a.). No functional studies on GFRα2b are available yet. GFRα2c is the shortest form and it consists of 330 amino acids, and it also has the same amino acid sequence as GFRα2a except the deletion of 133 amino acids (14-147 a.a.) at the N-terminal (Wong and Too, 1998). GFRα2c responds equivalently to both GDNF and NRTN in mediating the RET phosphorylation (Baloh *et al.*, 1997).

GFRα2 is expressed in the nervous system and other tissues during both the development and adulthood. It is expressed in the CNS (the spinal cord and the all areas of brain), the PNS (the sensory and autonomic ganglion), the developing heart, the developing limbs and the skeleton (Golden *et al.*, 1999). However, the

expression of GFRα1 decreases by P1, but the level of GFRα2 remains unchanged (Leitner *et al.*, 2005). GFRα2/NRTN may indeed have a role as a trophic factor for the maintenance of mature cells. This hypothesis is supported by expression data which show that a decreased expression of GFRα1 is also accompanied by an increased or maintained expression of GFRα2, in the sphenopalatine and otic ganglia of the parasympathetic nervous system (Enomoto *et al.*, 2000; Rossi *et al.*, 2000), as well as in the neurons of the ENS (Golden *et al.*, 1999).

1.2.2.1.3. GFRα3

Gfrα3 was actually cloned before the discovery of its ligand, ARTN. *Gfrα3* was identified based on the homology to *gfrα1* and *gfrα2*. GFRα3 is a protein of 397 amino acids which contains a hydrophobic signal peptide as well as a stretch of hydrophobic amino acids at its C-terminus that comprises a putative GPI-linkage sequence. It also contains three putative N-linked glycosylation sites. Rat GFRα3 displays 33 % amino acid identity with GFRα1 and 36 % identity with GFRα2. The fully glycosylated form has a molecular weight of 62 kDa (Jing *et al.*, 1997; Worby *et al.*, 1998; Widenfalk *et al.*, 1998; Masure *et al.*, 1998). No GFRα3 isoforms have yet been found.

GFRα3 is highly expressed by embryonic day 11 but is barely expressed in the adult mouse (Worby *et al.*, 1998). A prominent expression was found in the peripheral ganglia and in the nerves. No robust expression was found in CNS (brain and the spinal cord), but there was a strong expression in the DRG and in the trigeminal ganglia at all developmental stages investigated (Widenfalk *et al.*, 1998). *Gfrα3* encoding mRNA was also found in developing non-neuronal tissues, for instance in the kidney, liver, and heart (Jing *et al.*, 1997; Widenfalk *et al.*, 1998). Like GFRα1 and GFRα2, GFRα3 was found to be expressed in the developing carotid body (Leitner *et al.*, 2005).

The study on structure of GFRα3 and ARTN complex revealed that the complex consists of a single ARTN homodimer and two GFRα3 D2D3 molecules (Wang *et al.*, 2006). This "D2D3" modules are closely packed together to form a compact glubolar structure (Wang *et al.*, 2006) which is unexpected based on the speculation that the GFRαs fold into three independent domains (Leppänen *et al.*, 2004). The interface between the two domains of GFRα3 forms a large hydrophobic core involved ten highly conserved residues in all GFRα receptors. The binding domain for ARTN in GFRα3 is the D2 domain only, the D3 domain of GFRα3 has no interaction with ARTN (Wang *et al.*, 2006), which is in contrast to the speculation that it forms direct ligand contacts (Leppänen *et al.*, 2004). Together with the mutant chimeric receptors analysis, the data indicate that the role of the D3 domain of GFRα3 appears to stabilize the D2 domain (Wang *et al.*, 2006).

1.2.2.1.4. GFRα4

GFRα4, the preferred receptor for PSPN signaling, was first identified from chicken embryos (Thompson *et al.*, 1998). The chicken cDNA encodes a protein of 431 amino acids with an identity of about 40% to mammalian GFRα1 and GFRα2, but only with an identity of about 27% to mammalian GFRα3. Chicken GFRα4 also has the same pattern of conserved cysteines as the other GFRαs, a putative signal sequence and a putative sequence for a GPI-anchor (Thompson *et al.*, 1998).

Functional studies of chicken GFRα4 showed that it binds PSPN with a Kd of approximately 1 nM. By introducing *gfrα4* and *ret* into neurons, it was shown that GFRα4 and RET together can mediate PSPN signaling and support the survival of neurons (Enokido *et al.*, 1998). During the chicken development, GFRα4 is expressed in CNS, but also in non-neuronal tissues, such as kidney, heart and liver (Thompson *et al.*, 1998).

The mammalian GFRα4 was identified after the chicken GFRα4. Rat, human and mouse GFRα4 receptors were recently cloned by different laboratories. All the receptors lack the first Cys-rich domain, characteristic of the other GFRαs including the chicken GFRα4 (Lindahl *et al.*, 2000; 2001). It was shown that different mammalian GFRα4s can bind PSPN with high affinity. The Kds were about 6 nM, 100 pM and 2 nM for rat, human and mouse GFRα4, respectively (Masure *et al.*, 2000; Lindahl *et al.*, 2001 and I of this study). The rat *gfrα4* gene consists of six exons. Alternative splicing gives rise to two variants, GFRα4 A and GFRα4 B with different COOH-termini of the translated proteins. GFRα4 A protein has 273 amino acids with a predicted molecular weight of 30 kDa. It has two predicted possible GPI cleavage sites meaning that it may be a GPI-anchored protein on the cell surface. GFRα4 B protein has 258 amino acid residues with a molecular weight of 28 kDa, and has a shorter hydrophilic C-terminus, which indicates that it could represent a soluble GFRα4 form. Both forms have one putative N-linked glycosylation site (Masure *et al.*, 2000). The *gfrα4* encoding mRNA is expressed in the cortex, hippocampus, and substantia nigra as shown by *in situ* hybridization experiments in rats (Masure *et al.*, 2000). However, though rat GFRα4 binds PSPN with a high affinity, it does not mediate the activation of RET upon PSPN-stimulation (Masure *et al.*, 2000). It is therefore assumed that the discovered rat GFRα4 variant is biologically inactive.

Mouse *gfrα4* was identified from the mouse thyroid tissue based on the homology to other *gfrα*s (Lindahl *et al.*, 2000). The mouse *gfrα4* gene also consists of six exons, and the predicted full-length protein consists of 263 amino acid residues with a hydrophobic signal sequence and C-terminal sequence typical for GPI-anchored protein. The mouse protein is 53 % identical with the chicken GFRα4. Mouse GFRα4 also lacks the first cysteine-rich domain (D1) (Lindahl *et al.*, 2000). Alternative splicing of the mouse *gfrα4* gene gives rise to three variants, the putative GPI-anchored, transmembrane and soluble forms (described in more detail in the section of results and discussion and in the original publications I, II, and IV). The alternative splicing of the mouse *gfrα4* gene occurs tissue-specifically (Lindahl *et al.*, 2000).

Mouse *gfrα4* mRNA has been detected in different tissues, including the midbrain dopamine neurons (Lindahl *et al.*, 2000; Åkerud *et al.*, 2002). However, the expression of the GPI-anchored GFRα4 isoforms is very restricted. The isoforms are expressed in a tissue-specific manner, for instance, by semiquantitative RT-PCR analysis, the GPI-anchored form was detected in adrenal and thyroid from P0 to 6 weeks though the expression levels vary during different stages (Lindahl *et al.*, 2000). In pituitary, this isoform continues to be expressed until adulthood. In testis, the expression of the GPI-anchored form was found up to 4 weeks. The mRNA encoding the transmembrane form of GFRα4 has a higher expression level in the adrenal, thyroid (P0 and 6 weeks),

pituitary and testis as compared with the GPI-anchored form (Lindahl *et al.*, 2000). The expression pattern of the mRNA for soluble GFRα4 is similar with that of the transmembrane form, but there is in addition a very low expression in the brain (Lindahl *et al.*, 2000). The tissue-specific distribution of the three GFRα4 variants may indicate the various biological functions of them.

Human *GFRα4* was identified by using the information about mouse *gfrα4* (Lindahl *et al.*, 2001). The alternative splicing of the human *GFRα4* gene also gives rise to three different forms: *GFRα4*a, *GFRα4*b, and *GFRα4*c. *GFRα4*a encodes a protein of 290 amino acid residues and corresponds to the mouse GPI-anchored GFRα4. It contains a putative N-terminal hydrophobic signal, one N-linked glycosylation site, and a sequence for a GPI-anchore in the C terminus. Similary to rat and mouse GFRα4 human GFRα4 also lacks the first cysteine-rich domain (Lindahl *et al.*, 2001). The amino acid sequence alignment shows that the identity between mouse and human GFRα4 is 76%, whereas the identity between human and chicken GFRα4 is 54%. *GFRα4*b differs from *GFRα4*a with a small intron (79 bp) between exons 2 and 3 included in the transcript (Lindahl *et al.*, 2001). It also differs from *GFRα4*a in that the 3´-splice site of exon 4 is 11 bp upstream of the corresponding site in the *GFRα4*a transcript. Translation of this transcript leads to a protein of 299 amino acids, where N- and C-terminal ends are identical to those of the GFRα4a form. However, this "b" form has a unique stretch of 66 amino acids in the middle region (Lindahl *et al.*, 2001). GFRα4c is a protein of 236 amino acids, which is considered to be a soluble protein as the transcript includes two introns (between exons 2 and 3, and between exons 3 and 4) resulting in a frameshift with a stop codon located inside exon 5 (Lindahl *et al.*, 2001).

The expression of human *GFRα4* mRNA was detected at high levels in the adult thyroid gland and at lower levels in the fetal adrenal and fetal thyroid gland (Lindahl *et al.*, 2001). Interestingly, *GFRα4* mRNAs, both encoding the two GPI-anchored forms at the similar level and the soluble form at a low level, were expressed in the medullary thyroid carcinoma (MTC), particularly in the malignant C-cells, but not in other primary thyroid carcinomas (Lindahl *et al.*, 2001). This expression is pretty restricted, since no expression was detected in the accompanying connective tissue and blood vessels, nor in the adjacent apparently normal follicle cells or other thyroid tumors (Lindahl *et al.*, 2001).

1.2.2.2. *Ret* and its splice variants

Ret was originally identified as a novel oncogene (transforming gene) (REarranged during Transfection) activated by DNA rearrangement (Takahashi *et al.*, 1985). This chimeric oncogene encoded a fusion protein consisting of an N-terminal dimerizing domain and a C-terminal tyrosine kinase domain (Takahashi *et al.*, 1985; Takahashi and Cooper, 1987). The normal allele of *ret* encodes a receptor tyrosine kinase (RTK) with four cadherin-related motifs and a cysteine-rich region in the extracellular domain, a single membrane-spanning domain, and a tyrosine kinase domain in the intracellular domain (Takahashi and Cooper, 1987; Airaksinen *et al.*, 1999). RET is a glycosylated protein of 150 kDa and 170 kDa, the latter one is the fully glycosylated mature protein located on the cell surface which acts as the active signaling receptor for GFLs (Ponder, 1999). The 150 kDa

protein is considered as a partially glycosylated immature protein that resides in the ER (Ponder, 1999). The first cadherin-like domain in the extracellular portion has been shown to be required for the interaction with the GFRα/GFL complexes (Kjær and Ibáñez, 2003). The second and third cadherin-like domains form a calcium binding site (Anders *et al.*, 2001). Ca^{2+} is important for the transport of the fully mature RET protein to the plasma membrane (van Weering *et al.*, 1998), as well as for the GFL-dependent function of the receptor on the cell surface (Nozaki *et al.*, 1998).

RET is expressed widely in mammalian embryos. During embryonic development, RET is mainly expressed in the excretory and the nervous systems and plays diverse functions in the development (Pachnis *et al.*, 1993; Tsuzuki *et al.*, 1995). In the nervous system, RET is expressed in the progenitors of the ENS and is required for their migration. RET is important for the development of mouse enteric, autonomic and sensory neurons of the PNS, as well as for the motor neurons of the CNS (Natarajan *et al.*, 2002). Outside of the nervous system, RET is mainly expressed in the embryonic kidney (Pachnis *et al.*, 1993), and mice lacking RET show defects in the kidney and ENS development (Schuchardt *et al.*, 1994). RET is also expressed in the spermatogonia stem cells (Meng *et al.*, 2000). In adult humans, RET is expressed in cells of the nervous system, in C-cells, the adrenal medulla and the parathyroids (Nakamura *et al.*, 1994).

The intracellular domain of RET contains several tyrosine auto-phosphorylation sites. The phosphorylated tyrosine residues serve as docking sites for various intracellular signaling molecules in the target cells (Putzer and Drosten, 2004; Santoro *et al.*, 2004). Among the phosphorylated tyrosine residues, four are well studied (Tyr 905, Tyr 1015, Tyr 1062, and Tyr 1096) (Airaksinen and Saarma, 2002). The most interesting one is the Tyr 1062 which upon phosphorylation can bind at least six different molecules (Shc, FRS2, DOK4/5, IRS1/2, enigma, and PKCα) (Airaksinen and Saarma, 2002; Andreozzi *et al.*, 2003), and is important for the transforming ability of mutant RET (Asai *et al.*, 1996). Knock-in mice with mutated Tyr 1062 have a severe defect of enteric neurons in addition to kidney hypodysplasia, indicating that Tyr 1062 is important for the development of the enteric nervous system and the kidney (Jijiwa *et al.*, 2004). Tyr 905 binds to Grb7/10, Tyr 1015 to phospholipase Cγ (PLCγ), and Tyr 1096 to Grb2 (Pandey *et al.*, 1995; Borrello *et al.*, 1996; Alberti *et al.*, 1998). Recently, the Tyr 981 in RET has been characterized as a residue that recruits c-Src and thereby mediates neuronal survival (Encinas *et al.*, 2004). Recent studies also demonstrate that the same tyrosine residue, Tyr 1062, can contribute differently to the biological functions of the two RET isoforms (Degl'Innocenti *et al.*, 2004). A point mutation of this residue to Ala in RET51 did not abolish the ability of SK-N-MC to scatter, or the branching in MDCK cells. However, this mutation impairs both the scattering of SK-N-MC and branching of MDCK cells mediated by RET9 (Degl'Innocenti *et al.*, 2004). Besides the phosphorylated tyrosine residues, recent data show that the phosphorylation of Ser 696 is involved in the modulating the GDNF-induced activity of RET (Fukuda *et al.*, 2002). Importantly, the phosphorylation of Ser-696 in RET is not

induced by GDNF, but rather seems to be regulated by protein kinase A (PKA) in a GDNF-independent way.

By alternatively splicing of the 3' region, three RET variants, RET9 (1072 amino acids), RET43 (1106 amino acids), and RET51 (1114 amino acids), are generated. Of these three variants RET9 and RET51 are the major isoforms. The difference between these two forms is in their C-terminal tails. RET9 has a tail of nine amino acids, which is replaced by an unrelated tail of 51 amino acids in RET51 (Ishizaka *et al.*, 1989). Although the two forms behave similarly in a number of *in vitro* assays, *in vivo* assays with mutant mice demonstrate that when breeding of transgenic mice expressing either RET9 or RET51 with RET null mice, only RET9 can rescue the kidney phenotype of the RET null mice (Srinivas *et al.*, 1999). The importance of RET9 in embryonic development was confirmed by studies on transgenic mice with monoisoformic RET9 or monoisoformic RET51 (de Graaff *et al.*, 2001). Monoisoformic RET9 mice are viable and appear normal. In contrast, monoisoformic RET51 animals, which lack RET9, have kidney hypodysplasia and lack enteric ganglia from the colon. This indicates that RET51 is dispensable during embryogenesis, whereas RET9 is necessary and sufficient for normal development of the ENS and the excretory system (de Graaff *et al.*, 2001). However, RET51 may be involved in differentiation events later during kidney organogenesis (de Graaff *et al.*, 2001; Lee *et al.*, 2002). RET51, compared with RET9, associates more strongly with the ubiquitin ligase Cbl, which leads to faster turnover of RET51 (Sariola and Saarma, 2003; Scott *et al.*, 2005).

Interestingly, in addition to its function as the signaling receptor for GDNF family ligands, RET exhibits a pro-apoptotic property in the absence of GDNF. This pro-apoptotic activity can be blocked by the ligand implicating a possible novel functional mechanism, for example in ENS development (Bordeaux *et al.*, 2000). However, additional experiments and *in vivo* results are needed to understand the importance of RET-mediated apoptosis.

1.2.2.3. Alternative receptors

Throughout the nervous system, particularly in the forebrain and cortex, the expression of GFRα proteins is wider than that of RET (Trupp *et al.*, 1997; Yu *et al.*, 1998), indicating that GFLs in the complex with GFRαs may signal independently of RET by using alternative transmembrane proteins (Poteryaev *et al.*, 1999; Trupp *et al.*, 1999). One candidate for the alternative GFL signaling is the MET receptor. Exogenous GDNF can partially restore ureteric branching in *ret*-/- mice that exhibit severe renal hypodysplasia (Popsueva *et al.*, 2003). In MDCK cells expressing GFRα1, but lacking RET, GDNF stimulates branching but not chemotaxis. GDNF-dependent chemotaxis is only detectable after transfection of *ret* into these cells. MET can be phosphorylated upon GDNF stimulation in cells expressing GFRα1 (but lacking RET), indicating that the MET receptor might contribute to RET-independent GDNF signaling. However, no detectable interaction between GDNF/GFRα1 and MET is found, which makes a direct interaction unprobable (Popsueva *et al.*, 2003). Recently, another membrane protein, neural cell adhesion molecule (NCAM), has also been demonstrated to be an alternative receptor for GFL signaling (Paratcha *et al.*, 2003). NCAM ($p140^{NCAM}$ isoform) is a prominent cell adhesion molecule in the nervous

system, and participates in a number of developmental processes, for instance, cell migration, neurite outgrowth, and synaptic plasticity (Schachner, 1997; Crossin and Krushel, 2000; Ronn *et al.*, 2000). NCAM is coexpressed with GFRα1 in many tissues lacking RET (Crossin and Krushel, 2000). The intracellular domain of $p140^{NCAM}$ NCAM has been shown to associate with the Src family tyrosine kinase member Fyn (Beggs *et al.*, 1997). NCAM has been demonstrated to bind GDNF with a high-affinity in the presence of GFRα1 (Kd=1.1±0.32 nM) (Paratcha *et al.*, 2003). Interestingly, GDNF also directly binds to NCAM (Kd=5.2±1.8 nM) (Paratcha *et al.*, 2003). In neuronal cells and ganglia, GDNF can induce the activity of Fyn via NCAM (Paratcha *et al.*, 2003). *In vitro* assays showed that GDNF, only in the presence of GFRα1, can stimulate Schwann cell migration via NCAM, and GDNF can also mediate the axonal growth in primary neurons via NCAM and Fyn kinase. In both cases, the activity is independent of RET (Paratcha *et al.*, 2003). Another related study demonstrated that the *in vitro* and *in vivo* effects of GDNF on midbrain dopaminergic neurons are inhibited by NCAM-blocking antibodies, which further supports the physiological relevance of GDNF signaling through NCAM (Chao *et al.*, 2003).

In vitro accumulated evidence suggests that RET-independent GDNF/GFRα-mediated signaling plays an important physiological function. Either by capturing and concentrating diffusible GFLs and presenting them in *trans* to RET-expressing cells (Enomoto *et al.*, 2004), or by signaling through NCAM or MET *in trans* and *in cis* (Paratcha *et al.*, 2001; Ledda *et al.*, 2002; Paratcha *et al.*, 2003; Popsueva *et al.*, 2003).

1.2.3. GFL signaling pathways

The ligand/receptor complexes for GFLs include three components, the transmembrane receptor RET, the GPI-anchored GFRαs, and the GFLs. In this complex RET acts as the signaling transducer and GFRαs act as specific ligand-binding components. The four identified GFRα receptors, GFRα1, 2, 3, and 4 first bind their preferred ligands. However there may be weak cross-talks between GDNF-GFRα2 and NRTN-GFRα1, as well as between ARTN-GFRα1 (Airaksinen *et al.*, 1999; Airaksinen and Saarma, 2002) (Fig. 3), but the physiological function of these interactions is unclear .

The binding of various adaptor proteins to the different docking sites activates a variety of signaling molecules. The recruitment of Shc to Tyr 1062 triggers RAS-ERK(MAPK) and PI-3K/AKT pathways (Besset *et al.*, 2000; Hayashi *et al.*, 2000; Kurokawa *et al.*, 2001). The JNK and MAPK pathways are also activated mainly through Tyr1062 (Durick *et al.*, 1996; Hayashi *et al.*, 2000; Hennige *et al.*, 2000; Grimm *et al.*, 2001; Melillo *et al.*, 2001). Activation of MAPK and PI-3K/AKT pathways via Tyr 1062 has been shown to be important for the activation of the CREB and NFκB transcription factors (Hayashi *et al.*, 2000), as well as for the survival of PC12 cells (De Vita *et al.*, 2000). In addition, RAS activation is essential for RET-induced cell differentiation in PC12 cells (Califano *et al.*, 2000), whereas PI-3K signaling independent of AKT is necessary for lamellipodia formation that is a critical event during neuritogenesis (van Weering and Bos, 1997; van Weering and Bos, 1998). The role of PLCγ is to mediate Ca^{2+} release from intracellular pools. Recently, it has been shown that PLCγ

activation is involved in the enhancement of neurotransmission mediated by NT-3 (Yang *et al.*, 2001).

1.2.4. GFLs and lipid rafts

There is increasing evidence showing that lipid rafts are involved in growth factor triggered signal transduction. Lipid rafts are microdomains in the cell surface that consist of dynamic assemblies of cholesterol and sphingolipids (Simons and Toomre, 2000), as well as GPI-anchored proteins, certain transmembrane proteins, doubly acylated proteins, cholesterol-linked and palmitoylated proteins (Saarma, 2001). These lipid-associated proteins usually function in transmembrane signaling events. The close association of the proteins in the lipid rafts facilitate their interactions with each other and

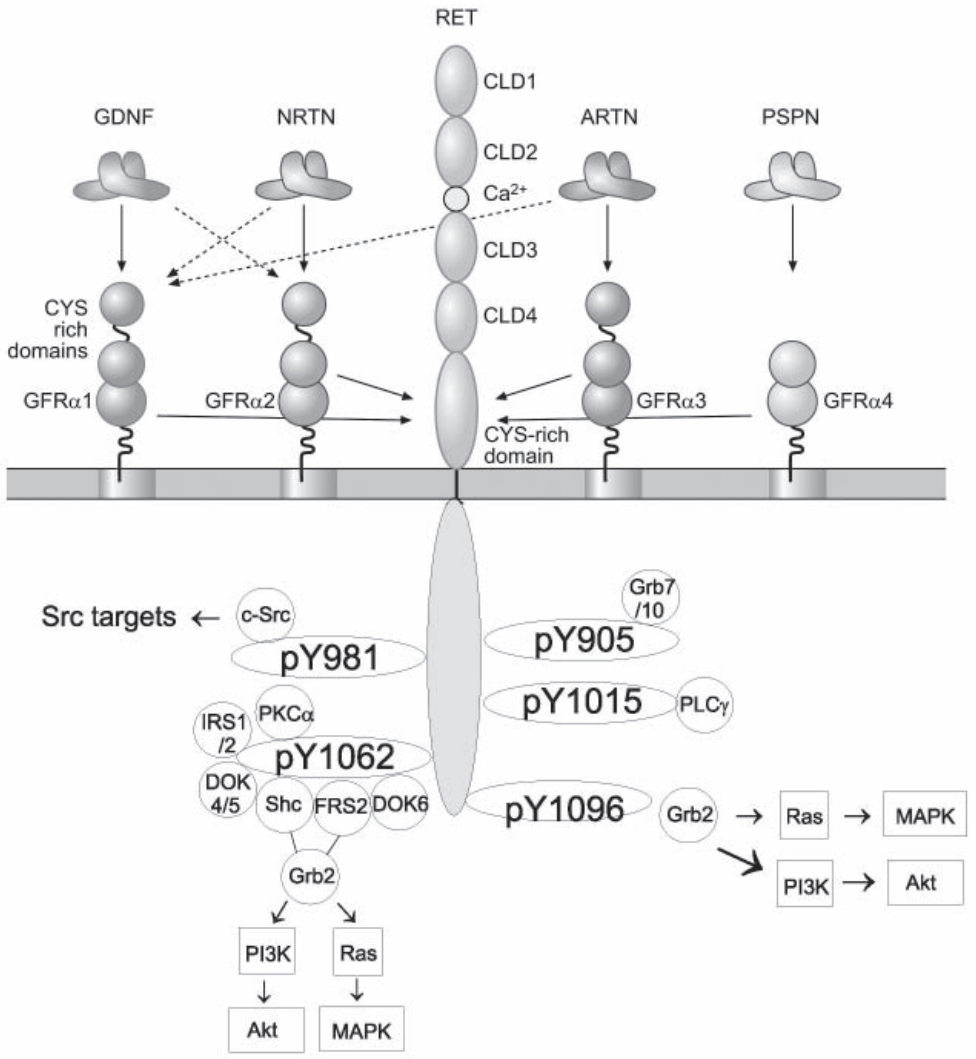

Fig. 3. Schematic representation of the interaction between GFLs and GFRs, RET. Note, the binding of enigma to Y1062 is independent of its phosphorylation (Modified based on the picture kindly provided by Professor Saarma).

prevent them from interacting with molecules outside the rafts. However, some transmembrane proteins associate with lipid rafts only transiently by entering or leaving lipid rafts in response to the ligand binding. This supports the idea that lipid rafts may be a platform for signal transduction (Paratcha and Ibáñez, 2002). The GFRα receptors, by virtue of the GPI-anchor, are located in lipid rafts, and therefore lipid rafts also have an important role for GFL-signaling (Poteryaev *et al.*, 1999; Tansey *et al.*, 2000; Paratcha *et al.*, 2001).

In cells coexpressing RET and GFRα1, GDNF stimulation triggers the localization of RET into lipid rafts. This recruitment is essential for GDNF-induced differentiation, neuronal survival and downstream signaling. Moreover, RET interacts with Src family kinases (SFK) (p60Src) only when localized to lipid rafts, and Src activity is necessary for an optimal GDNF-mediated differentiation, neuronal survival and signaling, (Tansey *et al.*, 2000; Encinas *et al.*, 2001). GDNF signaling has been shown to be dependent on the integrity of lipid rafts, because the depletion of cholesterol from cells with methyl-β-cyclodextrin, a treatment known to disorganize lipid rafts, reduces GDNF-dependent signaling (Tansey *et al*., 2000).

The non-overlapping expression of RET with GFRαs indicates that RET somehow may become activated by other means. Paratcha *et al.* and Worley *et al.* demonstrated that soluble GFRα1 which is formed by enzymatic cleavage of the GPI-anchor can promote RET signaling *in trans* in the presence of GDNF (Paratcha *et al.*, 2001; Worley *et al.*, 2000). Furthermore, soluble GFRα1 can also recruit RET into lipid rafts, by a novel mechanism that requires the activity of its intracellular kinase domain (phosphorylated Tyr 905, or Tyr 1062) (Paratcha *et al.*, 2001).

GFLs may signal independently of RET. *In vitro* assays show that GDNF promote biochemical and biological responses in cells expressing GFRα1, but lacking RET. In these cells, GDNF-stimulated GFRα1 activates Src family tyrosine kinases, triggers phosphorylation of phospholipase Cγ (PLCγ), MAPK and cAMP response element binding protein (CREB), up-regulates the transcription of *c-fos* encoding mRNA and also promotes cell survival (Poteryaev *et al.*, 1999; Trupp *et al.*, 1999). The discovery of MET and NCAM as alternative receptors explains how GFLs may signal independently of RET (Paratcha *et al.*, 2003; Popsueva *et al.*, 2003).

1.2.5. What are the knockouts of GFLs and their receptors telling us?

1.2.5.1. The defects in nervous system of GFL, GFRα and RET knockouts

*Gfr*α1-/-, and *ret*-/- mice, like *gdnf*-/- mice, die soon after birth. The phenotypes of GFRα1 and RET mutant mice are strikingly similar, but not identical with that of GDNF mutants (table 1), and display neuronal, spermatogonial and renal deficits. The motoneurons of the CNS are defective in GFRα1 mutant mice. For example, about 20-40 % of spinal and cranial motoneurons are missing in *gfr*α1-/-, which is similar with that of *gdnf*-/- mice (Cacalano *et al.*, 1998; Garces *et al.*, 2000). However, in *ret*-/- mice, significant losses are observed in all motoneuron populations examined (Airaksinen and Saarma, 2002). In PNS, no effects on the number of sensory neurons of spinal and trigeminal ganglia are detected in both *gfr*α1-/- and *gdnf*-/- newborns (Airaksinen

et al., 1999; Oppenheim *et al.*, 2000). In *gfr*α1-/- mice, no loss of SCG neurons was observed at birth, but about 30% loss was observed in *gdnf*-/- mice (Moore *et al.*, 1996; Cacalano *et al.*, 1998; Enomoto *et al.*, 1998). However, the migration of SCG precursor cells and initial axon growth are defective in *ret*-/- mice (Enomoto *et al.*, 2001). In parasympathetic neurons, the otic and sphenopalatine ganglia are missing in newborn of *gdnf*-/-, *gfr*α1-/- and *ret*-/- mice indicating that this ligand-receptor complex is required for the embryonic development of these parasympathetic neurons (Enomoto *et al.*, 2000; Rossi *et al.*, 2000). The enteric neurons are lacking below the stomach in *gdnf* -/-, *gfr*α1-/- and *ret*-/- mice (Manie *et al.*, 2001).

The *nrtn*-/- and *gfr*α2-/- mice, unlike the *gdnf* -/-, *gfr*α1-/- and *ret* -/- mice, are viable and fertile. In CNS, no gross defects of motoneurons are observed in *nrtn* - and *gfr*α2-deficient mice (Garces *et al.*, 2000), but a subtle deficit in the synaptic transmission of hippocampal neurons in *gfr*α2-/- mice has been reported (Nanobashvili *et al.*, 2000). In the parasympathetic nervous system, the cholinergic innervation is almost absent in the lacrimal and salivary glands and severely reduced in the small bowel. A lack of vagally stimulated secretion of pancreatic zymogens has been observed in *gfr*α2-/- mice. Also the parasympathetic innervation in the sublingual gland is missing and markedly reduced in the parotid gland that may explain why *gfr*α2-/- mice grow poorly (Rossi *et al.*, 1999; Rossi *et al.*, 2000; Rossi *et al.*, 2003). In other ganglia the reduced cell number, soma size as well the failed innervation are observed in *nrtn*-/- and *gfr*α2-/- mice (Airaksinen and Saarma, 2002). The data clearly demonstrates that NRTN-GFRα2 signaling via RET is required for the later target innervation of parasympathetic neurons and for the survival of many submandibular neurons during target innervation (Rossi *et al.*, 2000).

The expression of GFRα3 appears largely restricted to the PNS and ganglia. Accordingly, ARTN has the ability to enhance the survival, proliferation and neurite outgrowth of sympathetic neurons *in vitro* (Baloh *et al.*, 1998a; Baloh *et al.*, 1998b; Naveilhan *et al.*, 1998; Widenfalk *et al.*, 1998; Andres *et al.*, 2001), suggesting that ARTN/GFRα3 signaling influences the development and function of the sympathetic neurons. *In vitro* ARTN also stimulates the neurite outgrowth of juvenile DRG sensory neurons (Paveliev *et al.*, 2004).

The *artn*-/- and *gfr*α3-/- mice are viable and fertile (Nishino *et al.*, 1999; Honma *et al.*, 2002). It has been shown that, in *gfr*α3-/- mice the survival of SCGs in adult is impaired due to the massive apoptosis or marked reduction in size of SCG during postnatal development (Nishino *et al.*, 1999). However, in *artn* -/- mice, the size of SCG ganglia is normal except that the peripheral target is not innervated. This indicates that ARTN may be critical for sympathetic innervation to peripheral targets but not for sympathetic neuron survival *in vivo*. Therefore other target-derived trophic factors, such as NGF, must be critical for postnatal survival of sympathetic neurons once the axonal targets are innervated (Honma *et al.*, 2002). Using whole mount TH immunostaining, the rostral migration of SCG precursor cells was observed to be impaired in *gfr*α3-/- and *artn*-/- embryos (E11.5-E14.5). In addition, SCG ganglia are abnormally located and the trunk sympathetic chain ganglia in adult and P0 thoracic region are smaller and aberrantly segmented in both mutant mice. Although

GFRα3 is highly expressed in neurons of the peripheral sensory ganglia, including DRG and trigeminal, no deficiency is observed in these neurons in either mutant mice (Nishino *et al.*, 1999; Honma *et al.*, 2002).

The *pspn*-/- and *gfrα4*-/- mice appear normal in development and behavior (Tomac *et al.*, 2002; Lindfors *et al.*, 2006) though the *gfrα4* mRNA is expressed in many areas in both embryonic and adult rodents including the cortex and the hippocampus (Lindahl *et al.*, 2000; Masure *et al.*, 2000). The expression of PSPN and GFRα4 in the cortex and the hippocampus could suggest a potential function for PSPN/GFRα4 in memory and learning. *In vitro* data indeed showes that PSPN can promote the survival of rat motoneurons (Milbrandt *et al.*, 1998). But no *in vivo* data is in line with the *in vitro* observations (Tomac *et al.*, 2002). However, the ablation of the *pspn* gene in mice is increasing their sensitivity to stroke indicating that the *pspn*-/- mice developed a more severe cerebral infarct after ischemia (Tomac *et al.*, 2002). Furthermore, *in vitro* experimental data showed that a pretreatment with PSPN can markedly reduce the infarct volume of ischemia and protect cortical neurons from hypoxia-induced cell death (Tomac *et al.*, 2002).

1.2.5.2. The defects in non-nervous system of GFL, GFRα and RET knockouts

GDNF signaling through RET and the coreceptor GFRα1 is one of the main signaling pathways that promotes ureteric bud branching morphogenesis (Sariola and Saarma, 2003). Mice lacking GDNF die soon after birth, and show kidney agenesis owing to the failure to induce of ureteric buds. The enteric innervation is also defective (Pichel *et al.*, 1996; Moore *et al.*, 1996; Sanchez *et al.*, 1996). *In vivo* data from transgenic mice show that GDNF-dosage regulates the spermatogonial self-renewal and differentiation (Meng *et al.*, 2000; Meng *et al.*, 2001). Recent data confirms that GDNF-induced cell signaling is essential in spermatogonial stem cells (SSCs) self-renewal (Kubota *et al.*, 2004). Like the *gdnf*-/- mice, *gfr*α1-/- mice show renal abnormalities. Most of the homozygous animals lack both kidneys (Cacalano *et al.*, 1998; Enomoto *et al.*, 1998). In *ret*-/- homozygous, the animals show renal agenesis or several dysgenesis (Schuchardt *et al.*, 1994). However, unlike the *gdnf* +/- mice, where up to 30 % animals appear kidney abonormalities (Sanchez *et al.*, 1996), *gfr*α1+/- and *ret* +/- mice have normal kidneys comparing to the wild type animals (Schuchardt *et al.*, 1994; Enomoto *et al.*, 1998).

Nrtn-/- and *gfr*α2-/- mice have dry eyes with blinking eyelids due to the loss of innervation of the lacrimal gland. *gfr*α2-/-, but not *nrtn* -/- mice grow poorly after weaning (Heuckeroth *et al.*, 1999; Rossi *et al.*, 1999). This defect may due to the failure innervation of salivary gland leading to malnutrition (Rossi *et al.*, 1999).

The *artn*-/- and *gfr*α3-/- mice have the ptosis. The ptosis may result from the failure of sympathetic innervation to the superior tarsus muscle by the SCG. In both *artn*-/- and *gfr*α3-/- mice the SCG ipsilateral to the eye displaying ptosis is either missing or markedly reduced in size (Nishino *et al.*, 1999; Honma *et al.*, 2002).

Mammalian *gfrα4* gene was initially cloned from thyroid and thought to be required for the thyroid development. However, the functional ablation of *gfrα4* gene in mice does not cause any gross

defects in the thyroid, only mild testis degeneration was observed (Lindfors *et al.*, 2006). The expression of GFRα4 in the thyroid C-cells suggests that it may be important for the development of C-cells or some tumors derived from thyroid C-cells, for instance, medullary thyroid carcinoma (MTC). Recent studies on the *gfr*α4-/- mice crossed with RET^{MEN2B} knock-in mice showed that the number of C-cells, which are calcitonin positive as well, is reduced in RET^{MEN2B}/GFRα4 double mutant mice indicating that GFRα4 might be required for C-cell development (Lindahl, 2004). Moreover, the physiological role of GFRα4 has been assessed by measuring the thyroid calcitonin levels in GFRα4-deficient and wild type mice. In GFRα4 null mice, the calcitonin content is significantly reduced by about 60 % in newborn, by about 45 % in juvenile, but not in adult null (Lindfors *et al.*, 2006). The studies of *pspn*-/- mice show that PSPN may regulate the glutamate receptor-mediated calcium influx in a biphasic manner (high and low concentrations) (Tomac *et al.*, 2002).

1.2.6. GFLs, GFRαs and RET related diseases

1.2.6.1. Neurodegeneration diseases

Hirschsprung disease (HSCR) is a congenital disorder characterized by the absence of ganglion cells in the gastrointestinal tract. It is a disease which caused by the inactivity of the mutant genes and occurs in one in 5000 live births. Up to 90% of HSCR families are linked to *ret* mutations (mutations were dectected in up to 50% of familial patients and in 7%-35% of sporadic HSCR cases) (Lantieri *et al.*, 2006). In HSCR, the mutations of RET are dispersed throughout the whole gene, and the nucleotide changes include all inactivation mutants, microdeletions, insertions, splice variants, nonsense mutations and missense mutations. So far more than 100 missense mutations have been described, however none of them is sufficient for HSCR, meaning that the other mutant genes or factors, such as SOX10, EDNRB, EDN3 and ECE1, are needed. The molecular mechanism of HSCR pathogenesis is sustained by loss-of-function effects of RET mutations (Kashuk *et al.*, 2005; Lantieri *et al.*, 2006). Several mutations are also found in the *gdnf* gene from patients with HSCR. Only five of these are missense mutations and therefore result in a change in the amino acid sequence. The mutation at codon 93 with the replacement of arginine with tryptophan (R93W) has been reported in HSCR patients having RET mutations as well (Angrist *et al.*, 1996; Salomon *et al.*, 1996). This mutation has also been found in sporadic pheochromocytoma (Woodward *et al.*, 1997). The mutation at codon 150 (change from aspartate to asparagine) was identified in a case of sporadic HSCR in a patient with Down's syndrome (Salomon *et al.*, 1996). The replacement of threonine 154 with serine (T154S) was found as a *de novo* mutation in a case of sporadic HSCR (Ivanchuk *et al.*, 1996). In addition one mutation, proline 21 (P21S), was detected in the pre-pro-part of GDNF (Salomon *et al.*, 1996), and a mutation of isoleucine 211 to methionine has been reported (Martucciello *et al.*, 1998; Martucciello *et al.*, 2000). However, none of the GDNF mutations identified so far in HSCR patients are *per se* likely to result in HSCR. Functional studies of the effects of these mutations on GDNF function, for instance the ability to mediate the activity of RET, indicated that all mutations, except P21S which may have a function in the post-translation

processing of the protein (Salomon *et al.*, 1996), appeared not to affect the ability of GDNF to activate RET. However two of them (D150N and I211M) resulted in a significant reduction in the binding affinity of GDNF for GFRα1 (Eketjäll and Ibáñez, 2002). These data indicate that these two mutations may, in conjunction with other genetic lesions, contribute to the pathogenesis of this disease.

NRTN, the close homolog of GDNF, has also been considered to be a causative gene of HSCR. One mutation was identified at codon 96 with the replacement of alanine with serine (A96S). This amino acid substitution is very likely to alter the cleavage of the pre-pro-form of NRTN into mature NRTN, but the data showed that this mutation appears not sufficient to cause HSCR (Doray *et al.*, 1998). So far, there are no reports concerning ARTN- or PSPN-mutations which would be involved in the HSCR disease.

Although ten mutations in *GFRα1* have been detected, no disease specific mutations of this gene were found in a large population of HSCR patients. Of ten mutations, five of them resulted in amino acid substitutions, but none of these changes was found within predicted functional domain (Myers *et al.*, 1999). In *GFRα2*, six sequence variants were found, but four of them did not cause any amino acid change. The other two did affect the amino acid sequence, but no correlation between these variants and the HSCR was detected (Vanhorne *et al.*, 2001). With *GFRα3*, three mutations were identified, but like *GFRα1* and *GFRα2*, none of them correlates with the disease (Onochie *et al.*, 2000). Four sequence variants were also identified in *GFRα4*, but no relationship with the HSCR disease was detected. These data showed that none of the single mutations of *GFRα1-4* is strongly involved in HSCR (Borrego *et al.*, 2003).

Under the attempt to identify the gene responsible for Hallervorden-Spatz syndrome (NBIA1 syndrome, neurodegeneration with brain iron accumulation type 1), two variants of *GFRα4* that would alter the amino acid sequence were found from two NBIA1 families. In one family, a nucleotide substitution at residue 39, from thymine to adenine led to a change from one conserved cysteine to serine (C39S). In a second family, a cytosine to thymine change resulted in a proline to serine change at residue 145 (P145S). The P145S change seems not to be correlated with the disease, and the role of the C39S mutation is unclear (Zhou *et al.*, 2001).

1.2.6.2. Tumorgenesis

Besides the involvement in the neurodegeneration disorders, *GFRα* genes seem to have some correlation with MTC. Six mutations in *GFRα1*, one mutation in *GFRα2*, and two mutations in *GFRα3* have been identified, but only two variants in *GFRα1* seem to correlate with the sporadic MTC. One variant is at codon 6 with the replacement of leucine with proline (L6P), and the other sequence variant which is normally very rare is upstream of the coding sequence (at -193) (Borrego *et al.*, 2002). Recent studies (discussed in more detail below) showed that the mutations of *GFRα4* may contribute to the inherited multiple endocrine neoplasia type 2 (MEN 2) disease (Vanhorne *et al.*, 2005). It is an autosomal dominant cancer syndrome characterized by pheochromocytoma derived from the adrenal chromaffin cells and MTC, tumors arising from calcitonin-secreting C cells of the thyroid (Eng *et al.*, 1996; Brandi *et al.*, 2001; Machens *et al.*, 2003).

Although there are several mutations of genes contributing to MEN 2, the mutations of the *RET* proto-oncogene are the major contributor. However, there is considerable phenotypic variation within and among MEN 2 families with the same *RET* mutation. The mutation of *GFRα4* gene has recently been proposed as a modifier involved in the modification of the course of the disease (Vanhorne *et al.*, 2005). Vanhorne *et al.* detected two variants among total 10 sequence variants in the *GFRα4* gene. One is a single-base substitution upstream of the *GFRα4* coding region (52 bp upstream of the *GFRα4* translation initiation site) in patients with no known *RET* mutations. This mutation may affect the expression of *GFRα4*. Another one is a 7 bp insertion in exon 3 (GCGCCCC) in a patient with a mutation in RET (V804L). The latter mutation results in a reading frame shift for all *GFRα4* isoforms, and thereby changes their amino acid sequence. These *GFRα4* mutants may alter the formation of RET signaling complexes and could contribute to an MEN 2-like phenotype in the absence or presence of *RET* mutations (Vanhorne *et al.*, 2005).

RET proto-oncogene not only contributes to normal organ development as mentioned previously, but also contributes to the development of human diseases. *RET* was first recognized as a causative gene in MEN 2, then followed by papillary thyroid carcinoma (PTC), sporadic medullary thyroid carcinoma (sMTC) and then in HSCR disease (Edery *et al.*, 1994; Eng, 1999; Pacini *et al.*, 2000; Gonzalez *et al.*, 2003). The contribution of *RET* to human diseases is due either to a rearrangement of *RET* with a variety of other genes or to point mutations in *RET*. For instance, in sporadic and radiation-induced PTC, the genomic rearrangements lead to a fusion of the sequence encoding the intracellular domain of RET with the sequence of an activating gene. The activating gene is in all cases located 5´ to the sequence encoding the kinase domain of RET. A spontaneous dimerization of this N-terminal sequence leads to a ligand-independent dimerization and constitutive activation of the C-terminally located kinase domain (Klugbauer *et al.*, 2000; Salassidis *et al.*, 2000; Santoro *et al.*, 2004). Germ-line mutations, mainly point mutations, lead to a constitutive activation of RET, which are responsible for the development of MEN 2A and MEN 2B, and familial medullary thyroid carcinoma (FMTC) (Eng *et al.*, 1996; Brandi *et al.*, 2001; Machens *et al.*, 2003).

The association between *RET* mutations and MEN 2 and FMTC has been widely studied. It has been shown that most MEN 2A and FMTC mutations affect cysteines in the extracellular cysteine-rich domain of RET. MEN 2A is associated with six cysteine residues (codons 609, 611, 618,and 620 in exon 10, and 630 and 634 in exon 11) (Eng, 1999). The most frequent mutation is that codon 634 leading to a change from cysteine to arginine (C634R) (~85%) (Mulligan *et al.*, 1995; Eng *et al.*, 1996). This mutation reslts in the dimerization of RET via S-S bridges, and therefore causes the constitutive activity of RET leading to MEN 2A disease (Santoro *et al.*, 1995; Eng and Mulligan, 1997). FMTC mutations are evenly distributed among the various cysteines in the extracellular cysteine-rich domain of RET. In addition, FMTC can also be associated with residue changes in the tyrosine kinase domain at codons E768D (exon 13), L790F (exon 13), Y791F (exon 13), V804L (exon 14), V804M (exon 14), and S891A (exon 15) (Jhiang, 2000; Santoro *et al.*, 2004). The

mutations associated with MEN 2B are located at codon 883 (exon 15) and 918 (exon 16) in the kinase domain (Eng, 1999). Most patients (~95%) carry the methionine to threonine change at 918 (M918T), this mutation does not cause the dimerization of the protein, but alters the substrate specifity of the kinase, therefore affect the downstream signaling pathways, and thought to be the causative factor of MEN 2B (Santoro *et al.*, 1995; Eng and Mulligan, 1997). Only a small fraction of patients harbor the alanine to phenylalanine substitution at 883 (A883F) (Eng, 1999). These different point mutations may underlay the phenotypical differences between MEN 2A and MEN 2B patients (Santoro *et al.*, 1995; Jain *et al.*, 2004).

The studies of targeted overexpression of GDNF in undifferentiated spermatogonia demonstrated that the overdosage of GDNF can trigger the testicular tumorgenesis due to the abnormalities of spermatogenesis, spermatogonia differentiation, and spermatogonial clusters. It resembles the human classic seminomas (Meng *et al.*, 2001).

1.2.7. Use of GFLs in the treatment of diseases

GFLs were considered as potential therapeutic reagents for the treatment of some neurological diseases since their discovery. Most attention has paid to GDNF because of its survival-promoting effects on nigral dopaminergic neurons, which are undergoing degeneration in Parkinson´s disease (PD). The possibility to use the other GDNF family trophic factors in the treatment of diseases is still poorly investigated.

In animal models of PD, where nigrostriatal lesions are induced with 6 hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydopryridine (MPTP), injected GDNF can prevent the death of dopamine neurons and regenerate dopamine terminals *in vivo* (Kearns and Gash, 1995; Tomac *et al.*, 1995). The successful use of GDNF in animal models of PD prompted clinical trials in patients with PD. Although the results from Nutt *et al.* (2003), where GDNF was injected into the brain ventricles, were disappointing, the failure of this trial was most likely due to the failure of GDNF to penetrate into the putamen located deep in the brain. This region is the most affected region in PD and it is possible that it did not receive much of GDNF. In another trial GDNF was locally and continuously infused (intraparenchymal infusion) into the putamen region using mechanical pumps. In this limited trial with 5 patients GDNF protected the death of dopamine neurons and regenerated the dopamine terminals in PD patients (Gill *et al.*, 2003). Even after two years of continuos GDNF infusion, no serious clinical side effects were observed, and the patients showed significant clinical improvement (Patel *et al.*, 2005). The weak point in this trial was that only five patients took part in the trial, and there were no placebo controls. More recent double-blind placebo controlled trial with 34 patients GDNF showed no clinical benefit (Lang *et al.*, 2006).

Based on the reported data, the key question in the use of neurotrophic factors in treating diseases is the delivery of the factors. The currently used modes are: infusion of the recombinant proteins, cell-based delivery system, and viral vectors (Kirik *et al.*, 2004). Each of them has their own advantages and limitations. For example, the advantage in infusion method is that the dose can be well controlled and the infusion can be stopped in case of unwanted side effects. On the

other hand, the disadvantage is that the factor is infused from a point source and it makes the factor unavailable for the close tissues (Kirik *et al.*, 2004). The use of viral vectors, for instance, adeno-associated virus (rAAV) and lentivirus (rLV), brings highly efficient tools for the delivery of factors into CNS. However, the limitations of this method are the safety issues related to for instance their potential immunogenicity and their risks of mutagenesis (Kirik *et al.*, 2004).

In other neuronal diseases GDNF also exerts therapeutic functions. For example, *in vivo* data demonstrated that pretreatment of the cortical surface or the hippocampus with GDNF reduces the cerebral infarction caused by middle cerebral artery (MCA) occlusion in adult and newborns (Abe *et al.*, 1997; Kitagawa *et al.*, 1998; Miyazaki *et al.*, 1999; Ikeda *et al.*, 2000). Huntington´s disease is an autosomal dominant neurodegenerative disorder characterized by chorea, psychiatric disturbances and dementia, and caused by the expansion of a polyglutamine (polyQ) in the N-terminus of huntingtin protein (Landles and Bates 2004). In animal models of this disease, the infusion or viral delivery of GDNF into the striatum has significant neuroprotective effects (Araujo and Hilt, 1997; Alberch *et al.*, 2004; Kells *et al.*, 2004). Data from an animal model of amyotrophic lateral sclerosis (ALS) demonstrated that GDNF has a therapeutic potential also in this disease (Manabe *et al.*, 2003). Furthermore, GDNF has been shown to have some effects on drug addiction, particularly on chronic morphine and cocaine addictions. Infusion of GDNF into the rat VTA (the ventral tegmental area of the midbrain) blocks biochemical adaptations and behavioral responses to chronic drug exposure, whereas the functional inhibition of GDNF by GDNF function-blocking antibody enhances responsiveness to drug exposure. *Gdnf+/-* heterozygous mice show an enhancement of behavioral responses to drug exposure, indicating that GDNF has a potential to be used in the treatment of drug addiction (Messer *et al.*, 2000).

NRTN, like GDNF, can support the survival of midbrain dopaminergic neurons, which suggests its potential use in clinical treatment of PD as well. In animal models of Parkinson´s disease, NRTN promotes the survival of dopaminergic neurons exposed to 6-OHDA or MPTP (Horger *et al.*, 1998; Li *et al.*, 2003). Notably rodent DA neurons do not express GFRα2. NRTN is therefore most likely signaling via GFRα1/RET. Interestingly, recent studies show that unlike GDNF, the delivery of wild type NRTN by the lentiviral gene delivery method in the intrastriatal 6-hydroxydopamine lesion model of PD resulted in poor neuroprotective effects. However, the delivery of a pro-region deleted NRTN resulted in significantly higher neuroprotective activity compared to the wild type NRTN. The pro-region deleted variant of NRTN also showed efficient neuroprotection of lesioned nigral dopaminergic neurons, similar to GDNF, in the intrastriatal 6-OHDA lesion model of PD (Fjord-Larsen *et al.*, 2005). Moreover, in animal model of Huntington´s disease, NRTN showed also promising trophic effects on striatal projecting neurons, even more robustly than GDNF (Perez-Navarro *et al.*, 2000a).

As ARTN also has survival-promoting effects on midbrain dopaminergic neurons (Baloh *et al.*, 1998b), the injection of lentiviral vectors encoding ARTN increased the density of dopamine neurons in nigra and TH-immunoreactivity in striatum in rats exposed to 6-OHDA (Rosenblad *et al.*, 2000). This indicates that ARTN also

is a potent neuroprotective factor for the nigrostriatal dopaminergic neurons *in vivo*. Since the expression of GFRα3 in CNS is not detectable (Widenfalk *et al.*, 1998), it is possible that ARTN also signals via GFRα1.

2. Aims of the study

Recently three splice variants of mouse *gfrα4* were discovered. Interestingly, these splice variants are differently expressed in mouse tissues and organs. The aim of this study was to characterize if the splice variants mediate PSPN signaling in a similar or different way. The specific aims were:

A. First, to study the molecular structure, PSPN binding and function of the GPI-anchored mouse GFRα4; and to compare it to GFRα1.

B. Second, to elucidate if the transmembrane and soluble GFRα4 function similarly to, or differently from the GPI-anchored GFRα4.

C. As the mammailan GFRα4 receptors lack the N-terminal first cysteine-rich domain, which is present in all other GFRα receptors, my third goal was to determine what is the function of this domain in GFRα1.

3. Materials and methods

3.1. FLAG-tagging of GFRαs (I, II, III and IV)

Three splicing variants, GPI-anchored, transmembrane, and soluble forms, were previously cloned from the mouse thyroid (Lindahl *et al.*, 2000). The soluble form was cloned into a mammalian expression vector from the mouse adult thyroid tissue. Total RNA was isolated from adult mouse thyroid tissue by using Trizol reagent (Life Technologies). The RT-PCR was done with Superscript II (Life Technologies) for RT and the GC-rich PCR kit (Roche) for PCR. The primers used for mouse *gfrα4* soluble form were P1: 5´-CCA CCA TGG CCC ACT GCA TGG AGT C-3´ and P2: 5´-TTC AGC TCA GTG AGC AGT CAT CG-3´. The PCR products were then directly cloned into pCR-TOPO vector using the kit (Invitrogen) according to the manufacturer´s instructions. The cDNA was subcloned into the mammalian expression vector pcDNA3. The correct clone was verified by sequencing. The three variants were FLAG-tagged at the position following the last amino acid of the signal sequence. All constructs were sequenced throughout and the expression of the three FLAG-tagged proteins was tested individually with anti-FLAG antibody (Sigma) (I, II, and IV). The first domain-deleted rat GFRα1 was created by deleting the amino acid fragment 23-121. This truncted rGFRα1 was tagged by FLAG following the last amino acid of the signal sequence. The expression of this truncted rGFRα1 was verified by FLAG antibodies (III).

3.2. Generation of stable cell lines expressing different forms of mouse GFRα4s (I, II and IV)

In order to characterize the different forms of GFRα4 biochemically, I established stable cell lines expressing the variants individually. Mouse neuroblastoma Neuro 2a cells were transfected with pcDNA3 harboring inserts encoding FLAG-tagged m*gfrα4*s using FuGENE6 transfection reagent (Roche). The following day G418 was applied on the transfected cells at 400 μg/ml and the selection was continued for two weeks. Single clones were picked up and propagated. The expression of GFRα4s was verified by immunoblotting with anti-FLAG antibodies (I, II, and IV). The positive clones were then stored as stocks in liquid nitrogen.

3.3. Glycosylation and membrane association assays (I and IV)

Glycosylation of mouse GFRα4s was examined by culturing the established stable cells in medium with/without tunicamycin at 5 μg/ml (Sigma) for 16 h. The cells were washed and lysed on ice. The lysates were analysed by immunoblotting with antibodies to FLAG.

For the membrane association assay, the cells expressing GFRα4s were homogenised in lysis buffer, and the post-nuclear supnatant was mixed with 67 % sucrose to give a final concentration of 60 % sucrose. The sample was centrifugated in a gradient of 67 %, 60 %, 50 % and 5 % sucrose. After the centrifugation, the fractions were analyzed by Western blotting with antibodies to FLAG.

3.4. Immunofluorescence staining (IV)

Neuro 2a cells were grown on coverslips overnight and subsequently transiently transfected. 24 h after transfection the cells were fixed with 4 % paraformaldehyde (PFA), and then the cells were either permeabilised with 0.1 % Triton X-100 for 10 min at RT or were left intact. The cells were washed and stained with anti-FLAG antibodies for FLAG-tagged or anti-6xHis antibodies for His-tagged GFRα4. After washing, the cells were incubated with Cy^{3TM}-conjugated secondary antibody for 30 min at RT. Thereafter the cells were refixed and the coverslips were mounted on objective glasses (IV).

3.5. The binding and cross-linking of PSPN to mouse GFRα4s (I, II and IV)

Human recombinant PSPN (Pepro Tech EC Ltd) was enzymatically iodinated with ^{125}I/Na by the lactoperoxidase method (Lindahl *et al.*, 2001). The binding assay was essentially performed as previously described (Jing *et al.*, 1996; Lindahl *et al.*, 2001) for the GPI-anchored and transmembrane forms. Neuro 2a cells, transiently or stably transfected with mGFRα4-GPI and mGFRα4-TM, respectively, were used for the experiments. The transfected cells were seeded in pre-coated 24-well plates. The following day the cells were incubated with 1 nM ^{125}I-labelled PSPN in DMEM containing 0.2 % bovine serum albumin, 15 mM Hepes (pH 7.5) for 4 h on ice, either in the absence or presence of different concentrations of unlabelled PSPN. Cells were washed and lysed in 1M NaOH, and the amount of bound ^{125}I-labelled PSPN was measured using a Liquid Scintillation Counter (LAB, Wallac) (I and IV).

The binding assay for the soluble form of GFRα4 was done in a cell-free manner as follows: the supernatant of cells stably expressing FLAG-tagged soluble mouse GFRα4 was collected and spun down in order to get rid of cellular debris. ^{125}I-PSPN was added into the supernatant in the absence or presence of unlabeled PSPN. Thereafter the supernatant was rotated at + 4 ºC for 4 h. Anti-FLAG antibody was added into the tubes, the immunocomplexes were precipitated with protein G Sepharose beads (Amersham Pharmacia Biotech). The beads were then washed, and the amount of the bound ^{125}I-PSPN was measured using a Liquid Scintillation Counter as previously (II).

For the chemical cross-linking assays the cells expressing FLAG-tagged mGFRα4-GPI and FLAG-tagged mGFRα4-TM were incubated with 1 nM ^{125}I-labelled PSPN in TYROIDE buffer in the presence or absence 100 nM unlabelled PSPN (100x molar excess over the ^{125}I-labelled PSPN). For the soluble form the medium from the cells expressing soluble GFRα4 was used. The chemical cross-linker BS^3 (Perice) was added and then the cells were incubated at RT for 45 min. The ^{125}I-PSPN/soluble mGFRα4/RET complexes were brought down by immunoprecipitating with anti-FLAG antibodies. After running the SDS-PAGE, the gel was dried and analyzed with a PhosphoImager (FUJIFILM BAS-1500).

3.6. Receptor activity and signaling assays (I, II and IV)

The receptor activity was monitored using antibodies to phosphorylated tyrosine residues. The Neuro 2a cells co-expressing RET and mGFRα4s were starved, exposed to PSPN and lysed. RET was immunoprecipitated and the precipitate was analysed by Western blotting with anti-phosphotyrosine antibodies (I and IV).

To test the ability of soluble GFRα4 to activate RET, the *in vitro* kinase assay was carried out essentially as described by Kato, M. *et al.*, 2000. Naive Neuro 2a cells expressing RET were starved for 4-5 h in serum-free RPMI 1640 medium, then treated with medium with/without soluble mGFRα4, and lysed on ice. The post-nuclear supernatant was subjected to immunoprecipitation with anti-RET antibodies, and the immunocomplexes were collected with protein G Sepharose beads. The kinase reaction was done using myelin basic protein (MBP) as an exogenous kinase substrate. The activity of RET was visualised by autoradiography (II).

To analyze the activation of the PI-3 K/AKT pathway triggered by soluble GFRα4 via RET, naive Neuro 2a cells expressing RET were plated in 6 cm plates. The following day the cells were starved for 4-5 h in serum-free RPMI 1640 medium. The cells were treated with medium with/without soluble mGFRα4 (30, 60 min), and then lysed with the sample buffer. The phosphorylated AKT was monitored by anti-phospho-AKT antibodies after running the SDS-PAGE and blotting. The inhibition of the activation of PI-3K/AKT pathway was done using the selective inhibitor of PI-3 kinase, LY 294002. The starved cells were treated with LY 294002 at 20 μM for 1 h prior to the treatment of the cells with soluble mGFRα4 (II).

3.7. Lipid rafts localization analysis (I)

The different types of Neuro 2a cell lines going to the assay were starved for 4-5 h, and stimulated or not with GFLs, then collected into cold lysis buffer (10 mM Hepes pH 7.2, 250 mM sucrose, 2 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, and Complete Mini Protease Inhibitor Cocktail Tablets (Roche)) on ice and homogenised with a syringe. After addition of Triton-X100 (final concentration of 0.1 %) to the post nuclear supernatant the samples were incubated for 20 min and analysed on Optiprep density gradients. Fractions were collected and the localization of proteins was analysed by Western blotting with various antibodies to monitor different proteins.

3.8. Neurite outgrowth assay (I, II, III and IV)

The assay was done essentially as discrebed by Tansey *et al*. 2000 and Crowder *et al*. 2004. For GPI-anchored and transmembrane forms of GFRα4, the transfected PC6-3 or Neuro 2a cells were plated at 70,000 cells/well in Falcon 12-well dishes. In the next day the cells were switched to medium containing low serum concentrations, and supplied with or without GFLs. After 1-2 days (for Neuro 2a cells) or 3-4 days (for PC6-3 cells) of culture, cells bearing neurites at least one cell body diameters in length were counted from 3-4 random fields from each transfected cell lines in order to monitor the neurite outgrowth (I and IV).

For the soluble GFRα4-mediated neurite outgrowth assay the cells were, after 24 h plating, switched to medium containing soluble mGFRα4, and cultured for 4-5 days in order to induce the differentiation. The medium was changed every second day. For the statistical analysis, cells bearing neurites at least one cell body diameters in length were counted from 3 random fields from each treatment. The experiments were repeated three times, and for each treatment two parallel assays were done (II).

3.9. Neuronal culture, transfection, and survival assay (I, II and IV)

Primary cerebellar granule neurons (CGNs) were isolated from postnatal day 7 (P7) rats as described by Miller and Johnson, 1996. The cerebellum was dissected and chopped into pieces, washed and digested with trypsin. The cells were resuspended in the Basal Medium Eagle (BME) (Life Technologies) containing trypsin inhibitor and DNase. The cells were then triturated until the suspension became homogeneous. The neurons were then collected by centrifugating and resuspended in conditional BME medium and plated on poly-D-lysine precoated plates. After 24 h, cytosine β-D-arabinofuranoside (Ara-C) was added to the final concentration of 10 μM to the cells in order to inhibit the proliferation of non-neuronal cells (I,II, and IV).

The granule neuronal transfection was done as described essentially by Craig, A.M. (Culturing Nerve Cells, 1998). On day 5 of culture, the transfection was done with various cDNA constructs. EGFP was used to monitor for the transfected neurons. To assess the neuronal survival, the number of initial positively transfected neurons in designated fields were counted 24 h after transfection. The neurons were then switched to high potassium medium plus serum, or low potassium medium without serum in the presence of GFLs or soluble GFRα4 protein. The neurons were cultured for an additional 2.5~3.5 days, and the transfected cells remaining in the same fields were counted.

4. Results and discussion

4.1. Functional charaterization of the mouse GFRα4-GPI coreceptor (I)

Mouse *gfrα4* gene consists of six extrons. By alternative splicing the *gfrα4* gene gives three different forms which are developmentally regulated (Lindahl *et al.*, 2000). One of the variants is the putative GPI-anchored form (GFRα4-GPI) consisting of 260 amino acids harbouring a hydrophobic ER signal sequence, a glycosylation site and a putative C-terminal GPI-anchored region (Lindahl *et al.*, 2000). The other two variants are the putative transmembrane and soluble variants (discussed later) with identical N-terminal amino acid sequences as the GPI-anchored form. The difference between these three variants is in their C-termini (Lindahl *et al.*, 2000) (Fig. 4). The predicted molecular weight of the GPI-anchored form is 29 kDa, which is the same as the result from the Western blot analysis, where the lysates of Neuro 2a cells stably expressing FLAG-tagged GFRα4-GPI were immunoblotted with FLAG antibodies (I, Fig. 1A). As predicted, this protein is a glycosylated protein, in order to verify it, the cells expressing FLAG-targged GFRα4-GPI were treated with Tunicamycin, as showed in I of this study, the glycosylated band (upper band) disappeared after the treatment (I, fig. 1A). Next, we verified its GPI-anchored nature by PI-PLC treatment of the mouse GFRα4-GPI expressing cells. PI-PLC is a specific protease which can cleave the GPI-anchored proteins from the cell membrane, but not other membrane associated proteins. Our results showed that the putative GPI-anchored GFRα4

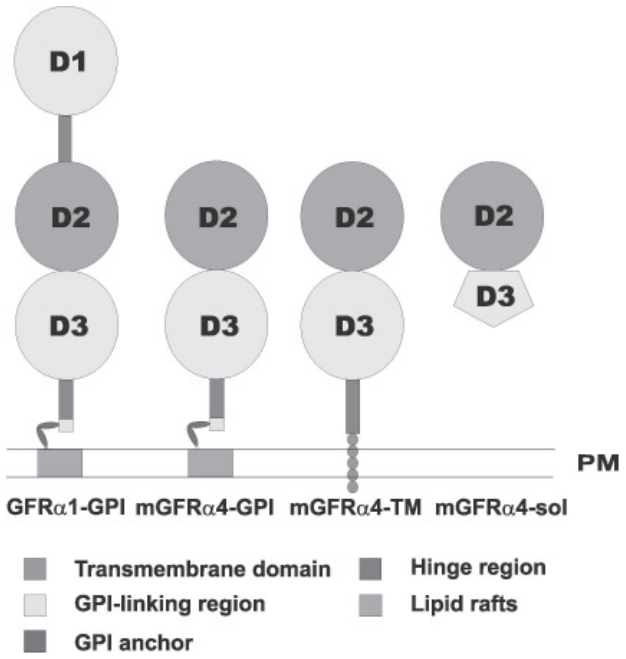

Fig. 4. Schematic domain structure of mouse GFRα4 variants comparing with GFRα1 (PM stands for plasma membrane)

can be released from the cell surface by the enzyme (I, fig. 1C). The membrane localization of this protein was further verified by the sucrose gradient and biotinylation assays (I, fig. 1B and D). It has been well studied that the initiation event of GFL-signaling is the binding of GFLs to their cognate receptors, GFRαs. With these new putative PSPN receptors, it was therefore of great interest to check if they have an equal affinity for their ligand, PSPN. Therefore I first performed the cell-based binding assay of PSPN to the GFRα4-GPI coreceptor with ^{125}I-PSPN, and determined the Kd of about 2 nM (I-II), which is more or less comparable with the Kds of other GFL-GFRα interactions except the mouse GFRα4-TM-PSPN (discussed later) (I).

In general, it is assumed that after the GFL-GFRα complex has formed, this complex interacts with RET and results in the dimerisation and phosphorylation of RET in order to induce signaling. Therefore I wanted to know if the PSPN/mGFRα4-GPI complex can interact with and activate RET. To answer this question, the cross-linking experiment was performed in Neuro 2a cells transfected with FLAG-tagged mGFRα4-GPI. The data showed that the complex of mGFRα4-GPI and ^{125}I-PSPN can form a complex with RET (I, fig. 1E). The consequence event of the triple complex formation, as also been shown with the human GFRα4/PSPN/RET complex, is to induce the phosphorylation of RET (Lindahl *et al.*, 2001), thus I studied the PSPN-mediated RET phosphorylation via mGFRα4-GPI. As expected when PSPN was applied to Neuro 2a cells co-expressing GPI-anchored GFRα4 and RET, PSPN mediated the activation of RET in a dose-dependent manner via mGFRα4-GPI (I, fig. 1F).

The biological activities of the mouse GFRα4-GPI variant were analysed by PSPN-induced neurite outgrowth and neuronal survival assays. Although it has been shown that PSPN can not induce motor axon outgrowth in organotypic spinal cord cultures (Bilak *et al.*, 1999), it does induce neurite outgrowth in rat midbrain dopaminergic neurons (Chen *et al.*, 2003). PC6-3 cells were transiently co-transfected with mGFRα4-GPI and RET and used for neurite outgrowth assays. Upon PSPN treatment, the mGFRα4-GPI showed clearly the ability to induce neurite outgrowth in cells (I, fig. 4B and C). GDNF and GFRα1 were used as a positive control. For the neuronal survival assays, the postnatal day 7 cerebellar granule neurons (CGN) were used as a model since no GFRαs and Ret expression was detectable, and furthermore, the neurons are undergoing apoptosis in the condition of low potassium. On day 5, the cultured CGNs were co-transfected with mGFRα4-GPI and RET encoding cDNAs, and in the next day, the transfected CGNs were switched to the low potassium medium with PSPN. The viable neurons were counted after 2.5 days. Our results showed that PSPN can support the survival of about 50 % of the transfected neurons via mGFRα4-GPI and RET receptors (I, fig. 4A).

Taken together our results show that the putative mouse GPI-anchored GFRα4 is a real co-receptor for PSPN, which can mediate the activation of RET and thereby also neuronal differentiation and survival *in vitro*. Interestingly, mGFRα4-GPI has a weaker capacity in recruiting RET into lipid rafts compared with GFRα1 (I; fig. 2). This recruitment was thought to be essential for RET signalling (discussed in more detail later).

4.2. The mouse soluble GFRα4 is an agonist of RET (II)

The mouse soluble GFRα4 consists of 190 amino acids (Lindahl *et al.*, 2000). It is a small (~21 kDa), secreted, and unglycosylated protein (II, fig. 1e and data not shown). Compared with the other two variants of mGFRα4 which have two domains, D2 and D3, the soluble form consists of the full D2 and partial D3 (II, fig.1 a-d), this may give it unique features. The crystal structure and sequence alignment assays of GFRα1 have located the GFL binding site to the D2 and to the cleft of D2 and D3 (Scott and Ibáñez, 2001; Leppänen *et al.*, 2004). In soluble GFRα4, the normally conserved structure of domain 3 is replaced by an unique sequence harbouring five cysteine residues. Although the folding of the conserved domian 2 is probably normal, the folding of the third domain is impossible to predict. The expression of GFRα4 protein is restricted to the calcitonin-secreting C cells of the thyroid (Lindfors *et al.*, 2006), where the MEN 2 disease arise from. As mentioned previously, due to the different location of the mutant residues in RET, MEN 2 has been classified into MEN 2A and MEN 2B (Eng, 1999). However, although more than 95% MEN 2 patients harbor the RET mutations, still about 5% of MEN 2 or MEN 2-like patients have no RET mutation identified (Vanhorne *et al.*, 2005). Also, the association between the different RET mutations and the various disease phenotypes is unclear suggesting that maybe some other molecules are possibly involved (Eng, 1999). Furthermore, the variation in age of the disease onset and types was found in the families even with a same specific RET mutation (Vanhorne *et al.*, 2005). All these data suggest that other related genes must contribute to or modify the phenotypes or severity of the disease. Actually, Vanhorne et al. 2005 have identified a mutation in human *GFRα4*, which may contribute to a more aggressive disease course of MEN 2. The mutation is derived from a 7 bp insertion, which causes the reading frame shift in all the human GFRα4 splice variants, and thereby seems to change the balance between the GPI-anchored and soluble human GFRα4 (Vanhorne *et al.*, 2005). Interestingly, this mutation was identified from the patients with MEN 2 or MEN 2-like phenotype, that do not have the RET mutations (Vanhorne *et al.*, 2005). Together with the restricted expression pattern of GFRα4 in thyroid, it suggests that GFRα4 may have a causative function in MEN 2 disease.

The RTKs not only are activated by their ligands, but also respond to their agonists. For instance, the EGF receptor (EGFR) can be tyrosine phosphorylated by its agonists, bradykinin and angiotensin (Luttrell *et al.*, 1999; Gschwind *et al.*, 2001). The mouse soluble GFRα4 is a small, secreted molecule, and exists as a homodimer independently of its ligand (II, fig. 1e). The homodimers probably are caused by the unpaired cysteines in the third domain. These may participate in the formation of an intermolecular bridge. To test if the soluble GFRα4 is biologically active, I first performed the binding assay with ^{125}I-PSPN, as showed in II, fig 2a, that the soluble GFRα4 can bind to PSPN with a Kd of about 1.6 nM. Since the soluble GFRα4 exists as a dimer and is an active receptor for PSPN (II, fig 1e and fig 2a), we asked if it can interact with RET independently of PSPN, and therefore promote the activation of RET? By coimmunoprecipiting I demonstrated that it can interact with RET (II, fig. 2c). Most interestingly soluble GFRα4 can promote the phosphorylation of RET independently

of PSPN (II, fig. 2d). To further study the biological activities of the soluble GFRα4, I investigated the intracellular signaling pathways mediated by RET upon its activation by soluble GFRα4 alone. In general, upon the interaction between RET and the complex of GFL/GFRα *in cis*, which means that RET and the GPI-anchored GFRα are expressed in the same cells as membrane-associated protein, the whole complex moves into lipid rafts, and triggers the raft-specific signaling, for example, the MAPK pathway (Airaksinen and Saarma, 2002). However, when the soluble GFRα together with GFL interacts with RET *in trans* meaning that the GFRα is cleaved from the cell surface and exists as soluble proteins, this complex is outside of lipid rafts (Airaksinen and Saarma, 2002), and due to the activation of RET, some adaptor proteins, for instance SHC, are recruited to its docking site (Tyr 1062), thereby triggering some other signaling pathways occurring outside rafts, such as the PI-3K/AKT pathway (Airaksinen and Saarma, 2002). From the lysates of Neuro 2a cells treated by the soluble GFRα4 protein alone, SHC protein was immunoprecipitated with the anti-RET antibodies (data not shown), also the PI-3K/AKT pathway was activated in those cells (II, fig. 3a). This result is in line with our findings that the GFRα4-GPI can not recruit RET into lipid rafts, but still has the biological function (I). On the cellular level, I tested if the soluble GFRα4 can promote the neuronal differentiation and survival. As expected, the soluble GFRα4 alone can induce neurite outgrowth in Neuro 2a cells and support the survival of CGN (Fig. 5 and II, fig.4).

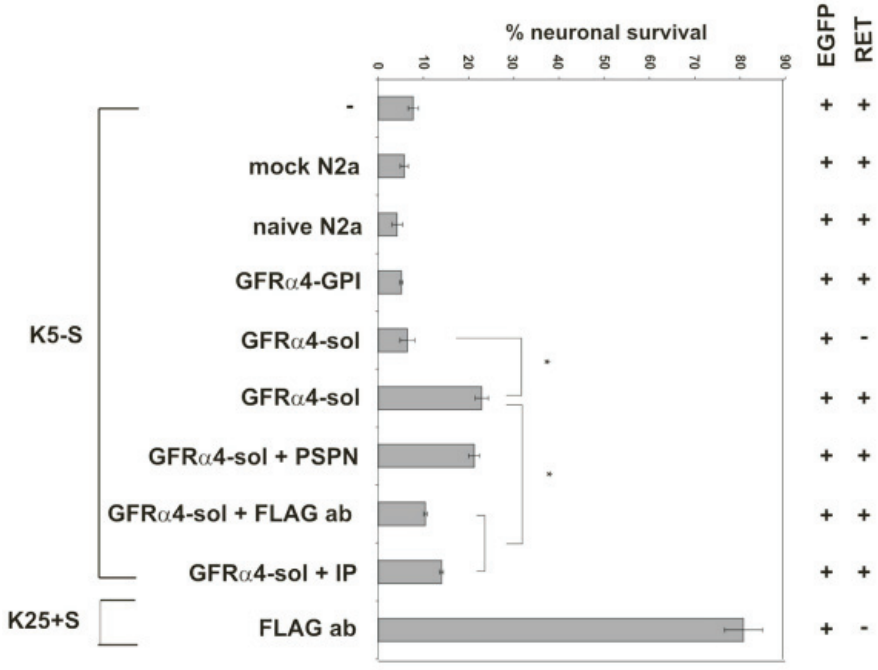

Fig. 5. Mouse GFRα4-soluble protein mediates the survival of CGN via RET

Our data are in line with the recent publication (Vanhorne *et al.*, 2005), and may provide a molecular mechanism of how the GFRα4 may modulate the course of the MEN 2 diseases. For example our results may explain why patients without RET mutations and with GFRα4 mutations can have MEN 2 disease. As Vanhorne *et al.* 2005 showed that the reading frame shift of GFRα4 due to the insertion of 7 nucleotides results in a predicted soluble GFRα4 protein, which may be the causative factor in MEN 2 or MEN 2-like disease in which no RET mutation is detectable. Also, based on the amino acid sequence alignment, we have showed that the cysteines in D2 domain are conserved between the mouse soluble GFRα4 and the human mutant GFRα4. This may suggest that the structure of these two proteins may be similar, indeed it is in line with our computational structure analysis of mouse soluble GFRα4 showing that it is very similar with the crystal stucture of human GFRα3 D2 (II, fig.1). Thus, combined together with our experimental data, we showed that, in the case of no RET mutation detectable in the MEN 2 or MEN 2-like patients, the soluble GFRα4 may act as an agonist to promote the phosphorylation of RET, therefore resulting in the constitutive activity of RET. Unfortunately, despite numerous serious attempts we were unable to purify the soluble GFRα4 protein to confirm these data with the purified protein. In summary, we showed for the first time that the soluble GFRα subunit can activate RET independently of the GFL ligand. Fig.6 shows a hypothetical scheme of how GFLs and the soluble GFRα4 signal.

The expression of PSPN *in vivo* is extremely low (Milbrandt *et al.*, 1998), and the *pspn* -/- mice show no gross

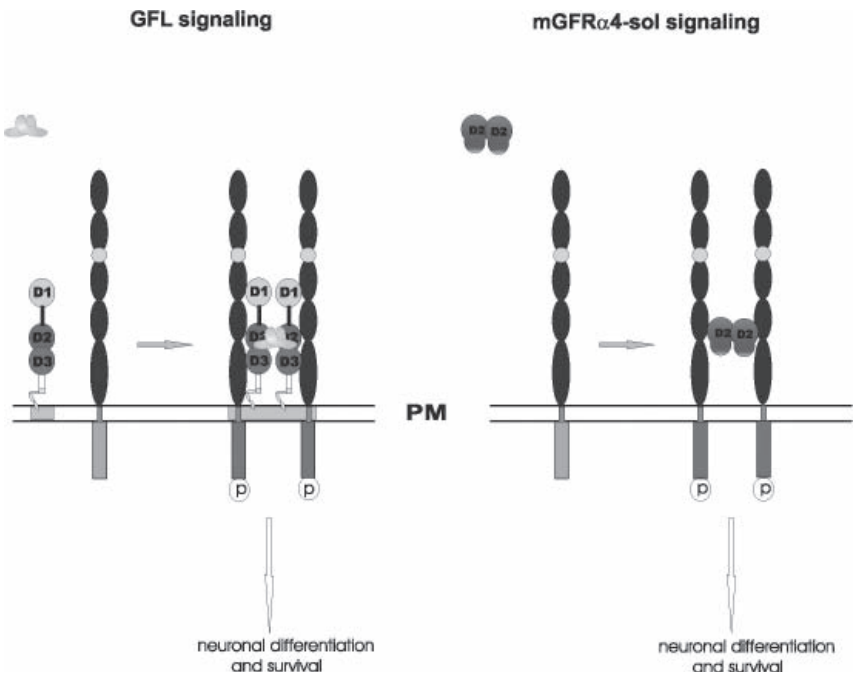

Fig. 6. Hypothetical scheme of mGFRα4-sol signaling

phenotype in CNS and PNS (Tomac *et al.*, 2002). Also, unlike the other GFLs, which can interact with the extracellular matrix components, for instance N-syndecan, for signaling, PSPN does not bind to N-syndecan (Bespalov and Saarma, unpublished data). These events suggest that PSPN and its coreceptor perhaps work differently from the other GFLs and GFRαs. Although it has been shown that the GFRα4 transcripts are expressed in the nervous system, most of them are presumed to be not functional due to either lack the functional signal sequence or translation as a truncated form, which is thought to be secreted (Lindahl *et al.*, 2000; Lindfors *et al.*, 2006). Our results show that indeed the truncated form is a secreted protein and active (II). It also indicates how this soluble GFRα4 may balance the shortage of PSPN *in vivo*. For instance, due to the very low level of PSPN, the soluble GFRα4 can act as a pleiotropic factor to activate RET independently of PSPN in order to facilitate the utilization of PSPN by the GPI-anchored GFRα4 whenever needed. On the other hand, since PSPN can not bind N-syndecan, which may help to concentrate the low level factors to the matrix, the soluble GFRα4 could trap PSPN and then bring it to RET-expressed cell and activate RET *in trans*. Moreover, as described previously, several forms of MEN 2 disease are derived from the mutant *Ret*, therefore *Ret* maybe the target for treating this disease. Our study demonstrated that as an agonist, the soluble GFRα4 can activate RET and mediate its signaling (II). This may give an opportunity to develop drugs for treating MEN 2 disease by designing small molecules or peptides based on the structure of the soluble GFRα4, that would block the activation of the mutant RET in the patients.

4.3. Functional charaterization of the mouse GFRα4-TM coreceptor (IV)

By alternative splicing involving the exon VI, the mouse *gfrα4* gene gives another variant, the putative transmembrane *gfr*α4 (m*gfrα4-TM*). Comparing with the m*gfrα4-GPI* which is mainly produced by the thyroid and parathyroid gland of 3-week old mice, the m*gfrα4-TM* is the major transcript in newborn and 6-week old mice (Lindahl *et al.*, 2000). The difference between this putative transmembrane form and the GPI-anchored form is that the mGFRα4-TM has a putative membrane-spanning region (Lindahl *et al.*, 2000). mGFRα4-TM consists of 293 amino acids with a predicted molecular weight 33 kDa, which is verified by Western blot with FLAG antibodies (IV, fig. 1C). To study the membrane association of the putative mGFRα4-TM, the FLAG-tagged m*gfrα4-TM* transfected cell lysate was analysed with the sucrose gradient. Western blotting analysis of the fractions showed that the FLAG-tagged mGFRα4-TM is located in the top fractions which are the membrane-associated fractions (IV, fig. 1E). To exclude the artificial membrane association of mGFRα4-TM, a portion of the initial membrane-associated fraction was treated with 50 mM Na_2CO_3 (pH 11.5) and centrifugated. After the soluble proteins were precipitated with acetone, the data show that the mGFRα4-TM is still in the membrane-associated portion (pellet) after Na_2CO_3 treatment (IV, fig. 1F). In order to further characterize the transmembrane feature of mGFRα4-TM, the cells expressing FLAG-tagged mGFRα4-TM were treated with PI-PLC enzyme and analysing the supernatant with anti-FLAG antibodies, we demonstrated that this putative mGFRα4-TM receptor is not a GPI-anchored protein since it can not be released from the cell surface

by the specific GPI-anchored protease (IV, fig. 1D). Although our biochemical data show that the mGFRα4-TM is a membrane-associated protein rather than GPI-anchored, the question we asked was whether mGFRα4-TM is localized to the cell membrane? To address this question, I performed the immunocytochemical analysis. With the aid of the C-terminal 6xHis-tagged mGFRα4-TM, I demonstrated that the mouse GFRα4-TM receptor is a real transmembrane receptor (IV, fig. 2).

As a putative coreceptor variant of PSPN, the binding of PSPN to the mGFRα4-TM and formation of the PSPN/mGFRα4-TM/RET complex were investigated by binding and cross-linking assays. Surprisingly, the transmembrane variant has a much weaker affinity for PSPN (Kd ≈ 180 nM) than the mGFRα4-GPI variant (IV, fig. 4A). Also in the cross-linking assay, due to the weaker binding capacity of PSPN to mGFRα4-TM, the formation of the PSPN/mGFRα4-TM/RET complex was dramatically reduced (IV, fig. 4B). Considerating the developmental regulation of the transcripts of mouse GFRα4, these data may indicate that indeed, under physiological conditions, the transmembrane form of GFRα4 is an inactive receptor, i.e. is not mediating PSPN effects because the expression level and tissue concentration of PSPN are very low *in vivo* (Milbrandt *et al.*, 1998). It also may reflect how the different variants of GFRα4 work *in vivo*. Sequence alignment shows that the rat, human, and mouse GFRα4 receptors, but not the chicken GFRα4, have a similar two-domain structure. The modelling of the electrostatic surfaces of human GFRα4 identified the amino acids arginine 96, arginine 97, and phenylalanine 101 as important amino acids for PSPN-binding (Lindahl, 2004). These amino acids are conserved in all mouse GFRα4 variants (II, fig. 1a). Therefore it remains unclear how the transmembrane domain of the mGFRα4 affects the binding of PSPN. Taken together these data indicated that mGFRα4-TM can bind PSPN and RET,

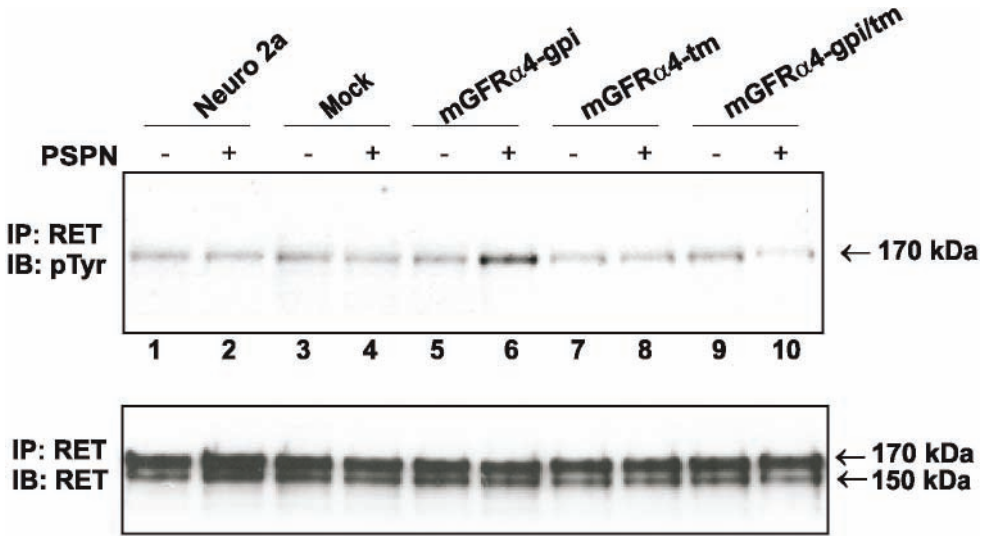

Fig. 7. mGFRα4-TM inhibits PSPN-mediated RET phosphorylation

but PSPN does mostly likely not signal via mGFRα4-TM through RET under physiological conditions.

In order to study the mouse GFRα4-TM in more detail, I further tested its biological functions such as its capacity to activate RET, induce neuronal differentiation and survival. To study the activation of RET by mGFRα4-TM, I used Neuro 2a cell expressing the FLAG-tagged GFRα4-TM. Upon the stimulation with PSPN at 100 ng/ml, no active RET was detected with the anti-phosphotyrosine antibodies (Fig. 7) compared to the mGFRα4-GPI coreceptor, even with the high concentration of PSPN (500 ng/ml) (data not shown). The neuronal differentiation assay was performed in Neuro 2a cells coexpressing the mGFRα4-TM and RET, the result shows that PSPN can not induce the neurite outgrowth via mGFRα4-TM and RET (IV, fig. 6). To study the effects of mGFRα4-TM on the neuronal survival, CGN were cultured and transfected as described in materials and methods. As expected, mGFRα4-TM can not support the survival in response to PSPN (IV, fig. 7). These data indicate that the mGFRα4-TM is indeed biologically inactive coreceptor of PSPN.

Because the mGFRα4-TM appears not to mediate PSPN signaling, we asked what the biological function of this variant could be? The identical N-terminal amino acid sequences between the mGFRα4-GPI and the mGFRα4-TM, the physiological data from the GFRα4-deficient mice, and also the expression pattern in tissues suggested that the mGFRα4-TM variant might interact with the mGFRα4-GPI and thus regulate its function. By immunoprecipitation assay, we showed that the mGFRα4-TM indeed interacts with the mGFRα4-GPI (IV, fig. 3A). This interaction seems to induce changes in the properties of the mGFRα4-GPI receptor and consequently, prevent its ability to bind PSPN (IV, fig. 4C). It also inhibits the PSPN-dependent phosphorylation of RET mediated by the GPI-anchored GFRα4 (Fig. 7). Consequently, the induction of neurite outgrowth and the promotion of neuronal survival are blocked (IV, figs 6 and 7). In addition, I found that the interaction between the mGFRα4-TM and the mGFRα4-GPI does not affect the interaction between mGFRα4-GPI and RET which was detected both in the presence and absence of PSPN (IV, fig. 3B). In addition, I showed experimentally in this study the existence of pre-formed complexes of GFRα and RET independently of GFL (IV, fig. 3B). Taken together, the data indicate that although the mGFRα4-TM and the mGFRα4-GPI are identical except the C-termini, their biological activities are quite different. This difference must be due to the difference in the receptor configuration caused by their C-termini.

Our data suggest that the mouse transmembrane GFRα4 may act as a dominant negative inhibitor of PSPN-mediated signaling pathway. It has been shown that the growth-factor-mediated signaling can be regulated through a dominant negative mechanism. For instance, the truncated *trkB* can negatively regulate the full length *trkB*-mediated signaling (Haapasalo *et al.*, 2001). The endogenous truncated EphA7 receptor can impair the activation of the active EphA7 receptor (full length), and alter its physiological actions during the neural tube development (Homberg *et al.*, 2000). Also the activation of fibroblast growth factor receptor and TGF-β have been reported to be regulated via a domainant negative way (Osterhout *et al.*, 1997; Onichtchouk *et al.*, 1999). The different

splicing of transmembrane and GPI-anchored GFRα4 is developmentally regulated (Lindahl *et al.*, 2000). The functional GPI-anchored form is expressed in juvenile thyroid C cells, and in contrast, the transmembrane GFRα4 is expressed in newborn and adulthood (Lindahl *et al.*, 2000; Lindfors *et al.*, 2006). The studies on *gfra4*-/- mice showed that *gfra4* does not affect the development of thyroid C cells, but the less calcitonin-positive cells (37%) in *ret*-/- mice comparing with the wild type indicates that the subpopulation of C cell development is *ret*-dependent and some other GFRαs may be involved in (Lindahl *et al.*, 2000). Actually the expression of GFRα1 in the C cells precursors during embryonic development suggests that maybe the RET-GFRα1 signaling is required for the earlier development of C cells (Lindfors *et al.*, 2006). There are two hypothetic mechanisms of how growth-factor-mediated signaling is negatively regulated. For instance, when the truncated TrkB is not expressed with the full-length TrkB in the same cells, the truncated TrkB can trap the ligand, therefore restrict the availability of ligand by the full length TrkB (Biffo *et al.*, 1995). However when they are expressed in the same cells, the truncated TrkB will interact with the full length TrkB and inhibit the activation of the full length TrkB. Our data demonstrated that *in vitro* the transmembrane GFRα4 acts as a dominant negative inhibitor of PSPN-mediated signaling via the GPI-anchored GFRα4 by interacting with the GPI-anchored GFRα4 when they are expressed in the same cells. The formation of this hetero dimer therefore restrict the utility of the ligand by the GPI-anchored GFRα4. Considering the overlapping expression pattern of the transmembrane and GPI-anchored *in vivo* (Lindahl *et al.*, 2000), our hypothesis is that the transmembrane GFRα4 may act as a domainant negative regulator of PSPN-mediated signaling *in vivo* as well. Recently, the mutations of human GFRα4 have been reported to be as modifiers of the MEN 2 disease which do not harbor RET mutants (Vanhorne *et al.*, 2005). It would be highly interesting to address whether the transmembrane GFRα4 can also impair the signaling in MEN 2 disease lacking the RET mutants mediated by the GFRα4 mutants. This may be the potential clinical advantage of the transmembrane GFRα4.

4.4. The difference between GFRα4 and GFRα1 (I, II and III)

4.4.1. The role of the first cysteine-rich domain of GFRα1, which is absent in mammalian GFRα4s (III)

As mentioned previously the first Cys-rich domain characterized in other GFRαs, including chicken GFRα4, is lacking in mammalian GFRα4s. GFRα1 has been postulated to consist of three similar Cys-rich domains, D1, D2 and D3 (Fig. 4). The domain 2 and cleft between D2 and D3 has been shown to be involved in the binding of GDNF. The D2 has be postulated to interact with RET (Airaksinen *et al.*, 1999; Scott and Ibáñez, 2001; Leppänen *et al.*, 2004;). Crystal structure study of the complex of GFRα3 and ARTN demonstrated that the D2 of GFRα3 interacts with ARTN, the D3 domain is not involved in the ligand binding but stablizes the D2 domain. The D2D3 region of GFRα3 is the binding surface for RET (Wang *et al.*, 2006). However, the D1-lacking GFRα4s (GPI-anchored and soluble variants) are GFRα receptors that can mediate the activation of RET, neuronal differentiation and survival (Lindahl *et al.*, 2001; I and II). These data, together with

the studies from Scott and Ibáñez (2001) and Wang *et al*. (2006) suggest that the D1 in GFRαs is dispensable. In order to investigate the real function of the D1, we used the D1-deletion variant of GFRα1 for the bioactivity assays (III). Our results showed that D1 of GFRα1 is important for stabilizing the binding of GDNF to GFRα1, but not for the interaction with RET. The truncated GFRα1 (D1-lacking) showed lower activities in mediating the phosphorylation of RET in low concentrations, in promoting the neurite outgrowth and neuronal survival as compared to the full-length GFRα1 (III, fig. 4 and 5). Unlike the D2 and D3 domains, which have a major contribution to the interaction of GDNF and RET, the D1 has a minor effect on this interaction, but has an important stabilizing role in GDNF binding and therefore is optimizing the function of GFRα1.

The hypothesis of how the D1 domain stabilizes the binding of GDNF to GFRα1 is that either by interacting with GDNF or both GDNF and GFRα1 subdomain. In the first case, D1 might bend its 30 amino acid hinge down in order to reach the bound GDNF and stabilize the GDNF-GFRα1 complex. In the latter case, the D1 might interact with the bound GDNF and the subdomain of GFRα1, therefore somehow link tightly the complex. Also it is possible that the D1 domain has some other still unidentified functions. Confirmation of these possibilities awaits the further studies, for instance the crystallization of the GDNF–GFRα1 complex.

4.4.2. The recruitment of RET to lipid rafts is mediated differently by GFRα1 and GFRα4 (I)

Lipid rafts participate at several different stages of signaling cascades and act as platforms for signal transduction integration. By virtue of their GPI anchor, the GPI-anchored GFRαs are localized into lipid rafts (Poteryaev *et al.*, 1999; Tansey *et al.*, 2000; Paratcha *et al.*, 2001). By comparing the GPI-anchored and an artificial transmembrane GFRα1, it was previously shown that the recruitment of RET molecules to lipid rafts trigged by GDNF is critical for the GDNF-mediated signaling. Using an artificial transmembrane GFRα1 it was shown that although it can not recruit RET to lipid rafts in response to GDNF stimulation, the proximal RET signaling events such as receptor complex formation and RET phosphorylation are unaffected (Tansey *et al.*, 2000). The authors also showed that in spite of its capacity to activate the phosphorylation of RET, the artificial transmembrane GFRα1 could not mediate full biological activity of GDNF. These data raised the question of whether the localization of RET to lipid rafts upon GFL treatment is required for GFL signaling? By examining the localization of the naturally existing mGFRα4-GPI and the recruitment of RET to lipid rafts mediated by the mGFRα4-GPI, we found that for some reasons the mGFRα4-GPI receptor is not associated with the lipid raft as tightly as the GPI-anchored GFRα1 does. In addition, PSPN/GFRα4 recruited much less of RET into the lipid raft as compared to GDNF/GFRα1 (I). This result is unexpected and in contrast with the data obtained on GFRα1 where GDNF signaling has been shown to depend on the integrity of lipid rafts, because cholesterol depletion with methyl-β-cyclodextrin, a treatment known to disorganize lipid rafts, reduces GDNF-dependent activation of MAPK and AKT kinases (Tansey *et al.*, 2000). Interestingly, PSPN still can mediate the neurite outgrowth and neuronal survival via the mGFRα4-GPI

and RET. Taken together, these data from mouse GPI-anchored GFRα4 indicate that RET can promote neuronal differentiation and survival although it is not strongly associated with lipid rafts.

4.4.3. PSPN-mediated signaling pathways

As the ligand for GFRα4, PSPN can induce the phosphorylation of RET receptor via GPI-anchored GFRα4, therefore promote the intracellular signaling pathways as neurite outgrowth and neuronal survival (Enokido *et al.*, 1998; Lindahl *et al.*, 2001 and I). Although it is unclear which tyrosine residues become phosphorylated after PSPN stimulation, indirect evidence showed that, most probably the Tyr1062 is phosphorylated upon PSPN stimulation since SHC and c-Src were recruited to the active RET mediated by PSPN via GFRα4 (data not shown). *In vitro* and *in vivo* studies also demonstrated that PSPN can promote the survival of many types of neurons except the peripheral neurons (Milbrandt *et al.*, 1998; Paveliev *et al.*, 2004). The evidence from the analysis of the splicing forms of mouse GFRα4 indicate that PSPN may promote different signaling pathways via different forms of its receptor. For instance, PSPN can activate MAPK and PI-3K/AKT signaling pathways via the GPI-anchored mGFRα4 receptor in Neuro 2a cells (Lindholm and Saarma, unpublished data). However, *in trans* together with soluble GFRα4, PSPN activates only PI-3K/AKT signaling pathway, but not the MAPK pathway (data not shown), possibly reflecting a different physiological role of soluble mGFRα4 *in vivo*.

Pspn-/- mice show hypersensitivity to focal cerebral ischemia or stroke, and the exogenous application of PSPN demonstrated that PSPN has the neuroprotective function in the ischemia/neurotoxicity models both *in vitro* and *in vivo* (Tomac *et al.*, 2002). However it is unknown through which forms of GFRα4 receptor PSPN exerts its neuroprotective roles. It is interesting to identify which form of GFRα4 is expressed in which particular neuronal types by the aid of available antibodies to the different forms. Also it will be very interesting to study the *gfrα4*-/- mice and see whether they have the same phenomenon as the *pspn*-/- mice. Combined with our current studies showing that the different forms of GFRα4 receptor function differently, the PSPN-mediated signaling, for example the regulation of the elevation of the intracellular calcium level in cortical neurons, maybe controlled by its different receptor forms.

5. Conclusions

I. The mouse GPI-anchored GFRα4 is a functional coreceptor for PSPN and mediates PSPN signaling via RET. It can promote the downstream signaling of RET and thereby, mediate PSPN-dependent neuronal differentiation and survival. However, upon ligand stimulation GPI-anchored GFRα4, contrary to the previously described GFRα1, fails to recruit RET to the lipid rafts.

II. The mouse secreted GFRα4 variant, independently of its ligand, PSPN, can function as an agonist of RET. This pluripotential protein can interact directly with RET, mediate the autophosphorylation of RET, and regulate the downstream forward signals needed for the stimulation of neuronal differentiation and survival.

III. The cysteine-rich D1 domain-deleted GFRα1 was used as a model to reveal the roles of the first domain which is absent in mammalian GFRα4. The data showed that D1 domain is involved in the stabilizing the binding of GDNF to GFRα1 receptor.

IV. The novel mouse transmembrane GFRα4, although having an identical N-end structure with GPI-anchored GFRα4, is biologically inactive in response to PSPN. The transmembrane form can inhibit the biological functions of the GPI-anchored GFRα4, block neuronal differentiation and survival mediated by the PSPN/mGFRα4-GPI/RET complex.

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