STRUCTURE-FUNCTION STUDIES OF GDNF FAMILY LIGAND-RET SIGNALLING

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ACADEMIC DISSERTATION

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To my family

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LIST OF ORIGINAL PUBLICATIONS

I.   Jianmin Yang, Maria Lindahl, Päivi Lindholm, **Heidi Virtanen**, Eleanor Coffey, Pia Runeberg-Roos, Mart Saarma (2004). PSPN/GFRα4 has a significantly weaker capacity than GDNF/GFRα1 to recruit RET to rafts, but promotes neuronal survival and neurite outgrowth. *FEBS Lett.* 569:267-71.

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III. Pia Runeberg-Roos, **Heidi Virtanen**, Mart Saarma (2007). RET(MEN 2B) is active in the endoplasmic reticulum before reaching the cell surface. *Oncogene* 26:7909-15.

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ABBREVIATIONS

AAV	adeno-associated virus
A-loop	activation loop
ARTN	artemin
BDNF	brain-derived neurotrophic factor
BMZF	bone marrow zinc finger
cAMP	cyclic adenosine monophosphate
CD2AP	CD2 (cluster of differentiation 2) -associated protein
CNS	central nervous system
CDNF	conserved dopamine neurotrophic factor
CLD	cadherin-like domain
CNTF	ciliary neurotrophic factor
CNTFR	CNTF receptor
CRD	cysteine-rich domain
EDNRB	endothelin receptor type B
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ENS	enteric nervous system
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ET-3	endothelin-3
FGFR	fibroblast growth factor receptor
FLT-3	Fms-like tyrosine kinase receptor 3
FMTC	familial medullary thyroid carcinoma
FRS2	FGFR substrate 2
GAB	GRB2-associated binding protein
GABA	gamma-aminobutyric acid
GDNF	glial cell line-derived neurotrophic factor
GFL	GDNF family ligand
GFRα	GDNF family receptor α
gp130	glycoprotein 130
GPCR	G protein-coupled receptor
GPI	glycosylphosphatidylinositol
GRB	growth factor receptor-bound protein
GZF1	GDNF-inducible zinc finger gene 1
HPT	hyperparathyroidism
HSCR	Hirschsprung's disease
IL	interleukin
IRS1	insulin receptor substrate 1
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kDa	kilodalton

LAR	leukocyte common antigen-related protein
LIF	leukemia inhibitory factor
LIFR	LIF receptor
MALDI-TOF	matrix-assisted laser desorption/ionisation - time-of-flight
MANF	mesencephalic astrocyte-derived neurotrophic factor
MAPK	mitogen-activated protein kinase
MEN 2	multiple endocrine neoplasia type 2
MPTP	1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine
MTC	medullary thyroid carcinoma
NCAM	neural cell adhesion molecule
NGF	nerve growth factor
NRTN	neurturin
NT	neurotrophin
6-OHDA	6-hydroxydopamine
OSMR	oncostatin M receptor
PC	phaeochromocytoma
PD	Parkinson's disease
PI3K	phosphatidylinositol-3-kinase
PI-PLC	phosphatidylinositol-specific phospholipase C
PLC	phospholipase C
PKC	protein kinase C
PNS	peripheral nervous system
PSPN	persephin
PTC	papillary thyroid carcinoma
PTPRJ	receptor-type protein tyrosine phosphatase J
RAS	rat sarcoma oncogene
RET	rearranged during transfection
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHC	Src homology 2 domain-containing protein
Shp	SH2-containing tyrosine phosphatase
Sos	son of sevenless
SOS	sucrose octasulfate
SPA	scintillation proximity assay
STAT3	signal transducer and activator of transcription 3
TGF-β	transforming growth factor β
TH	tyrosine hydroxylase
TK	tyrosine kinase
TM	transmembrane
TNFR	tumour necrosis factor receptor
Trk	tropomyosin-related kinase
WT	wild-type

ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) and its family members neurturin (NRTN), artemin (ARTN) and persephin (PSPN) are growth factors, which are involved in the development, differentiation and maintenance of many neuron types. In addition, they function outside of the nervous system, e.g. in the development of kidney, testis and liver. GDNF family ligand (GFL) signalling happens through a tetrameric receptor complex, which includes two glycosylphosphatidylinositol (GPI)-anchored GDNF family receptor (GFRα) molecules and two RET (rearranged during transfection) receptor tyrosine kinases. Each of the ligands binds preferentially one of the four GFRα receptors: GDNF binds to GFRα1, NRTN to GFRα2, ARTN to GFRα3 and PSPN to GFRα4. The signal is then delivered by RET, which cannot bind the GFLs on its own, but can bind the GFL-GFRα complex. Under normal cellular conditions, RET is only phosphorylated on the cell surface after ligand binding. At least the GDNF-GFRα1 complex is believed to recruit RET to lipid rafts, where downstream signalling occurs.

In general, GFRαs consist of three cysteine-rich domains, but all GFRα4s except for chicken GFRα4 lack domain 1 (D1). We characterised the biochemical and cell biological properties of mouse PSPN receptor GFRα4 and showed that it has a significantly weaker capacity than GFRα1 to recruit RET to the lipid rafts. In spite of that, it can phosphorylate RET in the presence of PSPN and contribute to neuronal differentiation and survival. Therefore, the recruitment of RET to the lipid rafts does not seem to be crucial for the biological activity of all GFRα receptors.

Secondly, we demonstrated that GFRα1 D1 stabilises the GDNF-GFRα1 complex and thus affects the phosphorylation of RET and contributes to the biological activity. This may be important in physiological conditions, where the concentration of the ligand or the soluble GFRα1 receptor is low. Our results also suggest a role for D1 in heparin binding and, consequently, in the biodistribution of released GFRα1 or in the formation of the GFL-GFRα-RET complex.

We also presented the crystallographic structure of GDNF in the complex with GFRα1 domains 2 and 3. The structure differs from the previously published ARTN-GFRα3 structure in three significant ways. The biochemical data verify the structure and reveal residues participating in the interactions between GFRα1 and GDNF, and preliminarily also between GFRα1 and RET and heparin.

Finally, we showed that, the precursor of the oncogenic MEN 2B (multiple endocrine neoplasia type 2) form of RET gets phosphorylated already during its synthesis in the endoplasmic reticulum (ER). We also demonstrated that it associates with Src homology 2 domain-containing protein (SHC) and growth factor receptor-bound protein (GRB2) in the ER, and has the capacity to activate several downstream signalling molecules.

REVIEW OF THE LITERATURE

1. Neurotrophic factors inside and outside of the nervous system

The development and maintenance of the nervous system is regulated by a great variety of molecules, including small secretory proteins called neurotrophic factors. The first growth factor that was shown to stimulate growth and support the survival of neurons was the nerve growth factor NGF (Levi-Montalcini and Hamburger, 1951, Cohen *et al.*, 1954). Subsequently, several other factors (discussed below) that can regulate survival and differentiation of nerve cells have been identified. Some of these growth factors are also active in non-neuronal tissues. Because of the therapeutic potential of growth factors, the field of neurotrophic factor research has expanded fast.

In the early stages of nervous system development, more neurons are produced than are present in an adult individual. In normal development, during a period of programmed cell death, a significant part of developing neurons will die. This process seems to give adaptability to the nervous system: When an excess of neurons is produced, they are available for adaptive use during neuronal development (reviewed in Oppenheim, 1991). The originally presented target-derived neurotrophic factor hypothesis (Thoenen and Barde, 1980) suggested that the survival of each population of neurons strongly depends on a single neurotrophic factor supplied by its target field and, without this factor, the neurons die by default. According to this model, neurotrophic factors are synthesised in limiting amounts so that only the required

number of neurons have sufficient access to the neurotrophic factor support to survive. Later, it has been recognised that, for many neuronal populations, the survival effect is regulated by a co-operation of many neurotrophic factors (reviewed in Davies, 1996).

Most trophic factors in the central nervous system can be grouped into families based on their structural homology. The families of neurotrophins, neurokines and glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) are named and classified as neurotrophic factor families because their first described and most prominent effects were neurotrophic. However, members of these families have later been shown to have important functions also outside of the nervous system. Moreover, several other factors classified as merely growth factors have been shown to have also neurotrophic effects.

All in all, the concept of a neurotrophic factor is very obscure. First, some inorganic molecules can promote the survival of neurons: e.g. high potassium concentrations support the survival of chick sympathetic neurons (Por and Huttner, 1984), so it has to be decided whether the term neurotrophic factor can be used for any molecule or only proteins or peptides. Mitsumoto and Tsuzaka propose in their review (1999) that "neurotrophic factors are signalling proteins that enhance neuronal survival, maintenance and differentiation, but they also can increase neurite growth and neurotransmitter production". This, however, leaves space for discussion of whether a factor should contribute to all of these functions in order to be defined as neurotrophic, or whether promoting

one of them is enough. One question is also whether a neurotrophic factor has to be a protein secreted by other cells, or whether it can function within the same cell, or, for example, from the surface of a neighbouring cell.

Another set of criteria to define a neurotrophic factor by Barde (1988) includes four requirements: in order to be defined as a neurotrophic factor, a molecule must 1) keep alive vertebrate neurons that would die in the absence of the factor, 2) be present in the biologically active form, synthesised and secreted from the target tissue of those neurons that will be saved, 3) be present in the target tissue in very small amounts and support the survival of a specific and limited set of neurons and 4) affect the development or maintenance of neurons *in vivo*. In conclusion, the current view seems to be that the minimal requirements of a neurotrophic factor are that the factor is secreted from the target tissue of the neurons and has the ability to support the survival of a certain neuron population *in vivo*.

1.1 Classification of neurotrophic factors

There are currently four main families of growth factors that, according to the classical view, are specific to the nervous system: neurotrophins, neurokines, the MANF (mesencephalic astrocyte-derived neurotrophic factor) family and GFLs. The classification is based on the homology in the amino acid sequences and the structures of the neurotrophic factors.

Neurotrophins and GFLs belong to a large superfamily of cysteine-knot growth factors, which also includes e.g. the transforming growth factor β (TGF-β) family of growth factors, human chorionic gonadotropin, platelet-derived growth factors and vascular endothelial growth factors (Butte 2001). All the members of this family contain a cystine knot that consists of three disulfide bonds. The neurotrophins form a non-covalently linked head-to-head dimer, whereas the monomers of GDNF and its family members are arranged head-to-toe and covalently linked by a disulfide bond. The three-dimensional structures of these factors are surprisingly similar in spite of their quite different amino acid sequences (Butte 2001). The structure of GFLs will be discussed in more detail later in this thesis.

Ciliary neurotrophic factor (CNTF) and most other neurokines are not cysteine-knot proteins, but consist of four helices forming a bundle. In the original crystal structure (McDonald *et al.*, 1995), CNTF seemed to be a dimer, but the authors note that this might be a crystallisation artefact. Later, it has been shown that CNTF exists primarily as a monomer, but significant dimer formation occurs at high protein concentrations (Narhi *et al.*, 1997). MANF family members MANF and CDNF (conserved dopamine neurotrophic factor) are secreted proteins with eight conserved cysteine residues. The crystal structures of both MANF and CDNF were solved very recently and they do not resemble the structure of any known growth factor. In both proteins the N-terminal domain is a saposin-like lipid-binding domain and the C-terminal domain contains a CKGC disulphide bridge like reductases and disulphide isomerases (Parkash *et al.*, 2009).

1.1.1 Neurotrophins

The mammalian neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF),

neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Neurotrophin signalling is critically involved in the development of the brain and nervous system, but neurotrophins also play a role at least in retinal, cochlear and heart development (Frade *et al.*, 1999, Fritzsch *et al.*, 1997, Tessarollo *et al.*, 1997). Moreover, neurotrophins, and BDNF in particular, are essential in the maintenance and plasticity of the adult neurons and their connections (reviewed in Chao, 2003, McAllister *et al.*, 1999 and Lykissas *et al.*, 2007). Neurotrophins are synthesised as large precursors, which are then processed to yield the pro-forms of neurotrophins and ultimately, the mature, neurotrophic proteins. The mature neurotrophins have a molecular weight of ~12-13 kDa and share about 50 % homology (Butte *et al.*, 2001). Interestingly, pro-forms of some neurotrophins have been found to have very different functions from the mature forms (Lee *et al.*, 2001, Teng *et al.*, 2005).

1.1.2 Neurokines

Neurokines, also called neuropoietic cytokines, or the CNTF-family, according to its best-characterised member, are small molecules that are structurally similar to cytokines and signal using common cytokine receptor components. CNTF was originally described as a growth factor that supports the survival of parasympathetic neurons from the chick ciliary ganglia (Adler *et al.*, 1979). Later, it has been found to have trophic and differentiating effects on different types of peripheral and central neurons and glia, but most prominently it affects the survival of motoneurons (reviewed in Vergara and Ramirez, 2004). In addition to CNTF, well-known members of this family are

interleukin 6 (IL-6), cardiotrophin 1 and 2, and leukaemia inhibitory factor (LIF). The actions of these cytokine family members on neurons are similar to CNTF's effects in some cases, but have much broader actions throughout the rest of the body (Ip and Yancopoulos, 1996).

1.1.3 MANF family

The very recently found MANF family consists of MANF, which is originally known as ARMET (arginine-rich, mutated in early stage tumours), and a homologous protein, CDNF (Shridhar *et al.*, 1996, Petrova *et al.*, 2003, Lindholm *et al.*, 2007). MANF was described recently as a survival promoting factor for embryonic midbrain dopaminergic neurons *in vitro* (Petrova *et al.*, 2003), and it is expressed widely in both the nervous system and non-neuronal tissues (Lindholm *et al.*, 2008). CDNF has been shown to function as a trophic factor for dopamine neurons *in vivo* and is expressed in the adult mouse heart, skeletal muscle and testis as well as in several neuronal cell types (Lindholm *et al.*, 2007). In addition, CDNF and MANF have been suggested to inhibit ER stress-induced cell death (Apostolou *et al.*, 2008, Parkash *et al.*, 2009). The receptors for MANF and CDNF are still unknown. Mammalian MANF and CDNF have an invertebrate homologue in *Drosophila melanogaster*, where this protein regulates the development of dopamine neurons (Palgi *et al.*, 2009).

1.1.4 GDNF and its family ligands

GFL family members GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN) belong to the TGF-β superfamily and are involved in the development, differentiation and maintenance of

many neuron types (Airaksinen *et al*., 1999). GDNF was first described as a neurotrophic factor that promotes the survival of midbrain dopaminergic neurons *in vitro* (Lin *et al*., 1993). This finding raised substantial interest because the symptomatic phases of Parkinson's disease (PD) are characterised by degeneration of dopaminergic neurons in the midbrain which innervate the striatum (German *et al*., 1992).

Later, GDNF has been found to be a potent trophic factor for spinal motoneurons (Henderson *et al*., 1994) and for central noradrenergic neurons (Arenas *et al*., 1995), and to play a critical role also outside the nervous system, e.g. in kidney development and spermatogenesis (Moore *et al*., 1996, Pichel *et al*., 1996, Sánchez *et al*., 1996, Meng *et al*., 2000). GDNF is expressed widely in the central and peripheral nervous system (Schaar *et al*., 1993, Strömberg *et al*., 1993, Golden *et al*., 1998), but also in a variety of other tissues and cell types (Trupp *et al*., 1995, Suvanto *et al*., 1996, Golden *et al*., 1999)

The most common isoform of GDNF is synthesised as a 211 amino acid precursor form, whereas the mature, secreted form consists of only 134 amino acids and has a molecular weight of ~20 kDa (Lin *et al*., 1993) (Figure 1). Both the secretion and proteolytic processing of GDNF are, however, quite poorly known. The precursors of some other neurotrophic factors, for example proNGF, have been shown to have other biological functions than the mature factors (Lee *et al*., 2001), so it can be speculated that also proGDNF and other proGFLs could have unexpected roles (Airaksinen and Saarma, 2002).

As is the case with a number of other neurotrophic factors, GDNF binds strongly to heparin (Lin *et al*., 1994, Alfano *et al*., 2007), which is thought to

retain GDNF close to its site of secretion within the tissue, and thus raise its local concentration. At low concentrations, heparin protects GDNF from proteolytic modification by an endoprotease (Rickard *et al*., 2003). Heparan sulphates, which are structurally related to heparin, are widely distributed on cell surfaces and in the extracellular matrix, and it has been claimed that GDNF signalling requires cell surface heparan sulphate glycosaminoglycans (Barnett *et al*., 2002, Davies *et al*., 2003). Davies *et al*. (2003) also report that low concentrations of exogenous heparin can block the neurite outgrowth induced in PC12 cells by GDNF and soluble GDNF family receptor α1 (GFRα1) protein. Similar results are reported by Ai *et al*. (2007), showing also that the activity of Sulfs, heparan sulphate modifying enzymes, decreases GDNF binding to heparan sulphates, promoting GDNF signalling. On the other hand, exogenous heparin has been shown to promote the activity of GDNF in the induction of tyrosine hydroxylase (TH) gene expression in neuroblastoma cells (Tanaka *et al*., 2002). Therefore, the role of heparan sulphates in GDNF signalling remains unclear.

Neurturin

NRTN is structurally related to GDNF, and its mature form shows 42 % sequence similarity with it. NRTN was first isolated on the basis of its ability to support the survival of sympathetic neurons in culture (Kotzbauer *et al*., 1996), and, like GDNF, it has been shown to promote the survival of dopaminergic neurons (Horger *et al*., 1998). Therefore, trials to protect dopaminergic neurons from extensive cell death have been carried out in various models of progressive PD, with

variable results (Rosenblad *et al*., 1999, Oiwa *et al*., 2002, Ceregene Press release 26.11.2008).

NRTN has also been shown to support survival and proliferation of several other neuron populations in the central and peripheral nervous system (Kotzbauer *et al*., 1996, Klein *et al*., 1997, Heuckeroth *et al*., 1998, Rossi *et al*., 1999, Golden *et al*., 2003). Most importantly, NRTN regulates the development of most of the parasympathetic neurons (Rossi *et al*., 1999). In addition, NRTN promotes epithelial branching, can induce branch initiation in developing kidney (Davies *et al*., 1999), directs liver bud migration (Tatsumi *et al*., 2007) and contributes to retinal function (Brantley *et al*., 2008). The prominent expression of NRTN in the gut, prostate, testicle and oviduct of adult mice also suggest some functions in these tissues (Golden *et al*., 1999).

The pre-pro-form of NRTN consists of 195 amino acids and it is cleaved to generate a 100 residue mature protein, which has a molecular mass of ~12 kDa (Kotzbauer *et al*., 1996) (Figure 1). Mature NRTN has also been shown to bind heparin, even with a higher affinity than GDNF (Alfano *et al*., 2007).

Artemin

Artemin is a survival and growth factor for sympathetic and sensory neurons *in vitro* (Baloh *et al*., 1998, Enomoto *et al*., 2001), and a potent neuroprotective factor for the rodent nigrostriatal DA neurons *in vivo* (Rosenblad *et al*., 2000). The ARTN sequence is more similar to the NRTN and PSPN sequences than to the GDNF sequence (Baloh *et al*. 1998). ARTN mRNA is expressed in brain and various other tissues, but the expression levels are highest in peripheral tissues including prostate, placenta, pancreas, heart and kidney (Masure *et al*., 1999). ARTN has been found to regulate sensory function (Wang *et al*., 2008) and is therefore being considered for the treatment of chronic pain (Gardell *et al*., 2003).

Like GDNF and NRTN, ARTN is also synthesised as a pre-pro-form which is processed to form a mature 113 amino acid protein with a molecular weight of ~12 kDa (Figure 1). Like GDNF and NRTN, ARTN has also been found to strongly bind heparin (Alfano *et al*., 2007).

Persephin

Persephin is related to other GFLs and shows about 40 % sequence identity to GDNF and NRTN. PSPN promotes the survival of ventral midbrain dopaminergic neurons in culture, supports the survival of motor neurons in culture and *in vivo* after sciatic nerve axotomy and, like GDNF, promotes ureteric bud branching *in vitro* (Milbrandt *et al*., 1998, Åkerud *et al*., 2002). Moreover, PSPN promotes the survival of embryonic basal forebrain cholinergic neurons *in vitro* (Golden *et al*., 2003). However, PSPN has not been found to support any peripheral neurons that have been examined. The expression of PSPN seems to be quite wide, but the detected mRNA levels are very low in most tissues (Milbrandt *et al*., 1998, Jaszai *et al*., 1998, Lindfors *et al*., 2006).

In addition, PSPN has been shown to promote both survival and neuritogenesis of midbrain dopamine neurons and thus it has been suggested that PSPN, like GDNF and NRTN, might have therapeutic potential in the treatment of Parkinson's disease (Åkerud *et al*., 2002). In addition, future therapeutic approaches may involve the use of PSPN in the treatment of stroke (Tomac *et al*., 2002).

The pre-pro-form of PSPN is 156 amino acids long and is cleaved to produce a 96 amino acids long mature protein with a molecular weight of 10-12 kDa (Milbrandt *et al.*, 1998) (Figure 1). Differently from other GFLs, PSPN is not able to bind to heparin and heparan sulphates (M. Bespalov, personal communication).

2. Neurotrophic factor receptors

Receptors are proteins that bind their specific ligands and mediate the ligand's messages of growth, differentiation, apoptosis or other functions. According to the target-derived neurotrophic factor hypothesis, neurotrophic factors are synthesised in limiting amounts so that only a limited number of neurons can
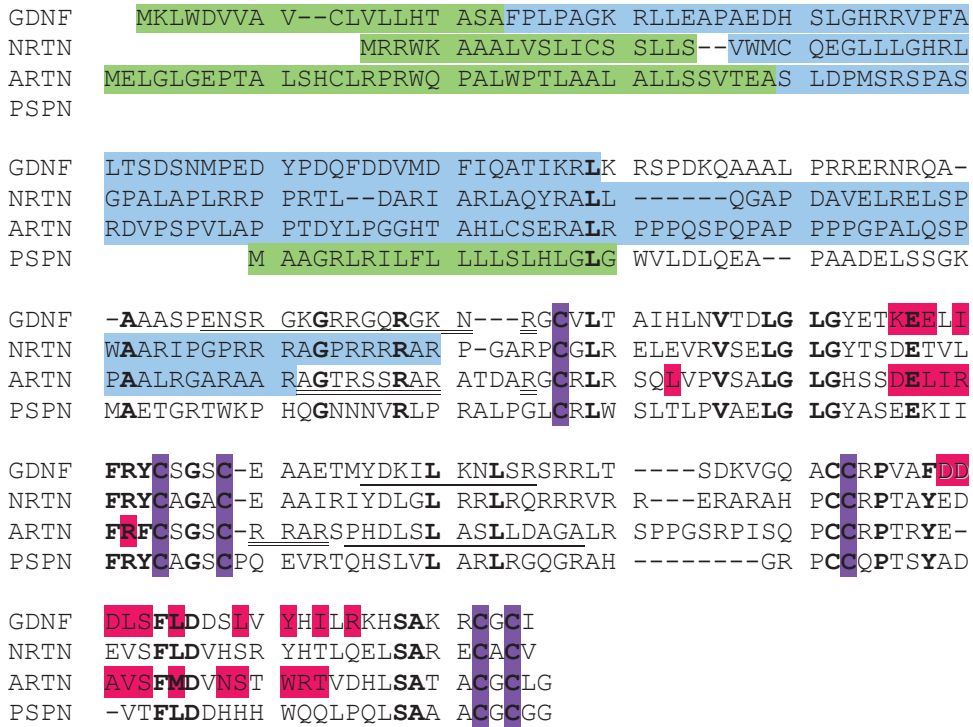
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GDNF   MKLWDVVA V--CLVLLHT ASAFPLPAGK RLLEAPAEDH SLGHRRVPFA
NRTN            MRRWK AAALVSLICS SLLS--VWMC QEGLLLGHRL
ARTN   MELGLGEPTA LSHCLRPRWQ PALWPTLAAL ALLSSVTEAS LDPMSRSPAS
PSPN

GDNF   LTSDSNMPED YPDQFDDVMD FIQATIKRLK RSPDKQAAAL PRRERNRQA-
NRTN   GPALAPLRRP PRTL--DARI ARLAQYRALL ------QGAP DAVELRELSP
ARTN   RDVPSPVLAP PTDYLPGGHT AHLCSERALR PPPQSPQPAP PPPGPALQSP
PSPN           M AAGRLRILFL LLLSLHLGLG WVLDLQEA-- PAADELSSGK

GDNF   -AAASPENSR GKGRRGQRGK N---RGCVLT AIHLNVTDLG LGYETKEELI
NRTN   WAARIPGPRR RAGPRRRRAR P-GARPCGLR ELEVRVSELG LGYTSDETVL
ARTN   PAALRGARAA RAGTRSSRAR ATDARGCRLR SQLVPVSALG LGHSSDELIR
PSPN   MAETGRTWKP HQGNNNVRLP RALPGLCRLW SLTLPVAELG LGYASEEKII

GDNF   FRYCSGSC-E AAETMYDKIL KNLSRSRRLT ----SDKVGQ ACCRPVAFDD
NRTN   FRYCAGAC-E AAIRIYDLGL RRLRQRRRVR R---ERARAH PCCRPTAYED
ARTN   FRFCSGSC-R RARSPHDLSL ASLLDAGALR SPPGSRPISQ PCCRPTRYE-
PSPN   FRYCAGSCPQ EVRTQHSLVL ARLRGQGRAH --------GR PCCQPTSYAD

GDNF   DLSFLDDSLV YHILRKHSAK RCGCI
NRTN   EVSFLDVHSR YHTLQELSAR ECACV
ARTN   AVSFMDVNST WRTVDHLSAT ACGCLG
PSPN   -VTFLDDHHH WQQLPQLSAA ACGCGG
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Figure 1. Sequence alignment of the GFLs. *GDNF, ARTN and PSPN sequences are rat sequences and NRTN is the mouse sequence (rat NRTN sequence is not available). Regions with high degree of sequence similarity are shown **bold** (according to MultAlin based on the algorithm described in Corpet, 1988). Structural and functional properties are highlighted as follows: Single underline; α-helix according to the crystal structure from Eigenbrot and Gerber, 1997 (GDNF) and Silvian et al., 2006 (ARTN). Double underline; heparin binding sequence/residue according to the experimental data from Alfano et al., 2007 (GDNF) and Silvian et al., 2006 (ARTN). Purple; cysteine residue thought to participate in the intramolecular cysteine bridges. Green; signal peptide (according to Uniprot). Blue; propeptide (according to Uniprot). Pink; residues interacting with GFRα according to Wang et al., 2006 (ARTN) and IV (GDNF).*

get enough of the neurotrophic factor support to survive. To avoid programmed cell death, neurons have to compete for the scarce quantities of trophic factors. Therefore, it is crucial that neurons express receptors that bind neurotrophic factors with high affinity and specificity.

2.1 Receptors of neurotrophins

The majority of trophic actions of neurotrophins are mediated by tropomyosin-related kinase (Trk)-type tyrosine kinase receptors: NGF signals via the TrkA receptors, BDNF and NT-4/5 signal via the TrkB receptors, and NT-3 signals via the TrkC receptors (Chao and Hempstead, 1995). Trk receptors are transmembrane proteins that span the membrane once and contain a heavily glycosylated extracellular domain, as well as a cytoplasmic domain consisting of a tyrosine kinase (TK) domain. The extracellular domain of each of the Trk receptors consists of a cysteine-rich cluster followed by three leucine-rich repeats, another cysteine-rich cluster and two immunoglobulin-like domains. These domains determine principally the affinity and specificity of binding of the neurotrophin (Pérez *et al*., 1995, Urfer *et al*., 1995, Ultsch *et al*., 1999).

Upon ligand binding, the tyrosines residing in the autoregulatory loop of Trk receptor TK domain become phosphorylated. This further leads to the phosphorylation and activation of the other intracellular tyrosine residues. The phosphorylated tyrosines function as docking sites for a number of cytoplasmic adaptor proteins, leading to the activation of various intracellular signalling cascades important for neuronal survival, differentiation and plasticity (reviewed by Reichardt, 2006). Intracellularly truncated

forms, and thus catalytically inactive forms of TrkB and TrkC receptors, also exist. Many of the functions of these receptors are unknown, but they might spatially restrict the actions of neurotrophins (neurotrophin scavengers) and inhibit Trk receptor signalling (dominant negative action) (Eide *et al*., 1996, Haapasalo *et al*., 2001).

Each neurotrophin is also capable of interacting with the low affinity p75 receptor, which belongs to the tumour necrosis factor receptor superfamily (TNFR) (Chao and Hempstead, 1995). The p75 receptor is widely expressed in the developing central and peripheral nervous system during the period of synaptogenesis and developmental cell death (Davies, 1991). The p75 receptor is a type I transmembrane protein with a molecular weight of ~75 kDa and consists of an extracellular domain that includes four cysteine-rich motifs, a single transmembrane domain and a cytoplasmic domain. The intracellular domain of p75 lacks catalytic activity, but contains a death domain motif similar to those found in other members of the TNFR family and their downstream targets (Liepinsh *et al*., 1997, He and Garcia 2004). One established function of p75 is indeed to promote cell death (Hempstead, 2002, Miller and Kaplan, 2001). However, TrkA-induced survival signalling protects neurons from p75-mediated developmental cell death (Majdan *et al*., 2001). Depending on the cellular context, the p75 receptors can also modulate the signalling of Trk receptors (Hempstead *et al*., 1991, Bibel *et al*., 1999, Esposito *et al*., 2001) or promote either atrophic or trophic cellular actions (Blöchl and Blöchl, 2007).

The p75 receptor-mediated cellular responses to mature neurotrophins are generally weak, but, interestingly, pro-

neurotrophins preferentially activate p75 receptors and thereby produce very different changes on neuronal functions compared to the mature neurotrophins (Lee *et al.*, 2001, Teng *et al.*, 2005). The authors in the study by Lee *et al.* (2001) stated that proNGF binds to p75 with a higher affinity than the mature NGF. However, Nykjaer *et al.* (2004) showed that actually the lack of processing reduces the affinity of proNGF for both p75 and TrkA, but increases the affinity for a p75 co-receptor, sortilin. Thus, sortilin and p75 co-operate in mediating proNGF-induced cell death. Like p75-deficient mice, sortilin 1-deficient mice show reduced neuronal apoptosis (Jansen *et al.*, 2007).

In contrast, the binding of mature NGF to p75 and TrkA is not significantly affected by sortilin (Nykjaer *et al.*, 2004). Later, it has also been shown that proBDNF is secreted from mouse neurons (Yang *et al.*, 2009) and it binds to a receptor complex formed by p75/sortilin leading to apoptosis (Teng *et al.*, 2005).

Neurotrophins bind as dimers to p75 receptors and Trk receptor family members. He and Garcia (2004) have reported that NGF binding to p75 results in a conformational change in NGF that alters the structure of the opposite side of the NGF dimer, preventing the binding of one NGF dimer to another p75 monomer. However, in another study (Aurikko *et al.*, 2005), the p75/NGF complex was found to have a 2:2 stoichiometry. The authors of this study propose the discrepancy to be due to the absence of glycosylation of p75 in He and Garcia (2004).

2.2 Receptors of neurokines

CNTF, LIF, IL-6 and many other cytokines belong to a family called interleukin-6-type cytokines. Members of this family

bind to receptors that can be classified in the non-signalling α-receptors – IL-6 receptor α, IL-11 receptor α, and CNTF receptor α – and the signal transducing receptors – glycoprotein 130 (gp130), LIF receptor (LIFR), and oncostatin M receptor (OSMR). The signal transducing receptors become tyrosine phosphorylated in response to cytokine stimulation and mediate the signal into the cell (Davis *et al.*, 1993a). Each of the IL-6-type cytokines recruits by ligand binding at least one gp130.

IL-6, IL-11 and CNTF first bind specifically to their respective α receptors. This binding induces the recruitment of two signalling receptors that are not associated at the cell surface before binding to the ligand–α receptor complex (Vergara and Ramirez, 2004). IL-6 and IL-11 signal through gp130 homodimers, whereas other IL-6 type cytokines signal through heterodimers of gp130 and LIFR, or gp130 and OSMR. LIF and oncostatin M bind their signalling receptors directly without an α receptor (Heinrich *et al.*, 2003). Upon binding of the ligand, the intracellular domains of signalling receptors become associated with a variety of signalling molecules, for example JAK (Janus kinase) tyrosine kinase family members, and activate them.

Non-signalling receptors, described above, are homologous and thus form a family of cytokine receptor family type 1. The extracellular region of members of this receptor family contains combinations of cytokine domains, fibronectin III-like domains and, in some cases, also immunoglobulin-like domains. All these cytokine receptors have a single 22–28 amino acid transmembrane domain and an intracellular domain, except for CNTF receptor (CNTFRα) (Vergara and Ramirez, 2004). CNTF

receptor is anchored to the membrane by a glycosylphosphatidylinositol (GPI)-anchor. Due to its GPI-linkage, it can be cleaved by phosphatidylinositol-specific phospholipase C (PI-PLC) to produce a soluble and functional form of the receptor (Davis *et al*., 1993b). Soluble forms of other cytokine receptors can be produced by alternative splicing or limited proteolysis of membrane bound proteins (Rose-John and Heinrich 1994).

## 2.3 Receptors of GDNF family ligands

The best-known GFL signalling happens through a tetrameric receptor complex, which includes two GFRα molecules and two RET (<u>re</u>arranged during <u>t</u>ransfection) receptor tyrosine kinases (Takahashi *et al*., 1985, Durbec *et al*., 1996a, Trupp *et al*., 1996). In this model of GFL signalling, GPI-anchored receptors GFRα1-4 bind their ligands GDNF, NRTN, ARTN and PSPN, respectively, but cannot mediate the signal through the cell membrane. The signal is then delivered by the transmembrane receptor RET that cannot bind GFLs on its own, but can bind the GFRα/GFL complex (Figure 2).

In several cell and tissue types GFRαs are expressed at high levels, whereas RET is undetectable (Trupp *et al*., 1997, Golden *et al*., 1999). This enigma could in theory be explained by GFL signalling through GFRα alone, or in association with some other receptor than RET. An alternative possibility is that the receptor complexes would be formed between RET and GFRα receptor from adjacent cells, or that the GFL-responsive cells would express RET with the co-receptor supplied in a soluble form. There is indeed evidence that these signalling modes are used by GFLs: RET can be activated by GFRα1 molecules

presented on the membrane of adjacent cells, which themselves do not express RET. Also released GFRα1 is capable of mediating GDNF signalling (Paratcha *et al*., 2001). When GFRα1 is present on both the cell surface and in a soluble form, it has been suggested that interactions between these forms of GFRα1 could act to potentiate the effects of GDNF (Worley *et al*., 2000). It has been shown that the neural cell adhesion molecule (NCAM) can function as a signalling receptor for GFLs (Paratcha *et al*., 2003). In addition, GDNF has been found to be able to signal through the Met receptor tyrosine kinase (Popsueva *et al*., 2003). It has also been shown that GFLs and GFRαs may have cellular functions independent of RET or NCAM, such as cell adhesion (Ledda *et al*., 2007), and regulation of differentiation and migration of cortical GABAergic neurons (Pozas and Ibáñez, 2005). However, these RET-independent signalling mechanisms are still relatively poorly known and this thesis concentrates on GDNF signalling via RET.

2.3.1 GFRα receptors

There are four different GFRα receptors: GFRα1 (Jing *et al*., 1996, Treanor *et al*., 1996), GFRα2 (Baloh *et al*., 1997, Buj-Bello *et al*., 1997, Jing *et al*., 1997, Klein *et al*., 1997, Suvanto *et al.*, 1997), GFRα3 (Jing *et al*., 1997, Baloh *et al*., 1998, Masure *et al*., 1998, Naveilhan *et al*., 1998, Nomoto *et al*., 1998, Trupp *et al*., 1998, Widenfalk *et al*., 1998, Worby *et al*., 1998) and GFRα4 (Thompson *et al*., 1998, Masure *et al*., 2000, Lindahl *et al*., 2000). The tissue expression pattern of GFRα receptors is very similar to their corresponding ligands, and each GFL seems to regulate the mRNA expression of its cognate co-receptor (Airaksinen
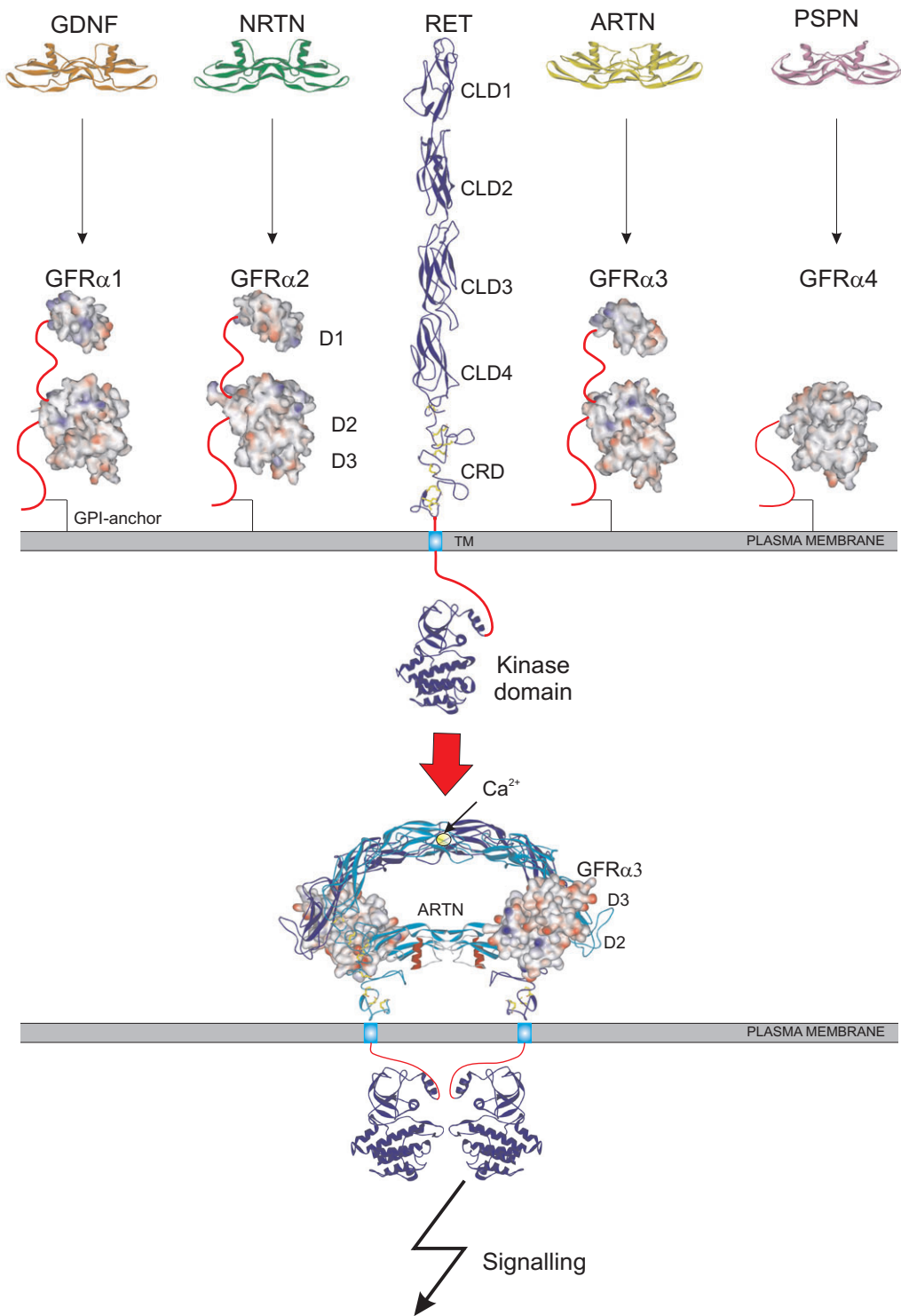
et al., 1999, Taraviras *et al.*, 1999). GFL knockouts and their corresponding GFRα knockouts also show very similar phenotypes, which suggests a specific pairing *in vivo* (Airaksinen *et al.*, 1999).

GFRα receptors are GPI-linked cell surface proteins that lack a cytoplasmic domain capable of mediating transmembrane signalling. The length of the unprocessed form of rat GFRα1 is 468 amino acids, of which the secretory and GPI-anchor mediating signals are removed (Jing *et al.*, 1996) (Figure 3). In general, the lengths of all GFRα receptors are around 400 amino acids, and they contain three putative N-glycosylation sites. The predicted molecular weight of GFRα1 in the absence of glycosylation is about 47 kDa (Jing *et al.*, 1996).

Although GFL structures have striking resemblance to structures of several TGF-β family proteins (Daopin *et al.*, 1993, Eigenbrot and Gerber, 1997, Silvian *et al.*, 2006), GFRα receptors have very little in common with the receptors of TGF-β family members. GFRα receptors also lack many of the domains most commonly present in other receptors, such as leucine repeats, immunoglobulin-like domains, and fibronectin-like domains (Scott and Ibáñez, 2001).

GFRα receptors are rich in cysteine with an unusual conserved spacing of these residues (Jing *et al.*, 1996) (Figure 3). In general, GFRα receptors consist of three homologous cysteine-rich domains (numbered 1-3 starting from the N-terminus). Domains 2 and 3 are located very closely, whereas domain 1 is separated from domain 2 by a quite long and flexible hinge region (Leppänen *et al.*, 2004). Domains 2 and 3 are present in all GFRαs, but all GFRα4s except for chicken GFRα4 lack domain 1 (Lindahl *et al.*, 2000, Masure *et al.*, 2000, Lindahl *et al.*, 2001).

GFRα receptors are initially linked to the plasma membrane from their C-terminus with GPI-anchors, but these anchors can be cleaved to generate soluble GFRαs. Soluble GFRα1 is able, together with GDNF, to induce RET phosphorylation in cells that do not express GFRα1 (Jing *et al.*, 1996).

Figure 2. Components of GFL–GFRα–RET complexes. GFL signalling happens through a tetrameric receptor complex, which includes two GPI-anchored GFRα molecules and two RET receptor tyrosine kinases. In the upper part of the figure only one RET and one GFRα receptor are shown. Each of the ligands binds preferentially one of the four GFRα receptors. The complete structure of the GFL$_2$-GFRα$_2$-RET$_2$ complex has not been solved, but to illustrate the activated receptor complex, known and predicted structures of GFLs, GFRαs and RET have been used. Images of RET extracellular domain, NRTN, PSPN, and GFRα2 and GFRα4 are generated by homology modelling. NRTN and PSPN images are based on the GDNF crystal structure. GFRα1 and GFRα3 models are based on the crystal structure of GFRα1 and the structure of the ARTN-GFRα3 complex, respectively (Wang et al., 2006 and IV). RET cadherin-like domains (CLD, blue) and cysteine-rich domain (CRD, blue line) are modelled using E-cadherin and the laminin γ1 chain, respectively (Söding et al., 2005). RET intracellular domain models are based on the crystal structure of the RET kinase domain (Knowles et al., 2006). Calcium ions (yellow circle in the lower figure) are necessary for RET signalling. Modified from Bespalov and Saarma, 2007.

```
GFRα1           MFLATLYFA LPLLDLLMSA EVSGG-DRLD CVKASDQCLK
GFRα2  MILANAFCL FFFLDETLRS LASPSSPQGS ELHGWRPQVD CVRANELCAA
GFRα3  MGLSWSPRPP LLMILLLVLS LWLPLGAGNS LATENRFVNS CTQARKKCEA
GFRα4

GFRα1  EQSCSTKYRT LRQCVAGKET NFSLTSGLEA KDECRSAMEA LKQKSLYNCR
GFRα2  ESNCSSRYRT LRQCLAGRDR NTML-----A NKECQAALEV LQESPLYDCR
GFRα3  NPACKAAYQH LGSCTSSLSR PLPLEESA-M SADCLEAAEQ LRNSSLIDCR
GFRα4

GFRα1  CKRGMKKEKN CLRIYWSMYQ SL-QGNDLLE DSPYEPVNSR LSDIFRAVPF
GFRα2  CKRGMKKELQ CLQIYWSIHL GLTEGEEFYE ASPYEPVTSR LSDIFRLASI
GFRα3  CHRRMKHQAT CLDIYWTVHP ARSLGDYELD VSPYE----- --DTVTSKPW
GFRα4                                             MAHCMESAL

GFRα1  ISDVFQQVEH ISKGNNCLDA AKACNLDDTC KKYRSAYITP CTTSMS-NEV
GFRα2  FSGTGADPVV SAKSNHCLDA AKACNLNDNC KKLRSSYISI CNREISPTER
GFRα3  KMNLSKLNML KPDSDLCLKF AMLCTLHDKC DRLRKAYGEA CS-----GIR
GFRα4  LLLLLLGSAS FTDGNRCVDA AEACTADERC QQLRSEYVAR CLGRAAPGGR

GFRα1  -----CNRRK CHKALRQFFD KVPAKHSYGM LFCSC--RDI ACTERRRQTI
GFRα2  -----CNRRK CHKALRQFFD RVPSEYTYRM LFCSC--QDQ ACAERRRQTI
GFRα3  -----CQRHL CLAQLRSFFE KAAESHAQGL LLCPCAPEDA GCGERRRNTI
GFRα4  PGPGGCVRSR CRRALRRFFA RGPPALTHAL LFCGC--EGS ACAERRRQTF

GFRα1  VPVCSY--EE RERPNCLSLQ DSCKTNYICR SRLADFFTNC QPESRSVSNC
GFRα2  LPSCSY--ED KEKPNCLDLR SLCRTDHLCR SRLADFHANC RASYRTITSC
GFRα3  APSCAL--PS -VTPNCLDLR SFCRADPLCR SRLMDFQTHC HP--MDILGT
GFRα4  APACAFSGPG LVPPSCLEPL ERCERSRLCR PRLLAFQASC APAPGSRDRC

GFRα1  LKENYADCLL AYSGLIGTVM TPNYVDSS-- SLSVAPWCDC SNSGNDLEDC
GFRα2  PADNYQACLG SYAGMIGFDM TPNYVDSNPT GIVVSPWCNC RGSGNMEEEC
GFRα3  CATEQSRCLR AYLGLIGTAM TPNFISKVNT TVALS--CTC RGSGNLQDEC
GFRα4  PEEGGPRCLR VYAGLIGTVV TPNYLDNV-- SARVAPWCGC AASGNRREEC

GFRα1  LKFLNFFKDN TCLKNAIQAF GNGSDVTMWQ PAPPVQTTTA TTTTAFRVKN
GFRα2  EKFLKDFTEN PCLRNAIQAF GNGTDVNMSP KGPTFSATQA PRVEKTPSLP
GFRα3  EQLERSFSQN PCLVEAIAAK MRFHRQLFSQ DWADSTFSVV QQQNSNPALR
GFRα4  EAFRKLFTRN PCLDGAIQAF DSLQPSVLQD QTAGCCFPRV SWLYALTALA

GFRα1  -KPLGPAGSE NEIPTHVLPP CANLQAQKLK SNVSGSTHLC LSDSDFGKDG
GFRα2  -DDLSDSTS- --LGTSVITT CTSIQEQGLK ANNSKELSMC FTELTTNISP
GFRα3  LQPRLPILSF SILPLILLQT LW
GFRα4  LQALL

GFRα1  LAGASSHITT KSMAAPPSCS LSSLPVLMLT ALAALLSVSL AETS
GFRα2  GSKKVIKLYS GSCRARLSTA LTALPLLMVT LA
GFRα3
GFRα4
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In vivo soluble GFRα1 is released by enteric nervous system (ENS) neurons, neuronal cells, Schwann cells and injured sciatic nerve. It has been shown that RET stimulation by soluble GFRα1 potentiates downstream signalling, neurite outgrowth and neuronal survival (Paratcha *et al.*, 2001). It has also been suggested that alternative splicing could produce soluble GFRα4, but so far there is *in vivo* evidence only at mRNA level (Lindahl *et al.*, 2001). However, there is evidence that soluble GFRα4 can associate *in vitro* with, and induce, phosphorylation of RET (Yang *et al.*, 2007).

2.3.2 RET receptor tyrosine kinase

RET is a receptor tyrosine kinase (RTK) superfamily member that can function as a growth factor receptor or as an oncogenic protein. RET is expressed during vertebrate development in the developing excretory system, in the peripheral nervous system (PNS), and in motor and catecholaminergic neurons of the central nervous system (CNS) (Pachnis *et al.*, 1993, Avantaggiato *et al.*, 1994, Durbec *et al.*, 1996b). In adult humans, RET is expressed at least in several neuronal cell types and chromaffin cells of the adrenal medulla (Nakamura *et al.*, 1994). In addition, RET is expressed in human tumours of neural crest origin (Santoro *et al.*, 1990). RET dysfunction is connected to several congenital diseases, which will be discussed later in this thesis. It is also possible that RET can function in the absence of GDNF as a dependence receptor that induces apoptosis (Bordeaux *et al.*, 2000) and thus, in some conditions, prevent tumour growth (Cañibano *et al.*, 2007).

Under normal circumstances, RET requires GPI-anchored co-receptors for ligand binding and activation. The current view is that RET is synthesised as a non-phosphorylated monomer, which can get into contact with its ligands and co-receptors only at the cell surface. RET harbours 12 putative N-linked glycosylation sites. The N-linked core glycosylation of RET takes place in the ER, and the molecular weight of RET initially increases from approximately 120 kDa to 150 kDa (Takahashi *et al.*, 1991). Further modification of glycosylation takes place in the Golgi apparatus and the molecular weight of mature RET is about 170 kDa.

Due to alternative splicing of the RET primary transcript, RET is expressed as several isoforms (Tahira *et al.*, 1990, Lorenzo *et al.*, 1995, Ivanchuk *et al.*, 1997). The two best-studied and clearly most abundant isoforms differ in their C-termini. They share the first N-terminal 1063 amino acids, but the tail is different: The short isoform (RET9) has a C-terminal

Figure 3. Sequence alignment of the GFRαs. *GFRα1 sequence is the rat sequence (used in this work) and GFRα2-4 sequences are mouse sequences (rat NRTN and rat ARTN sequences are not available). Regions of high degree of sequence similarity are shown in* **bold** *(according to MultAlin based on the algorithm described in Corpet, 1988). Structural and functional properties are highlighted as follows:* <u>Single underline</u>*; GFRα1 domain 1 (according to Leppänen et al., 2004).* <u>**Thick underline**</u>*; GFRα1 domain 2 (according to the crystal structure, Leppänen et al., 2004).* <u>Double underline</u>*; GFRα1 domain 3 (according to the crystal structure, Leppänen et al., 2004). Green; signal peptide (according to Uniprot). Blue; GPI-anchor signal sequence (according to Uniprot). Pink; residues interacting with GFL according to Wang et al., 2006 (GFRα3) and* **IV** *(GFRα1).*

tail of nine amino acids and the whole protein consists thus of 1072 amino acids. The long isoform of RET (RET51) has a tail of 51 amino acids and it contains thus in total 1114 amino acids (Tahira *et al.*, 1990, Lorenzo *et al*, 1995).

RET isoforms are differentially expressed in mouse embryos and in adult mice (Lee *et al.*, 2003), and it has been shown by RT-PCR that in mouse tissues RET9 is predominantly expressed (Yoong *et al.*, 2005). RET9 has been shown to be critical for the development of the mouse kidney and the enteric nervous system (de Graaff *et al.*, 2001). On the other hand, RET51, but not RET9, is required for the metabolism and growth of mature rat sympathetic neurons (Tsui-Pierchala *et al.*, 2002a) and may contribute more significantly to the tumour development associated with multiple endocrine neoplasia 2 (MEN 2) than RET9 (Asai *et al.*, 1996).

The biochemical differences which account for these functional differences between these isoforms are only partly known. Interestingly, Tyr1062, which is phosphorylated during the RET activation process and functions as an important docking site for adaptor proteins, is located right next to the last C-terminal residue that is common for both isoforms. It has indeed been suggested that the distinct activities of RET9 and RET51 result from the differential regulation of Y1062 by C-terminal flanking sequences (Wong *et al.*, 2005). In addition, RET51 also has two additional tyrosine residues, Tyr1090 and Tyr1096, which may participate in signalling events. The two isoforms of RET interact differently with adapter proteins like Src homology 2 domain-containing protein (SHC), growth factor receptor-bound protein 2 (GRB2) and Enigma (Lorenzo *et al.*, 1997, Alberti *et al.*, 1998, Borrello *et al.*, 2002). Activated RET51 associates more strongly than RET9 with the ubiquitin ligase Cbl, which leads to faster turnover of RET51 (Scott *et al.*, 2005).

3.   The GFL-GFRα-RET complex

The signal of GFLs is delivered into the cell by RET receptor tyrosine kinase, with the help of GFRαs that bind the GFL. These componets form the signalling complex on the cell surface. The GFL-GFRα-RET complex is assumed to consist of a disulphide-linked GFL dimer and two GFRα molecules bound to two molecules of RET. According to the original model, the initial event of GDNF-GFRα-RET complex formation is the binding of dimeric GDNF to GFRα1, in either monomeric or dimeric form. The GDNF-GFRα1 complex then interacts with two RET molecules, thereby inducing their homodimerisation and tyrosine autophosphorylation (Jing *et al.*, 1996). It is believed that also other GFL members interact with their cognate co-receptors and activate RET in a similar manner to GDNF.

However, clear and direct evidence of the complex formation has not been established. It is possible that GFL and GFRα form together a binding surface for RET, or that the binding of GFL changes the conformation of GFRα, creating thus a binding site for RET. In these models, GFRα and RET would not bind each other without the presence of GFLs. On the other hand, it is possible that monomeric GFRα and monomeric RET form a pre-associated complex to which GFLs can then bind. The formation of the complex could happen in three different ways (Figure 4): 1) GFL$_2$ (GFL homodimer) binds first to GFRα, this complex recruits

a second co-receptor, and the GFL$_2$-GFRα$_2$ complex recruits two molecules of RET. Alternatively, after binding of GFL$_2$ to GFRα, one molecule of RET is recruited, and then a second monomeric GFRα and RET are recruited to the GFL$_2$-GFRα-RET complex. 2) GFL$_2$ binds to a pre-associated GFRα-RET heterodimer and recruits another GFRα-RET pair. 3) Upon GFL$_2$ binding, a pre-associated GFRα$_2$-RET$_2$ heterotetramer undergoes a conformational change and gets activated.

Different views on the structure and kinetics of the GFL/GFRα/RET complex have been proposed on the basis of partially controversial results of the interactions between the complex members. Jing *et al*.

(1996) proposed that RET is not involved in the initial binding of GDNF to the complex. However, it has been shown that these two components can be cross-linked in the presence of GFRα1, which indicates that these two molecules are at least in close proximity in the complex (Trupp *et al*., 1996, Amoresano *et al*., 2005). There is also evidence of pre-association between GFRα1 or GFRα2, and RET (Sanicola *et al*., 1997, Treanor *et al*., 1996). The fact that some GDNF mutants with impaired ability to interact with GFRα1 can still activate RET in the presence of GFRα1 (Eketjäll *et al*., 1999) suggests that, either there is a preformed GFRα1/RET complex that has a higher affinity to GDNF than

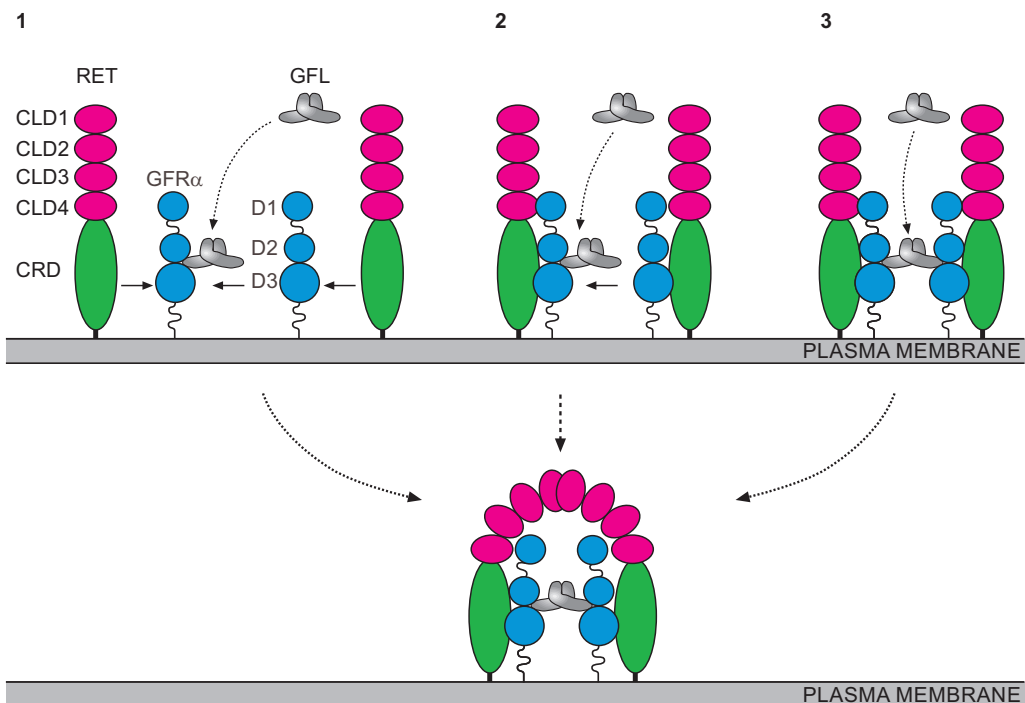***Figure 4. Putative mechanisms of GFL–GFRα–RET receptor complex activation.*** *Only the extracellular parts of RET are shown. The cadherin-like domains (CLD) of RET are red and the cysteine-rich domain (CRD) is green. GFL$_2$ is grey and GFRα receptor blue. The formation of the complex could happen in three different ways described in the text. The model of the GFL$_2$-GFRα$_2$-RET$_2$ complex shown in the lower part of the figure is loosely based on the results of Kjaer and Ibáñez, 2003, but also other models have been proposed. Modified from Bespalov and Saarma, 2007.*

GFRα1 alone, or then RET just stabilises the GDNF/GFRα complex. In addition, Cik *et al*. (2000) found a high-affinity GDNF-binding site on GFRα1 only in the presence of RET. Yet Tansey *et al*. (2000) report that in their assays RET does not co-immunoprecipitate with GFRα co-receptors in the absence of ligand.

Many receptor tyrosine kinases are activated when two receptor monomers are brought together and thus are subjected to transphosphorylation. However, in some biochemical and structural studies with dimerising receptors, it has been found that only part of the dimers have such conformations, that they can participate in trans-autophosphorylation and stimulation of downstream signalling proteins (Lemmon and Schlessinger, 1994). Dimerisation is thus not always sufficient for the activation. It is thought that receptor monomers are in equilibrium with receptor dimers and that active dimers exist even in the absence of ligand. Ligand binding to the extracellular domain of the receptor stabilises the formation of active dimers and consequently stimulation of downstream signalling molecules (Schlessinger 2000). This model has also been proposed for RET, with the addition of GFRα (Bespalov and Saarma, 2007). In this model, the equilibrium is formed between monomers of RET and GFRα, inactive and active forms of RET dimers and inactive $GFRα_2$-RET_2 heterotetramers. In addition to ligand binding, the equilibrium can be pushed towards the active dimers by increasing the receptor density on the cell surface. This model is supported by the finding that ligand-independent activation of RET occurs when RET is over-expressed, as is demonstrated in this study (**III**).

3.1   The structure of GFLs

GFLs belong to the cystine knot growth factor family based on their amino acid sequence and spatial structures (Lin *et al*., 1993, Eigenbrot and Gerber, 1997, Silvian *et al*., 2006). The members of this family contain seven cysteine residues with the same relative spacing. The structures of GDNF and ARTN have been solved (Eigenbrot and Gerber, 1997, Silvian *et al*., 2006), but both lack the structure of the flexible N-terminus. In GDNF, this N-terminus is prominent and covers about 1/4 of the total sequence. The analysis of these structures has demonstrated that, in spite of the low amino-acid sequence homology, GFLs resemble structurally members of the TGF-β superfamily. Moreover, the head-to-tail dimerisation, supported by an interchain disulphide bond, is similar to other TGF-β superfamily members.

The structure of both GDNF (Figure 5) and ARTN contain the same basic elements. The monomer consists of a central well-ordered α-helix, the "wrist" or "heel" of the monomer, which is surrounded by flexible, less-ordered stretches. From these stretches start the two "fingers", regions formed mainly of β-sheets. The N-terminal finger 1 contains two uninterrupted anti-parallel β-sheets, separated by a turn of 3_{10}-helix; the C-terminal finger 2 contains four β-sheets, interrupted by short stretches. The dimer arrangement is anti-parallel, which creates symmetry and suggests symmetric binding sites for a dimerised receptor (Eigenbrot and Gerber, 1997, Silvian *et al*., 2006). This is confirmed by the $GDNF_2$-$GFRα1_2$ complex structure (**IV**).

However, there are some differences: the shape and possibly the flexibility of the elongated homodimer differs. Unlike

GDNF, ordered segments in the pre-helix and post-helix stretches of ARTN increase the hinge angle between the "fingers" and "wrist" of each monomer (Silvian *et al.*, 2006). In addition, the pre-helix stretch of ARTN contains a positively charged heparin consensus sequence XBBXBX, where B is a basic residue and X is any residue. This region has been shown to contain the key heparin-binding residues in ARTN (Silvian *et al.*, 2006). The same region in GDNF is negatively charged, but, in the post-helix region, there is a putative heparin-binding sequence SRSRRL (Lin *et al.*, 1993, Silvian *et al.*, 2006). Moreover, Alfano *et al.* (2007) have localised a heparin-binding region in the N-terminal part of GDNF (see Figure 1).

Mutagenesis has been performed on GDNF to determine its interfaces with RET and GFRα1 (Eketjäll *et al.*, 1999, Baloh *et al.*, 2000). Eketjäll *et al.* found four negatively charged (D52, E61, E62 and D116) and four hydrophobic residues (I64, L114, Y120 and I122) in GDNF which

seem to be crucial for GFRα1 binding. In addition, they found some residues (such as E58, K60 and L118) where mutations caused smaller effects, and which may also participate in forming the interface. Baloh *et al.* (2000) identified two critical regions in GDNF for the interaction with GFRα1 and one region critical for the alternate GDNF-GFRα2 interaction. The identified regions are not continuous in the primary sequence of GDNF, but, in the crystal structure, they are directly adjacent and located in the finger 2. Moreover, they identified an additional region in the heel region that is critical for the NRTN-GFRα2 and ARTN-GFRα3 interactions.

3.2 The structure of GFRαs

The first secondary structure predictions suggested that GFRαs are mainly α-helical and consist of three conserved cysteine-rich domains that are joined together by less conserved adapter sequences (Airaksinen *et al.*, 1999). Later Scott
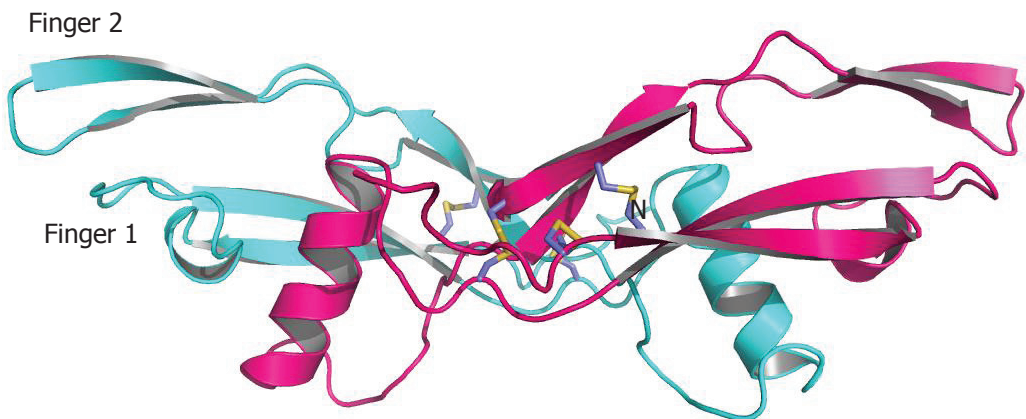
Figure 5. The structure of a GDNF homodimer. *The crystal structure of each GDNF monomer (light blue and pink) contains residues 34-134. Fingers 1 and 2 and the N-terminal end of the structure (N) are marked in the figure. The tips of the fingers are predicted to be important in binding to GFRα1. The cystine knot (disulphide bridges shown in purple and yellow) is located in the centre of the homodimer. Figure kindly provided by Vimal Parkash.*

and Ibáñez (2001) proposed that the region containing domains 2 and 3 forms only one domain. They also mapped the ligand binding sites in GFRα receptors by analysing the ligand binding and signalling capacities of a variety of chimeric and truncated receptors. The major determinant of ligand binding was found in the central region of the GFRα receptors, which is the most conserved region. The authors suggested this region to comprise of four α-helices and two β-strands.

Two motifs, the hydrophobic triplet MLF in the first predicted β-strand and the basic triplet RRR, were found to mediate the binding to GDNF, but not to RET. The N-terminal domain (domain 1) was found to be dispensable for both specific ligand binding and RET phosphorylation, and the authors speculated that it could possibly have a function in subcellular sorting or in the interaction with other transmembrane molecules (Scott and Ibáñez, 2001). In another study (Wang *et al.*, 2004), residues N152, N153, R259, S316, N317 and S318 in the GFRα1 central region were found to be critical for GFRα1 binding to GDNF and eliciting downstream signal transduction.

The first crystal structure of the domain 3 (D3) of GFRα1 receptor revealed a novel protein fold (Leppänen *et al.*, 2004). D3 forms a bundle of five α-helices with five disulphide bridges. Three helices (α1, α2 and α4) form a central spiral and the core of the bundle contains hydrophobic residues from these helices. The three most buried phenylalanines (F263, F328 and F332) are highly conserved among mouse GFRα sequences (Lindahl *et al.*, 2000). Three hydrophobic patches that might be involved in interactions with other domains, GDNF, RET or other proteins were identified in D3.

The structure of GFRα1 domain 3 was also used to model the homologous domain 2 (D2) (Leppänen *et al.*, 2004). This model suggested that D2 and D3 are clearly separate and D2 consists of five α-helices, but has a more compact structure than D3 (Figure 1). The surface of D2 was found to have two large positively charged areas. One of them is defined by R224, R225, R217, H207 and K150, and the second positively charged area is characterised by a potential heparin-binding motif BBBXBBXXB (residues 189–197).

A model of the GDNF-GFRα1 interface was built using the D3 crystal structure, D2 model, and previous information about the interacting regions in GDNF and GFRα1 (Eketjäll *et al.*, 1999, Scott and Ibáñez, 2001). This model served as a basis for a site-directed mutagenesis strategy and subsequent biochemical characterisation of the mutants. Four mutants with impaired GDNF-binding or effects on RET phosphorylation (F213A, R224A, R225A and I229A) were found (Leppänen *et al.*, 2004). These residues were thus suggested to be located in the GFRα1-GDNF interface. In addition, one mutant (R217E) showed similar binding in the presence and absence of RET, which indicates that it may be involved in the allosteric properties of GFRα1 or in binding RET. This data confirmed that the triplet RRR suggested by Scott and Ibáñez (2001) is indeed important, but of the hydrophobic MLF triplet, only F213 interacts with GDNF. However, the results of this study (Leppänen *et al.*, 2004) do not support the findings of Wang *et al.*, (2004).

Recently, the structure of ARTN complexed with its receptor GFRα3 was published (Wang *et al.*, 2006). The structural study was complemented with

biochemical assays. It was shown that D2 and D3 are separate, but together form a compact globular structure. Each domain consists of five α helices (α1–α5 for D2, α6–α10 for D3) that form one roughly triangular spiral in each domain. The interface between the two domains forms a large hydrophobic core where the residues involved in the hydrophobic interactions include L200, F204, L216, L217, L289, Y292, L293, I296, F304 and I345, which are highly conserved in all GFRα receptors. The disulphide pattern of D2 is similar to that of D3.

D3 does not form any contacts to the ligand, in contrast to the speculations in Leppänen *et al.* (2004). The interaction between ARTN and GFRα3 occurs so that the finger tips of ARTN insert into the pocket created by the triangular spiral of α-helices. 16 residues from ARTN and 19 residues from GFRα3 form the interface, which buries a total surface area of about 1500 Å^2. ARTN-GFRα3 contact interface contains both apolar and polar residues that are conserved in GFLs and GFRα receptors (Wang *et al.*, 2006). The authors suggest that these residues serve as the common anchor points in all GFL-GFRα pairs, which are then surrounded by specificity determinants unique to each GFL-GFRα pair.

Based on previous studies of the RET-binding interface of GFRαs and conserved residues that are exposed on the surface of GFRαs, the authors propose that a surface of GFRα3 including residues from helices α2, α3, α7, α8, α9 and α10 (both from D2 and D3) forms part of the RET-binding surface. This RET-binding surface would be located adjacent to the two fingers in ARTN, which could form part of the composite RET interaction surface. However, these predictions have not been confirmed experimentally.

3.3   The structure of RET

In spite of several attempts, the complete crystal structure of RET has not been solved yet. However, the intracellular TK domain structure of both the non-phosphorylated and phosphorylated forms is available (Knowles *et al.*, 2006). According to a molecular modelling, mature RET comprises four cadherin-like domains (residues 29-516), a cysteine-rich domain (residues 517-635), a transmembrane domain (residues 636-657), a juxtamembrane domain (residues 658-723), a kinase domain (residues 724-1016), and a C-terminal tail (Anders *et al.*, 2001, Runeberg-Roos and Saarma, 2007) (see Figures 2 and 7B).

The domain structure of RET resembles that of other RTKs, but the extracellular domain is exceptional in that it consists of four cadherin-like domains (CLDs) (Anders *et al.*, 2001). Cadherins need calcium for their function: bound calcium ions linearise and rigidify the molecule, promote dimerisation and protect the cadherin from proteolytic degradation (Nagar *et al.*, 1996). Binding of calcium to RET between each cadherin-like domain may also induce linearisation and rigidification of the whole extracellular region of RET, and promote its dimerisation (Anders *et al.*, 2001). Calcium has indeed been shown to be important for the correct folding of RET (van Weering *et al.*, 1998) and its ligand-induced activation (Nozaki *et al.*, 1998). It has been suggested that the three first N-terminal cadherin-like domains of human RET contain an extended ligand binding surface and that the GFRα1 binding interface is located in the first N-terminal cadherin-like domain (Kjaer and Ibáñez, 2003). However, in another study, it was found that CLD4 and

cysteine-rich domain (CRD) are required for binding of RET to GFRα1/GDNF, and RET did not get cross-linked to GDNF in the absence of GFRα1 (Amoresano *et al*., 2005).

The cysteine-rich domain located next to the four cadherin-like domains consists of 117 amino acids of which 16 are cysteines. Of the total 28 cysteine residues that are present in the extracellular domain of RET, 27 are conserved in the human and mouse RET proteins (Takahashi *et al*., 1989), suggesting that most of these cysteine residues could be involved in the formation of intrachain disulfide bonds that contribute to the formation of the tertiary structure of the RET protein (Asai *et al*., 1995). In addition, the cysteine-rich domain has been suggested to participate in GFRα binding (Amoresano *et al*., 2005).

The transmembrane domain of RET has been suggested to be involved in non-covalent interactions between two RET molecules, which may contribute to keeping receptor molecules in the proximity of each other and allow RET homodimers to be formed (Kjaer *et al*., 2006). The intracellular juxtamembrane domain is located right between the cell membrane and the kinase domain. Thus it has not been expected to participate in RET signalling directly. However, one Tyr687 in this part of RET has been shown to get phosphorylated *in vitro* (Liu *et al*., 1996) and both this tyrosine and Ser696, which functions as a protein kinase A binding site, are involved in the modulation of RET kinase activity by cyclic adenosine-3',5'-monophosphate (cAMP) (Fukuda *et al*., 2002). By studying S697A knock-in mice it has been found that Ser697 (Ser696 in human RET) is required for the migration of enteric neural crest cells in mouse developing gut and a S697A

mutation leads to the absence of enteric nervous system in the distal colon (Asai *et al*., 2006).

Most of the intracellular part consists of the kinase domain, the structure of which has been recently solved (Knowles *et al*., 2006) (see also Figure 2). The RET kinase domain adopts a characteristic protein kinase fold consisting of a smaller N-lobe (residues 713-805) and a larger C-lobe (residues 812-1013), connected by a linker (residues 806-811). This domain is followed by a C-terminal tail, the length of which differs in different RET splice variants.

4. RET-dependent GFL-signalling

All GFLs signal through the receptor tyrosine kinase RET, which is activated only if the GFL binds a GFRα receptor. GDNF binds preferably to GFRα1, NRTN to GFRα2, ARTN to GFRα3 and PSPN to GFRα4. However, some cross-talk between the ligands and receptors can occur, although its significance *in vivo* is not clear (Airaksinen and Saarma 2002). Binding of GFL and GFRα to RET leads to phosphorylation of the intracellular tyrosine kinase domain of RET. Phosphorylated tyrosine residues then function as docking sites for various adapter proteins mentioned below, which in turn activate further signalling proteins belonging to cellular signalling cascades that regulate cell survival, differentiation, proliferation, migration, chemotaxis, branching morphogenesis, neurite outgrowth and synaptic plasticity.

4.1 Activity of the RET kinase domain

RET has 18 intracellular tyrosine residues that form putative phosphorylation sites. In

a study, in which *in vitro* phosphorylation of RET was followed by mass spectrometric analysis, it was found that tyrosines 806, 809, 900, 905, 981, 1062, 1090 and 1096 (in human RET) are putative RET autophosphorylation sites (Kawamoto *et al*., 2004). In addition, Liu *et al.* (1996) have indentified four additional *in vitro* tyrosine phosphorylation sites: Tyr687, Tyr826, Tyr1015 and Tyr1029. However, only five tyrosines that are located in the kinase domain and C-terminal tail of RET (Tyr905, Tyr981, Tyr1015, Tyr1062 and Tyr1096) have – with the aid of specific antibodies – been shown to be activated by ligand-stimulation (Salvatore *et al*., 2000, Tsui-Pierchala *et al*., 2002a, Coulpier *et al*., 2002, Encinas *et al*., 2004).

Traditionally, the activity of the kinase domain of RET is believed to depend on a transphosphorylation reaction between two adjacent RET molecules. The majority of the previously characterised non-phosphorylated RTK forms have low catalytic activity because of a suboptimal conformation of a so called activation (A)-loop that interferes with either the ATP- or substrate-binding structures of RTKs (reviewed in Schlessinger, 2003). Phosphorylation of the A-loop causes structural changes that relieve this autoinhibition of kinase activity.

Comparison of the crystal structure of the kinase domain of phosphorylated and non-phosphorylated RET reveals that the three-dimensional structure of the kinase domain of RET does not depend significantly on the A-loop phosphorylation state (Knowles *et al*., 2006). Moreover, enzyme kinetic data show that the tyrosine phosphorylated form of RET is only slightly more active than the non-phosphorylated form. The authors thus suggest that there could be some other inhibitory mechanism for the autoregulation of RET kinase activity (Knowles *et al*., 2006). However, in this study, RET was only phosphorylated on tyrosine residues, so the regulation may happen through serine/threonine phosphorylation.

4.2 Downstream signalling

Several signalling pathways are activated by GFL-stimulated RET (Figure 6). Of the phosphorylated tyrosines that form docking sites for adaptor proteins, phosphorylated Y1062 is a binding site for the largest variety of adaptor proteins. Phosphorylated Y1062 binds SHC (Asai *et al*., 1996, Arighi *et al*., 1997), FGF receptor substrate 2 (FRS2) (Kurokawa *et al*., 2001), insulin receptor substrate 1 (IRS1) (Melillo *et al*., 2001), Dok1/4/5/6 (Murakami *et al*., 2002, Grimm *et al*., 2001, Crowder *et al*., 2004), Enigma (Durick *et al*., 1998) and protein kinase C α (PKCα) (Andreozzi *et al*., 2003). Y1062 has been shown to be important for the transforming ability of mutant RET (Asai *et al*., 1996) and for self-renewal of spermatogonial stem cells and regulation of their differentiation (Jijiwa *et al*., 2008).

Of other phosphotyrosines that function as docking sites for adaptor proteins, phosphorylated Y905 is a binding site for GRB 7/10 (Pandey *et al*., 1995, Pandey *et al*., 1996), phosphorylated Y981 for Src (Encinas *et al*., 2004), phosphorylated Y1015 for phospholipase Cγ (PLCγ) (Borrello *et al*., 1996) and phosphorylated Y1096 for GRB2 (Alberti *et al*., 1998). Moreover, it has been shown that the oncogenic multiple endocrine neoplasia (MEN) 2A form of RET activates signal transducer and activator of transcription 3 (STAT3) via Y752 and Y928 (Schuringa *et al*., 2001).

In addition to phosphorylated tyrosines, the elevation of cAMP levels causes Ser696 phosphorylation. This serine phosphorylation promotes lamellipodia formation of neuronal cells (Fukuda *et al*., 2002), regulates the c-Jun N-terminal kinase (JNK) signalling pathway and controls the migration of enteric neural crest cells in the developing gut (Asai *et al*., 2006).

Upon ligand stimulation, at least two distinct protein complexes assemble on phosphorylated Y1062 via SHC. The recruitment of GRB2/Sos (son of sevenless) leads to activation of the RAS/ERK (rat sarcoma oncogene/ extracellular signal-regulated kinase) pathway, while the recruitment of GRB2/ GAB1 (GRB2 associated binding protein 1) leads to the activation of the PI3K (phosphatidylinositol-3-kinase)/AKT pathway, which has been shown to be involved in the formation of lamellipodia (van Weering *et al*., 1997) and cell survival (Maeda *et al*., 2004). The GRB2/ GAB1 complex can also assemble directly onto phosphorylated Tyr1096, offering an alternative route to PI3K activation by GDNF (Besset *et al*., 2000, Hayashi *et al*., 2000). Moreover, the JNK pathway is activated mainly through tyrosine 1062 (Chiariello *et al*., 1998, Hayashi *et al*., 2000).

The downstream signalling capacity of RET has been shown to be negatively regulated by Sprouty2 protein, which is a common regulator of downstream signalling initiated by RTKs (Chi *et al*., 2004, Ishida *et al*., 2007). In addition, RET interacts with protein phosphatases LAR (leukocyte common antigen-related protein, Qiao *et al*., 2001), Shp-1 and Shp-2
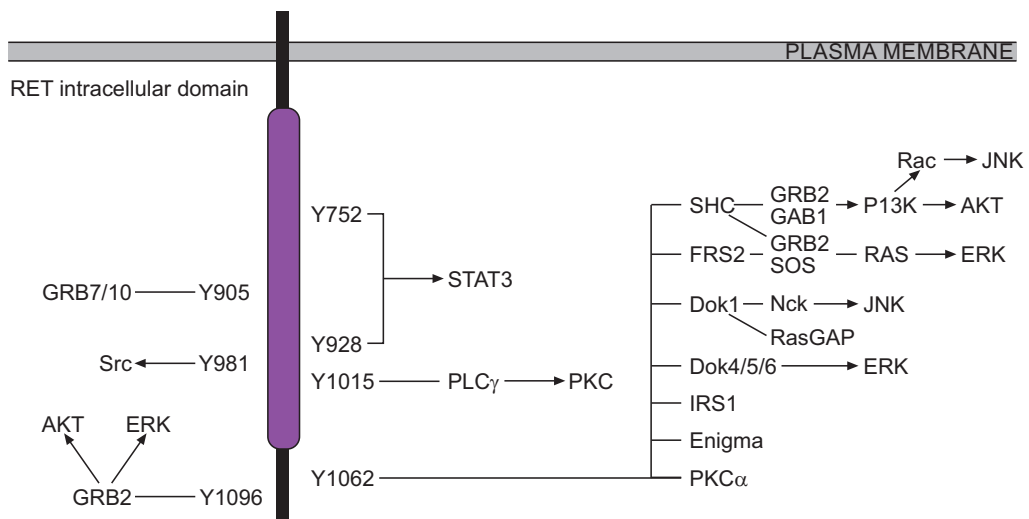

Figure 6. *Signalling pathways mediated by RET.* *Five tyrosines in the kinase domain of RET (purple) have been shown to get phosphorylated by ligand stimulation. In addition, the oncogenic MEN 2A form of RET activates STAT3 via Y752 and Y928. Phosphorylated Y1062 forms as a docking site for a large variety of proteins. It has been shown to bind SHC, FRS2, IRS1, Dok1/4/5/6, Enigma and PKCα, which activate RAS/ERK, GRB2/GAB1, JNK and PI3K/ AKT pathways. Phosphorylated Y905 is a binding site for GRB7/10, phosphorylated Y981 for Src, phosphorylated Y1015 for PLCγ, and phosphorylated Y1096 for GRB2. Modified from Kodama et al., 2005.*

(SH2-containing tyrosine phosphatase 1 and 2, Incoronato *et al.*, 2004), and PTPRJ (receptor-type protein tyrosine phosphatase J, Iervolino *et al.*, 2006). Both LAR and PTPRJ seem to reduce the constitutive tyrosine autophosphorylation and kinase activity of RET[MEN 2A], but not RET[MEN 2B] (Qiao *et al.*, 2001, Iervolino *et al.*, 2006).

Although many signalling pathways activated by RET have been identified, until quite recently, most of the information of the genes that are targeted by the signalling molecules activated by RET has concerned the oncogenic function of RET (Califano *et al.*, 1995, Carlomagno *et al.*, 1996, Watanabe *et al.*, 2002, Jain *et al.*, 2004). However, more information about the modulation of gene expression by normal GDNF/RET signalling is emerging. It is now known that GDNF elevates the expression of the tyrosine hydroxylase gene at both mRNA and protein levels in cells expressing RET (Xiao *et al.*, 2002). The expression of a zinc finger protein GZF1 (GDNF-inducible zinc finger gene 1) has been found to be induced during GDNF/RET signalling and it plays a role in renal branching morphogenesis (Fukuda *et al.*, 2003, Morinaga *et al.*, 2005). Very recently, it was reported that expression of the transcriptional repressor BMZF3 (bone marrow zinc finger 3) is also induced by GDNF (Suzuki *et al.*, 2008). The signalling pathways leading to these transcriptional modulations are, however, still unknown.

4.3   Signalling by different ligands

All members of the GDNF ligand family utilise RET as a signalling receptor and specificity is achieved by their binding to different GFRα molecules. It could be expected that they could activate RET

in different ways, for example through differential tyrosine phosphorylation, or differences in strength and duration of phosphorylation. However, it is still unclear whether GFLs differ in their signalling via RET.

Phosphorylation and dephosphorylation of the key tyrosine residues in RET (Y905, Y1015, Y1062 and Y1096) after stimulation with GFLs that utilise different GFRα receptors has been studied thoroughly, but no significant differences were found (Coulpier *et al.*, 2002). This observation suggests that the RET receptor is unable to discriminate among different ligands or GFRα co-receptors. In a later study, differences in the phosphorylation profile as well as cellular responses were found after GDNF and NRTN stimulation of cells that express only GFRα1 (Lee *et al.*, 2006), but further studies are needed to confirm these preliminary results. Even if no differences in RET signalling could be confirmed, possible differences in the biological activities of different GFLs could still be explained by the differential tissue-specific and developmentally regulated expression pattern of GFLs, and the existence of RET-independent signalling mechanisms.

4.4   Subcellular localisation of RET

In many cell types, RET is expressed as two forms – a 150 kDa form and a 170 kDa form – which differ in their subcellular localisation. Both forms of RET are produced from a single polypeptide of 120 kDa by posttranslational glycosylation (Takahashi *et al.*, 1991). Both 150 kDa and 170 kDa forms have tyrosine kinase activity (Takahashi *et al.*, 1993). The 150 kDa isoform is endoglycosidase H-sensitive, showing that it is an incompletely processed form of RET,

present in the endoplasmic reticulum (Asai *et al*., 1995). Cell fractionation experiments have shown that the 170 kDa isoform of RET is present at the plasma membrane, indicating that the 170 kDa protein represents the mature glycosylated form of RET (Takahashi *et al*., 1993).

It has been shown that single point mutations in the calcium-binding cadherin-like domains of RET, or calcium depletion, completely abolish cell surface expression of the mature form of RET (Asai *et al*., 1995, van Weering *et al*., 1998). Both seem to lead to improper processing of RET in the ER. Decreased expression of mature RET during embryonic development underlie the defects observed in Hirschsprung's disease (HSCR) patients (Schuchardt *et al*., 1995). Many mutations in the extracellular part of RET also decrease significantly the transforming activity of RET with MEN 2A mutations (Carlomagno *et al*., 1996, Iwashita *et al*., 1996).

Like other RTKs, RET can be downregulated after ligand-induced activation through targeted degradation of the receptor itself. However, whereas many other RTKs are degraded in the lysosome, degradation of RET happens predominantly by a proteasome-dependent way, which includes polyubiquitination of the receptor (Scott *et al*., 2005, Pierchala *et al*., 2006). Two molecules, Cbl-3 and CD2-associated protein (CD2AP) that are critical regulators of this RET downregulation have been found (Tsui and Pierchala, 2008). In some conditions, ubiquitinated RET has been found to colocalise with an early endosome and clathrin-coated vesicle marker after internalisation (Richardson *et al*, 2006). In addition to RET downregulation, RET internalisation is required for the complete activation of ERK1/2, but not for the

activation of AKT (Richardson *et al*., 2006). Consistent with this, it has been shown that normal endocytic trafficking of epidermal growth factor receptor (EGFR) is important for the full activation of ERK1 and 2 (Vieira *et al*., 1996).

Like the level of RET, also GFRα1 and GFRα2 levels on the cell surface decline fast after ligand stimulation. This suggests that either RET is internalised in a complex with GFRα or that GFRαs are released from the cell surface by cleavage of their GPI-anchor (Pierchala *et al*., 2006).

4.4.1 GFL signalling and lipid rafts

Lipid rafts are considered to be special cell membrane domains that are enriched in sphingolipids and cholesterol. They are believed to function as platforms for specific proteins (Simons and Ikonen 1997). It has been suggested that lipid rafts could have important functions in signal transduction, membrane trafficking, cell adhesion and migration, synaptic transmission and cytoskeletal organisation (Brown and London 1998, Simons and Toomre 2000, Harris and Siu 2002, Tsui-Pierchala *et al*., 2002c). GPI-anchored proteins have been shown to be sorted to lipid rafts and it has been suggested that they cluster on the rafts (Brown and Rose, 1992, Sharma *et al*., 2004). Many GPI-anchored proteins are co-receptors for transmembrane growth factor receptors and it has been proposed that they could bring the transmembrane protein into contact with raft-associated cytosolic signalling molecules like Src family kinases (Brown 1993, Simons and Toomre 2000). However, the detailed properties of lipid rafts are still unclear and even the existence of lipid rafts has been questioned (see Munro, 2003, for a review).

Lipid rafts have been proposed to be involved in GFL signalling in several ways. First, Poteryaev *et al.* (1999) reported that GDNF activates Src family kinases RET-independently through a GFRα1-mediated pathway and suggested that this would happen in lipid rafts where GFRα1 and Src cluster. Later, Tansey *et al.* (2000) found that GPI-anchored GFRα1 recruits RET to lipid rafts after GDNF stimulation. To determine the functional relevance of RET localisation to lipid rafts, the authors used an artificial transmembrane (TM) form of GFRα1 which is not localised on lipid rafts. GFRα1-TM could not recruit RET to lipid rafts and led to a significantly attenuated activation of AKT and mitogen-activated protein (MAP) kinases compared to cells expressing wild-type GFRα1. This led to a decrease in the ability of GDNF to cause differentiation and neuronal survival. However, as discussed later (**I, II**), there are some severe technical problems in the experiments with GFRα1-TM, and the conclusions have partly been contradicted later by Paratcha *et al.* (2001).

As mentioned earlier, GFRαs can bind ligand and activate RET when provided exogenously in soluble form. Therefore, it has been suggested that GFRαs could capture and concentrate diffusible GDNF family ligands from the extracellular space, and then present these factors from the surface of one cell to afferent RET-expressing cells (Trupp *et al.*, 1997). It has been shown that soluble GFRα1 mediates recruitment of RET to lipid rafts via a mechanism that requires the kinase domain activity of RET itself (Paratcha *et al.*, 2001). In the same study, it was also shown that activated RET associates with different adaptor proteins in membrane parts with different degree of ordering.

In addition, it has been shown that RET localisation to lipid rafts enhances its association with Src and that Src activity is necessary to elicit optimal GDNF-mediated signalling, neurite outgrowth, and survival (Tansey *et al.*, 2000, Encinas *et al.*, 2001). Therefore, it has been thought that recruitment of RET to lipid rafts may be a critical determinant of RET signalling efficiency. RET in lipid rafts is not degraded by the proteasome, and thus lipid rafts appear to cluster and protect RET from degradation. The authors of this study therefore suggest that one function of lipid rafts may be to sequester active receptors from downregulation (Pierchala *et al.*, 2006).

4.5 Cross-talk with other pathways

It has been found that receptor tyrosine kinases like EGFR and Trk-type tyrosine kinases can be activated by different stimuli even in the absence of their ligands (Carpenter *et al.*, 1999, Lee and Chao 2001). One of these activation mechanisms depends on G protein-coupled receptor (GPCR) activation. This kind of cross-talk with another signalling pathway is believed to be common for receptor tyrosine kinases. Tsui-Pierchala *et al.* (2002b) postulate three possible advantages of cross-talk between RTKs in the nervous system. First, they suggest that, through cross-talk, a growth factor can activate signalling pathways that cannot be activated through its own RTK and thus expand the biological activity of the factor. Second, activation of multiple RTKs by a single growth factor may amplify the signal, and third, prolong the activity of a signalling pathway.

Cross-talk of GDNF-RET signalling with other signalling pathways has been found on three levels: First, cross-talk can occur through direct interactions between RET and another signal mediating

protein. An example of this is the cross-talk between RET and EGFR. It has been shown very recently that EGFR can form a complex with RET and contribute to RET kinase activation, signalling, and growth stimulation (Croyle *et al.*, 2008). In addition, RET binds directly at least PKCα, and activates PKC α, δ and ζ. PKCα activation then inhibits RET kinase activity by promoting RET phosphorylation on serine/threonine. This modulation forms a negative feedback loop that controls RET signalling (Andreozzi *et al.*, 2003).

Another mechanism for cross-talk is modulation of protein amounts by regulation of transcription, or degradation of the proteins participating in the cross-talk. A well-studied cross-talk of this kind happens between RET and NGF/TrkA. It has been shown that TrkA activation by NGF promotes RET phosphorylation in a GFL-independent manner in mature sympathetic neurons (Tsui-Pierchala *et al.*, 2002b). However, NGF-induced RET phosphorylation both occurs and decreases considerably more slowly than GFL-induced RET phosphorylation. The cross-talk seems to be unidirectional, since GDNF does not promote TrkA phosphorylation. The mechanism by which the cross-talk happens has been found recently: NGF inhibits the ubiquitin-dependent degradation of RET that clears activated RET from the cell surface and thus increases the amount of phosphorylated RET (Pierchala *et al.*, 2007).

RET phosphorylation has been shown to be regulated by a cAMP-dependent protein kinase A (Fukuda *et al.*, 2002, Asai *et al.*, 2006). This observation suggests that RET activity could be regulated by GPCRs. There is indeed evidence that endothelin-3 (ET-3), which functions through endothelin receptor type B (EDNRB), a G-protein coupled receptor, modulates the action of GDNF (Hearn *et al.*, 1998). These results were obtained by testing the growth, survival, migration, or neurogenesis in response to GDNF and ET-3 in a culture of precursor cells of the enteric nervous system, and the mechanism of this cross-talk remains unknown. It is possible that this cross-talk happens on a transcriptional level and affects the expression levels of the proteins of the RET signalling complex.

The third level of cross-talk occurs through modulation of localisation. It has been shown that the neurotrophic effect of GDNF both *in vitro* and *in vivo* requires the presence of TGF-β (Krieglstein *et al.*, 1998). In a later study, it was found that TGF-β neither upregulates GFRα1 and RET genes, nor participates in the direct activation of RET. Instead, it seems to recruit GFRα1 molecules to the plasma membrane. It was also shown that the presence of soluble GFRα1 replaces TGF-β, supporting the results (Peterziel *et al.*, 2002).

5. The GFL/GFRα/RET signalling pathway and human diseases

5.1 Gain-of-function mutations in RET

RET is expressed in many human tumours of neural crest origin (Santoro *et al.*, 1990). Specific autosomal dominant gain-of-function mutations in RET cause medullary thyroid carcinoma (MTC), a tumour of the neural crest-derived parafollicular C cells responsible for the production of calcitonin. MTC can be found in three hereditary cancer syndromes: multiple endocrine neoplasia (MEN) type 2A and 2B, and familial medullary thyroid carcinoma (FMTC).

MTC is also the most common cause of death in patients with MEN 2A, MEN 2B and FMTC (Skinner *et al*., 2005). The disease phenotypes correlate with the location of the mutation of RET (Figure 7B). The most common mutations in MEN 2A and FMTC are located in the cysteine-rich domain, but FMTC mutations can also be found in the tyrosine kinase domain. Mutations in MEN 2B are found exclusively in the tyrosine kinase domain (Mulligan *et al*., 1995, Eng *et al*., 1996). Recently, it has also been suggested that mutations of GFRα4 contribute to MEN 2 in the absence of RET mutations or modify the RET mutation phenotype (Vanhorne *et al*., 2005).

MEN 2A is the most common of these cancer syndromes and it is characterised by MTC, phaeochromocytoma (PC), and hyperparathyroidism (HPT). About 50 % of patients develop phaeochromocytomas, while only 15-30 % of patients develop HPT or parathyroid adenomas (Howe *et al*., 1993, Hansford and Mulligan, 2000). MEN 2A is associated most frequently with RET mutations of cysteine 634, but also of 609, 611, 618 and 620 (Mulligan and Ponder, 1995). These substitutions leave one cysteine in the cysteine-rich domain of RET without a pair, in which case it is not able to form a normal intramolecular cysteine bridge. Abnormal cysteine bridges are then thought to form between two RET proteins, leading to dimerisation and constant activation (Santoro *et al*., 1995) (Figure 7A). Since folding of proteins takes place in the ER, it can be thought that the MEN 2A mutants of RET dimerise and are activated already during their synthesis in the ER, before they reach the cell surface.

Also MEN 2B patients suffer from MTC, and about half of them develop PC. However, HPT is rare in MEN 2B.

MEN 2B is further characterised by developmental abnormalities including marfanoid habitus, thickened corneal nerves, and ganglioneuromatosis of the buccal membranes and in the gastrointestinal tract. MEN 2B is considered to be the most aggressive of the MEN 2 subtypes, and its symptoms begin on average 10 years earlier than in MEN 2A (reviewed in Hansford and Mulligan, 2000). The most frequent RET mutation in MEN 2B is a single mutation M918T, which is found in more than 90 % of cases (Eng *et al*., 1994, Hofstra *et al*., 1994). In addition, mutations in residue 883 have been reported (Smith *et al*., 1997). Both amino acids 883 and 918 are located in the kinase domain of RET, within the substrate binding pocket of RET, and their mutations are believed to result in altered substrate specificity of the kinase domain (Songyang *et al*., 1995, Santoro *et al*., 1995) (Figure 7A). As with MEN 2A, oncogenic MEN 2B forms of RET can be expected to be activated during their synthesis already in the ER (see also Results and discussion, 3).

The third subtype of MEN 2, FMTC, is characterised by MTC in four or more family members as its only disease phenotype. FMTC is generally considered the least aggressive of the three cancer syndromes with a later onset than MEN 2A or 2B (Hansford and Mulligan, 2000). The mutations in RET are often the same as in MEN 2A: mutations of cysteine residues 609, 611, 618, 620 and 634 are found in more than 80 % of FMTC families (Mulligan *et al*., 1995). However, these mutations are all pretty common and there is no emphasis on one of the residues. The most common mutation of MEN 2A, C634R is not found in FMTC. In some cases, MEN 2A might be difficult to distinguish from FMTC, if the family
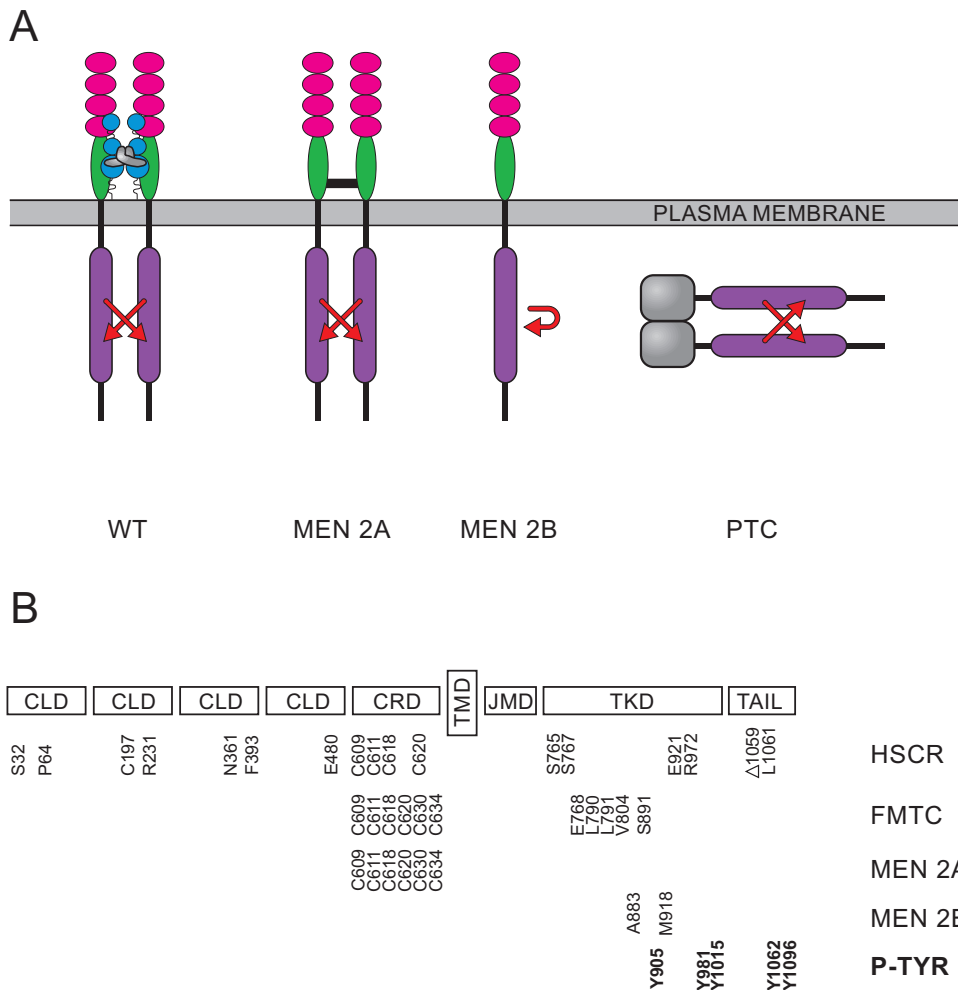
Figure 7. Schematic representation of RET and its oncogenic variants. A) Wild-type RET is phosphorylated on the cell surface in the presence of GFLs and GFRα receptors. The kinase domains of the dimerised RET molecules phosphorylate each other. The dimeric GFL is shown in grey and GFRα receptors in blue. The cadherin-like domains of RET are red, the cysteine-rich domain is green and the kinase domain purple. The most common multiple endocrine neoplasia type 2A (MEN 2A) mutations are located in the cysteine-rich domain of RET. The MEN 2A variants are activated through the formation of abnormal covalent disulphide bridges (the black bar) between the extracellular domains of two RET molecules. The MEN 2B mutations are located in the intracellular kinase domain and affect the kinase activity. MEN 2B mutants may activate signalling cascades either as monomers or as non-covalently associated dimers. The papillary thyroid carcinoma (PTC) variants are cytosolic, and the activation of their tyrosine kinase domains is driven by the dimerisation of an N-terminally fused unrelated protein (grey). B) The extracellular part of RET consists of four cadherin-like domains (CLD) and a cysteine-rich domain (CRD). The transmembrane domain (TMD) connects the extracellular and intracellular parts. The intracellular part consists of a juxtamembrane domain (JMD), a tyrosine kinase domain (TKD) and a C-terminal tail. The most common mutations linked with HSCR, FMTC, MEN 2A and MEN 2B are listed under RET. In addition, the positions of phosphorylated tyrosines are shown on the lowest row. Modified from Runeberg-Roos and Saarma, 2007.

is small, and PC and HPT develop late (Hansford and Mulligan, 2000).

Another cancer type caused often by mutations in RET is papillary thyroid carcinoma (PTC). In the thyroid gland, RET is highly expressed in parafollicular C-cells but not in follicular cells, where it can be activated by chromosomal rearrangement. In this rearrangement, the kinase domain encoding part of the RET gene is fused to a dimerising domain, encoding part of different unrelated genes (Grieco *et al*., 1990). The fusion usually causes formation of a RET/PTC, in which the non-RET part tends to dimerise thereby spontaneously forming cytoplasmic dimers (Figure 7A). As the tyrosine kinase domain of the RET receptor is left intact, the RET/PTC oncoprotein can bind adaptor molecules and activate signalling cascades in the cytoplasm (Knauf *et al*., 2003). RET/PTC is found on average in about 20 % of adult sporadic papillary carcinomas and in general RET/PTC incidence is high in tumours from patients with a history of radiation exposure (Nikiforov, 2008).

5.2 Loss-of-function mutations in RET

During embryogenesis, RET is expressed in many cell types derived from the neural crest. Among these are the vagal neural crest and the myenteric ganglia cells, which colonise the entire gut (Pachnis *et al*., 1993). Hirschsprung's disease (HSCR) is a congenital disorder that occurs in 1/5000 live births. It is characterised by the absence of enteric ganglia along a variable length of the intestine, which leads to intestinal obstruction or chronic constipation (see Amiel and Lyonnet, 2001, for a review).

About 20 % of HSCR cases are familial ones, with considerable genetic diversity. Studies of the genetic bases of HSCR have identified several genes linked to the disease, including RET, GDNF, NRTN, ET-3, EDNRB, and the transcription factors Sox10 and SMAD-interacting protein-1 (SIP1) (Luo *et al*., 1993, Romeo *et al*., 1994, Ivanchuk *et al*., 1996, Doray *et al*., 1998, Parisi and Kapur, 2000). Heterozygous germline mutations in the RET gene are associated with HSCR in up to 50 % of familial cases and in 15 % of sporadic cases (Hofstra *et al*., 2000). These mutations include deletions, insertions, missense, nonsense, and frameshift mutations and occur throughout the RET gene (Edery *et al*., 1994, Sancandi *et al*., 2000). The majority of these mutations causes either a reduction in the amount of RET or the loss of RET function (Iwashita *et al*., 2001).

Functional consequences of HSCR mutations correlate with their position in the coding sequence. Mutations in the extracellular domain interfere with RET maturation and impair its translocation to the plasma membrane (Kjaer and Ibáñez, 2003). Mutations in the kinase domain may either reduce the catalytic activity, or effect the binding of adaptor molecules to activated RET (Iwashita *et al*., 2001, Geneste *et al*., 1999). Some mutations in the extracellular cysteines that cause HSCR have also been identified in MEN 2A and FMTC, and MEN 2A/FMTC and HSCR co-segregate in a fraction of families (Mulligan *et al*., 1994). This is proposed to result from the dual effect of the mutation to RET: due to the mutation in an extracellular cysteine, the folding of RET may not happen normally. The impaired maturation of the mutant RET leads to a reduced RET level on the cell surface, and this level may not be sufficient for keeping the enteric neurons alive. On the other hand, intermolecular disulphide

bridge formation and the resulting dimerisation of mutant RET molecules causes the constitutive activation of RET, which can be sufficient in some tissues to cause abnormal proliferation and lead to the formation of tumours (Takahashi *et al.*, 1999).

Some GDNF mutations that contribute to HSCR have been reported (Ivanchuk *et al.*, 1996). However, it seems that these mutations alone do not lead to HSCR, but together with other mutations, for example in the RET gene, contribute to the pathogenesis of the disease (Salomon *et al.*, 1996, Eketjäll and Ibáñez, 2002). Also the reported mutation in NRTN contributing to HSCR is not sufficient to cause the disease (Doray *et al.*, 1998). The study, which evaluated the link between different polymorphisms in GDNF, NRTN, ARTN and PSPN, and susceptibility to HSCR, failed to find any disease-contributing mutations in ARTN or PSPN (Fernandez *et al.*, 2008). Mutations in GFRα receptors do not seem to be important contributors to HSCR (Borrego *et al.*, 2003), but abnormal expression of GFRαs in the enteric nervous system of some patients may be involved in the pathogenesis of HSCR (Lui *et al.*, 2002).

5.3 Parkinson's disease

PD is a neurodegenerative disorder that is, according to the classical view, characterised by the progressive degeneration of the nigrostriatal dopaminergic pathway, resulting in the loss of dopamine (DA) in the basal ganglia. However, new studies postulate that PD may progress in six neuropathological stages, during which different neuron populations are affected (Braak *et al.*, 2004). Lewy bodies, abnormal aggregates of proteins consisting mainly of misfolded

α-synuclein (Spillantini *et al.*, 1997), begin to form at defined induction sites and their formation advances to certain cell types in a known order. α-Synuclein exists in many neurons of the human nervous system and in order to become involved in PD, neurons have to express sufficient levels of normal α-synuclein (Braak *et al.* 2000).

The first two stages of PD are presymptomatic, and during this period, inclusion body pathology is confined to the medulla oblongata/pontine tegmentum and olfactory bulb/anterior olfactory nucleus. At stages 3-4, during which the substantia nigra and other nuclear grays of the midbrain and forebrain become the focus of pathological changes, most individuals probably move from the presymptomatic phase to the symptomatic phase of the illness (Braak *et al.*, 2003). In the final stages 5-6, the process enters the mature neocortex, and the patients manifest the full range of PD-associated clinical symptoms (Braak *et al.*, 2004).

The locomotor symptoms of PD arise from selective loss of dopamine neurons in the substantia nigra pars compacta. These neurons project to the putamen and the caudate (the striatum) where they release dopamine. A loss of greater than 50-60 % of these neurons is required for the manifestation of the symptoms, which include resting tremor, akinesia, rigidity and bradykinesia (Sian *et al.*, 1999). Most symptoms of PD can usually be effectively treated with L-dopa, but it does not attenuate neuronal degeneration. Moreover, PD is progressive and the majority of patients show a gradual loss of L-dopa efficacy (Lewitt 2008).

GDNF signalling is important in the survival of both dopamine neurons and motor neurons *in vitro* (Lin *et al.*, 1993, Henderson *et al.*, 1994). GDNF-, GFRα1- and RET-deficient mice do not have

significant differences in the substantia nigra area compared to wild-type mice (Schuchardt *et al.*, 1994, Moore *et al.*, 1996, Pichel *et al.*, 1996, Sánchez *et al.* 1996, Cacalano *et al.*, 1998, Enomoto *et al.*, 1998). However, as these knock-out mice die at birth, it has been difficult to investigate whether GDNF signalling is required for the postnatal survival of dopaminergic neurons. Granholm *et al.* (2000) avoided this problem by transplanting fetal neural tissues from GDNF -/-, GDNF +/-, and wild-type (WT) mice into the brain of adult wild-type mice and showed that survival of ventral mesencephalic DA neurons is dependent on GDNF.

In another study, mice with regionally selective RET ablations that allow the postnatal survival were used to investigate the significance of GDNF-RET signalling in adult mice nervous system (Kramer *et al.*, 2007). It was found that deficiency of RET causes progressive and late loss of DA neurons in the substantia nigra pars compacta, degeneration of DA nerve terminals in striatum and reduced levels of evoked dopamine release. Aged mice lacking RET showed a phenotype similar to presymptomatic PD. Thus the results suggest that RET is an important signalling receptor for the maintenance of adult nigrostriatal DA system.

A study (Kowsky *et al.*, 2007), in which mice from Kramer *et al.* (2007) were used, demonstrated that RET signalling does not promote the survival of dopaminergic neurons in the MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) model of Parkinson's disease, but it facilitates the regeneration of dopaminergic axon terminals. Pascual *et al.* (2008) reported that their conditional GDNF knock-out mice showed a severe catecholaminergic cell death that affected

the locus coeruleus, the substantia nigra and the ventral tegmental area. These results are in line with the observation that the constitutive activity of RET in knock-in MEN 2B mice increases the number of dopaminergic neurons in the substantia nigra pars compacta (Mijatovic *et al.*, 2007).

On the other hand, the studies of Jain *et al.* (2006) on RET conditional mice contradict these results. Their results indicate that RET is not required for survival of midbrain dopaminergic neurons in adult mice and RET deficiency in these neurons does not cause major sensorimotor abnormalities. However, the differences in the results might be explained by the fact that Kramer *et al.* (2007) followed their mice for a significantly longer time than Jain *et al.* (2006).

Genetic variation of RET does not seem to cause reduced GDNF-RET signalling in PD as Lücking *et al.* (2008) could not find any association between RET polymorphisms and PD. The authors suggest that GDNF-RET signalling could be disturbed on some other level than genetic. In conclusion, GDNF-RET signalling seems to be important in aging PD model mice, but the connection between GDNF-RET signalling and PD in humans has not been confirmed yet. In addition, no association between HSCR and PD has been found (Lücking *et al.*, 2008).

6. Therapeutic use of GFLs

Since their identification as neurotrophic factors, GFLs have been considered as potential therapeutic agents for treatment of neurological diseases. Because of its potential in the treatment of PD, GDNF has drawn most attention. However, lately also NRTN has been shown to

have similar potential. In addition, it has been found that ARTN reverses nerve injury-induced pain behaviour and has therapeutic potential (Gardell *et al*., 2003, Sah *et al*., 2005). PSPN may have clinical applications in the treatment of stroke (Tomac *et al.*, 2002).

There have been numerous *in vivo* studies investigating the therapeutic potential of GDNF. These studies have primarily focused on the rodent partial lesion model of PD, where the lesion is usually caused with 6-hydroxydopamine (6-OHDA) and on mouse and the non-human primate model, where 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) is commonly used (Hong *et al*., 2008).

Many studies have shown that GDNF injections into the striatum before or after 6-OHDA-administration protects nigral dopaminergic cells that would otherwise undergo cell death (Sauer *et al*., 1995, Kearns and Gash, 1995, Shults *et al*., 1996, Rosenblad *et al*., 1998). The neuroprotective effects of GDNF seem to result from a reduction in oxidative stress (Smith and Cass, 2007). Studies in the non-human primate model of PD also support the findings of the neuroprotective effects of GDNF (Gash *et al*., 1996, Zhang *et al*., 1997, Oiwa *et al*., 2006). However, it has been shown that there is a need for continuous GDNF infusion to maintain dopaminotrophic effects (Zhang *et al*., 1997). Thus, alternative techniques like gene therapy with the use of various viral vector systems have been considered and investigated. Viral delivery of GDNF into the brain has been shown to produce beneficial anatomical and functional effects (Lawlor and During, 2004). Moreover, lately many pharmacological agents, that have proven useful in the symptomatic treatment of PD, have been recognised as

modulators of GDNF expression, as well of other neurotrophic factors (reviewed in Saavedra *et al*., 2008).

Clinical trials in patients with PD have given conflicting results. Transplantation of GDNF-treated fetal ventral mesencephalon cells increased the survival of dopaminergic cells and improved the condition of the patients significantly (Mendez *et al*., 2000). In an open-label study by Gill *et al*. (2003), GDNF was administered via a catheter into the putamen, and it was found that the dopamine uptake of cells in the putamen increased and PD symptoms improved significantly in all five patients. Also Slevin *et al*. (2005) administered GDNF into the putamen and found considerable improvement in the patients. However, in a study, in which GDNF was administered via a catheter into the right lateral ventricle of a patient, there was no evidence of regeneration of endogenous dopaminergic nigrostriatal neurons, and the symptoms continued to worsen (Kordower *et al*., 1999). In a randomised, double-blind study of Nutt *et al*. (2003), GDNF was administered through an implanted intracerebroventricular catheter. Also in this study, GDNF did not seem to have any impact in the symptoms. The authors note, however, that the reason for this may be that GDNF never reached the target tissues (putamen and substantia nigra). In addition, the latest randomised double-blind study (Lang *et al*., 2006), where GDNF was administered into the putamen of PD patients, showed no significant improvement in symptoms. Moreover, several adverse effects including nausea, loss of appetite, hallucinations and depression have been found in some studies (Nutt *et al*., 2003).

The reason for very conflicting results is probably the differences in the administration methods used: Salvatore

et al. (2006) have tested the infusion protocol of the phase 2 clinical trial that failed in showing any impact for GDNF (Lang *et al*., 2006), and their results show that the catheter used did not distribute GDNF efficiently, and the bioavailability of GDNF was limited to a small portion of the human putamen. A problem in the delivery of both GDNF and NRTN into the brain is that their diffusion in the target tissue is very limited. This seems to be caused by the binding of these ligands to heparin in the extracellular matrix, and heparin can be used to improve the distribution (Hamilton *et al*., 2001).

In addition to GDNF, NRTN has been shown to enhance survival of dopaminergic neurons in rodent and monkey models of PD (Horger *et al*., 1998, Rosenblad *et al*., 1999, Oiwa *et al*., 2002, Li *et al*., 2003). Quite recently, Fjord-Larsen *et al*. (2005) also reported about a successful lentiviral gene transfer of a modified NRTN, which protected nigral dopaminergic neurons in rats. A phase I clinical trial by Ceregene showed promising results (Peterson and Nutt, 2008), but the very recent phase II clinical trial of NRTN for PD failed to demonstrate an appreciable difference between patients treated with an adeno-associated virus (AAV) vector carrying the gene for NRTN versus those in the control group (Ceregene Press release 26.11.2008).

AIMS OF THE STUDY

This study aimed at characterising the biochemical and cell biological function as well as the structure of components of the GFL signalling system. It had been found that the mammalian GFRα1 and GFRα4 receptors differ in that GFRα4 lacks the N-terminal domain 1, which is present in all other GFRα receptors. Before this study, the significance of domain 1 was unclear. The biochemical and cell biological properties of mouse GFRα4 were not very well characterised, and it was not known how the difference in the domain structure of GFRα1 and GFRα4 affects their function. Detailed structural information of the interactions of GDNF and GFRα1 was also not available. In addition, the activity of RET which functions as the signal mediating component of the complex had not been studied during its synthesis in the endoplasmic reticulum.

The specific aims of the study were to:

- Characterise the biochemical and cell biological features of the mouse GFRα4 receptor and to compare these characteristics to those of GFRα1.

- Study the function and significance of domain 1 in GFRα1.

- Set up methods and use them to study the activation of RET precursors in the endoplasmic reticulum.

- Determine the structure of GFRα1 in the complex with GDNF and to verify this structure with biochemical experiments. Particularly, we were interested in studying the interactions between GFRα1, RET and GDNF.

MATERIALS AND METHODS

Methods that I have used myself are presented here in detail. Methods that I have not used or in which my contribution has been minor are listed in Table 1 with a reference to the publication in which they have been used and described.

1. RET phosphorylation assays (used in I-IV)

1.1 With soluble GFRα1 receptors

RET phosphorylation assays with soluble GFRα1 receptors were done in two different cell lines. PC6-3 cell line that expresses low amount of RET, but does not express GFRα1 was used in **II**. MG87RET cell line which is stably transfected with RET long isoform, but does not express GFRα1 was used in **II** and **IV**. Cells were starved for 4 h in serum-free DMEM at +37 °C and subsequently stimulated with 0-1000 ng/ml of the soluble GFRα1 proteins and 100 ng/ml of GDNF for 60 min at +37 °C. Cells were lysed in the lysis buffer containing TBS, 10 % glycerol, 1 % Triton X-100, 1 % Nonidet P-40, 2 mM EDTA, 1 mM Na_3VO_4 and Complete Mini EDTA-free (Roche GmbH, Germany). The nuclei were removed by centrifugation (2500 g) and antibodies to RET (Ret C-20, Santa Cruz Biotechnology, USA) were added to the post-nuclear lysates.

In **II,** immunocomplexes were collected with protein G sepharose (Amersham Biosciences, UK) and analysed by Western blotting with phosphotyrosine antibodies (anti-phosphotyrosine 4G10, Upstate Biotechnology Millipore, USA). Membranes were restained with antibodies to RET to ensure that equal amounts of RET were present. In **IV,** lysates were used in pRET ELISA (enzyme-linked

immunosorbent assay) assays (described later). The assays were repeated at least three times with similar results.

1.2 With GPI-anchored GFRα1 receptors

In RET phosphorylation assays with GPI-anchored GFRα1 receptors, two different cell lines were used: MG87RET cells (**II)** and PC6-3 cells (**II, III**). MG87RET cells were transfected with GFRα1 constructs and PC6-3 cells were transfected with GFRα1 and RET constructs. Transfections were done with Lipofectamine 2000 (Invitrogen, USA).

After transfection, cells were starved at least for 4 h in serum-free DMEM at +37 °C and subsequently stimulated with 100 ng/ml of GDNF (PeproTech UK) for 60 min at +37 °C. Cells were lysed with the lysis buffer described above. The nuclei were removed by centrifugation (2500 g) and antibodies to RET (Ret C-20, Santa Cruz Biotechnology) were added to the post-nuclear lysates. Immunocomplexes were collected with protein G sepharose (Amersham Biosciences) and analysed by Western blotting with phosphotyrosine antibodies (anti-phosphotyrosine 4G10, Upstate Biotechnology). Membranes were restained with antibodies to RET to ensure that equal amounts of RET were present. The assays were repeated at least three times with similar results.

2. Neurite outgrowth assays (I-II)

2.1 With soluble GFRα1 receptors

PC6-3 cells were transferred to RPMI medium containing 1 % horse serum

and 1 % penicillin/streptomycin (Gibco, Invitrogen, USA) on plates coated with collagen (Becton Dickinson, USA). Soluble GFRα1 receptors (1000 ng/ml) and GDNF (100 ng/ml) or NGF (50 ng/ml, Promega, USA) were added and neurites were counted after 3-4 days from living cells under the microscope. Only those neurites that were longer than the cell soma were counted. 200-300 cells were counted per sample and the experiments were repeated three times with different batches of purified GFRα1 proteins.

2.2 With GPI-anchored GFRα1 receptors

PC6-3 cells on collagen-coated plates were transiently transfected with constructs encoding GFRα1, GFRα4 and RET, and transferred to RPMI medium containing 5 % horse serum, 2.5 % fetal calf serum and 1 % penicillin/streptomycin. GDNF or PSPN (100 ng/ml) were added and neurites two times longer than the cell soma were counted after 4-5 days as described above.

3. Co-immunoprecipitation of SHC/RET and GRB2/RET (III)

PC6-3 cells were transiently transfected (6 h) with constructs encoding RET and GFRα1. In one assay, cells were stimulated for 10 min with GDNF (100 ng/ml). Cells were lysed with the lysis buffer described above. The nuclei were removed by centrifugation (2500 g). Each sample was divided into two parallel parts before the immunoprecipitation. From one part, SHC-associated proteins were co-immunoprecipitated (IP) with antibodies to SHC (#610081, Transduction Laboratories, USA). The SHC-immunoprecipitated samples were analysed by Western

blotting with antibodies to RET and SHC. From the other part of the sample RET was precipitated with Ret C-20 antibody (Santa Cruz Biotechnology). Samples were analysed by Western blotting with antibodies to phosphotyrosine (4G10, Upstate Biotechnology) and RET. GRB2/RET co-immunoprecipitation was performed similarly but instead of antibodies to SHC, antibodies to GRB2 (C-7, Santa Cruz Biotechnology) were used, and the transfection time was increased to 10 h.

In another assay cells were stimulated for 10 min with GDNF (100 ng/ml) in the presence of Brefeldin A (5 µg/ml, Epicentre Biotechnologies, USA) and the samples were lysed with lysis buffer described above. RET was immunoprecipitated from the samples and the precipitates were analysed by Western blotting with antibodies to SHC. The membrane was restained with antibodies to RET. The assays were repeated at least three times with similar results.

4. AKT phosphorylation assay in the presence of Brefeldin A (III)

AKT phosphorylation assay was done in PC6-3 cells which were transiently transfected with RET constructs both in the absence and presence of Brefeldin A. Cells were transfected with Lipofectamine 2000 (Invitrogen) or MATra-A reagent (IBA GmbH, Göttingen, Germany). For MATra-A transfection the transfection procedure was modified from the manufacturer's instructions: higher concentrations of beads and shorter incubation time worked better for PC6-3 cells. After an 8 h expression, cells were lysed and the lysates were divided into two parts. One part was used as a total lysate

for Western blot analysis of phospho-AKT (#9271, Cell Signaling Technology, USA). The Western blot membrane was then reprobed with antibodies to AKT (#9272, Cell Signaling Technology). The other part was used to check the levels of total RET and phosphorylated RET in the samples. RET was immunoprecipitated and the precipitate was analysed by Western blot analysis with antibodies to RET (Ret C-20, Santa Cruz Biotechnology) and phosphotyrosine residues (anti-phosphotyrosine 4G10, Upstate Biotechnology). The assays were repeated at least three times with similar results.

5.   AKT, ERK and STAT3 phosphorylation assays with ER-retained RET (III)

PC6-3 cells were transiently transfected with RET constructs. Western blot analyses were carried out from total lysates. The antibodies to ERK (K-23), P-ERK (E-4) and STAT3 (C-20) were from Santa Cruz Biotechnology. The antibodies to AKT and P-STAT3 (#9131) were from Cell Signaling Technology. The assays were repeated at least three times with similar results.

6.   Phospho-RET ELISA assays (IV)

The cleared lysates from RET phosphorylation assays (described above) were applied on a 96 well plate (OptiPlate 96 F HB, Black, Wallac), which had been previously coated with 0.5 µg/ml of RET C-20 antibody (Santa Cruz Biotechnology) and blocked with 2 % BSA in TBS, and the plate was incubated in +4 °C for 1 h. Phosphorylated RET was detected by anti-phosphotyrosine (4G10, Upstate

Biotechnology, 1:1000), anti-mouse HRP (DAKO A/S, 1:3000) and enhanced chemiluminesence reaction (Femto ELISA ECL Kit, Pierce). All washes between the incubations were done with the same washing buffer (TBS, 1 % Triton X-100). The signal was detected by counting on MicroBeta luminometer (PerkinElmer). The assays were repeated at least three times with similar results.

7.   Other methods

Table 1. List of methods used by co-authors in articles I-IV.

Method	Used and explained in
Creation of GPI-anchored GFRα constructs	*I, II*
RET phosphorylation assay with GPI-anchored GFRα4	*I*
Generation of stable cell lines expressing GFRα4	*I*
Glycosylation assays	*I*
Membrane association assays	*I*
Neuronal survival assay	*I*
Binding and cross-linking of PSPN to mouse GFRα4	*I*
Expression and purification of soluble GFRα1 variants and RET^{ED}	*II*
Characterisation of purified soluble GFRα1 variants	*II*
GDNF binding to GPI-anchored GFRα1 variants	*II*
Scintillation proximity assays	*II*
Expression and purification of the GDNF$_2$-GFRα1$_2$ complex	*IV*
Site-directed mutagenesis of GFRα1	*IV*
MAP kinase activity assays	*IV*
Crystallography	*IV*

RESULTS AND DISCUSSION

1. Functional characteristics of the mouse GFRα4-GPI receptor (I)

In this work we characterised the mouse GFRα4 receptor biochemically and cell-biologically. Previously, it had been shown that in the presence of GDNF, GFRα1 recruits RET to lipid rafts, and it was suggested that this recruitment is essential for GDNF-induced downstream signalling, differentiation and neuronal survival (Tansey *et al.*, 2000). PSPN had been found to promote the survival of sympathetic neurons, microinjected with GFRα4 and RET (Lindahl *et al.*, 2001), but the biochemical and cell biological features of mouse GFRα4 had not been thoroughly characterised. In addition, nothing was known about the capacity of GFRα4 to recruit RET to lipid rafts in the presence of PSPN.

1.1 Biochemical and functional characterisation of the mouse GFRα4 receptor

In our first experiments, we transfected FLAG-tagged mouse GFRα4 into Neuro 2a cells (ATCC) endogenously expressing RET. The GFRα4 receptor was expressed in the presence and absence of tunicamycin, a chemical which prevents the N-glycosylation of proteins, and the cell lysates were then analysed by Western blotting with FLAG antibodies. In the presence of tunicamycin, we found one band with a molecular weight of approximately 31 kDa, whereas in the absence of tunicamycin, two bands with molecular weights of about 31 kDa and 33 kDa were detected (**I**, Figure 1A). The FLAG antibody did not recognise

any bands from the lysates from non-transfected cells. We concluded that the 31 kDa band represents a non-glycosylated precursor of the mature 33 kDa GFRα4 receptor. The glycosylation site is predicted to be N184 (Uniprot), but this has not been confirmed experimentally.

The membrane flotation assay and subsequent Western blot analysis of the fractions showed that the FLAG-tagged mouse GFRα4 receptor is located in the top fractions of the gradient (**I**, Figure 1B). We therefore concluded that the receptor is membrane-bound. In addition, the cell surface localisation was confirmed with biotinylation of cell surface proteins (**I**, Figure 1D). PI-PLC treatment of the cells detached the 33 kDa form of GFRα4 from the cell surface, which indicates that it is bound to the cell surface with a GPI-anchor (**I**, Figure 1C). Cross-linking of cell surface proteins to [125]I-PSPN in cells that were transfected with mouse GFRα4 and expressed endogenously RET, and subsequent immunoprecipitation with RET antibodies revealed two major bands that could be displaced with unlabelled PSPN (**I**, Figure 1E). Based on their sizes, these bands could represent PSPN-GFRα4 and PSPN-GFRα4-RET complexes. We concluded that the FLAG-tagged mouse GFRα4 is a GPI-anchored, cell surface located receptor for PSPN.

Our RET phosphorylation assay in mouse GFRα4-transfected Neuro 2a cells, stimulated with PSPN, showed a clear dose-dependent RET phosphorylation (**I**, Figure 1F). In untransfected cells, PSPN did not cause RET phosphorylation. Therefore, our results show that PSPN activates RET through GFRα4. However, GDNF caused a clear RET phosphorylation in untransfected Neuro 2a cells (**I**, Figure

1G), which indicates that GFRα1 is present in these cells in contrast to what has been proposed in previous studies (Tansey *et al.*, 2000, Scott and Ibáñez, 2001). This result was verified by PCR (data not shown).

1.2 Recruitment of RET to lipid rafts by the GFRα4 receptor

To determine whether GFRα4 can recruit RET to lipid rafts in the presence of PSPN, we made cell lysates of cells that had been transfected with mouse GFRα4 and stimulated with PSPN. Triton X-100, which dissolves most of the cell membranes, but leaves sphingolipid- and GPI-anchored protein-enriched membrane parts insoluble in +4 °C (Brown and Rose, 1992), was added to the post-nuclear lysates. The detergent-resistant membrane parts can be found in the top fraction in a membrane flotation assay. As a marker for the Triton X-100 insoluble fraction, we used GFRα1 receptor, and as a marker for Triton X-100 soluble fractions, the transferrin receptor (Tansey *et al.*, 2000). As a positive control for RET recruitment, we used GFRα1-transfected cells, which were stimulated with GDNF.

Although the control GDNF-GFRα1 sample showed a clear re-localisation of RET to the Triton X-100 insoluble fraction, and PSPN stimulation caused RET phosphorylation in GFRα4-transfected cells, we found that only very little RET was recruited to the Triton X-100 insoluble fraction by GFRα4 (**I**, Figure 2). In addition, GFRα4 seemed to be less tightly associated with the Triton X-100 insoluble fraction than GFRα1.

We checked by Western blotting with RET antibodies that the levels of phosphorylated RET were equal in both the GDNF/GFRα1 and PSPN/GFRα4 samples. We also confirmed that the

FLAG-tag does not disturb the interaction of GFRα4 with RET by repeating the experiment with human non-tagged GFRα4 (Lindahl *et al.*, 2001). Taken together, these results show that the localisation of GFRα1 and GFRα4 as well as their ability to recruit RET in the Triton X-100 insoluble fraction are different. This difference may cause distinct signalling of GDNF/GFRα1 and PSPN/GFRα4 through RET, since the signalling molecules, which dock in the active RET, may be different in membrane parts with different order of lipid organisation. Moreover, there might be differences in RET internalisation and inactivation.

Our results do not explain what causes the difference in the localisation of GFRα1 and GFRα4, or the difference in their abilities to recruit RET to lipid rafts. It can be speculated that the difference could be caused by different GPI-anchors. The GPI-anchor has a complex structure that can be modified with phosphoethanolamine groups and sugars, but definitive conclusions that relate GPI-anchor structure and function have been difficult to draw (Paulick and Bertozzi, 2008). There is no published data on the GPI-anchor types of GFRα receptors, so further characterisation is needed to find evidence of this possibility. Another explanation for the difference may be that the protein interactions in the PSPN/GFRα4/RET complex could be more sensitive to Triton X-100 treatment than in the GDNF/GFRα1/RET complex, possibly because of the lack of D1 in GFRα4, since D1 seems to stabilise the interaction between GDNF and GFRα1 (**II**). RET could therefore be released from the complex and the rafts during the lysis. However, this would not affect the association of GFRα4 with the Triton X-100 insoluble fraction alone.

More studies are also needed to examine these explanations.

1.3 The biological activity of the GFRα4 receptor

Because it had been suggested that RET recruitment to lipid rafts is essential for GDNF-induced cell differentiation and neuronal survival (Tansey *et al.*, 2000), we wanted to test whether PSPN/GFRα4 can mediate these effects. The capacity of PSPN/GFRα4 to mediate neuronal survival was tested in rat cerebellar granule neurons transfected with mouse GFRα4. Normally, when switched from a culture medium containing high K^+ levels to one containing a low K^+ concentration, differentiated granule neurons degenerate and die (D'Mello *et al.*, 1993). In these conditions, only about 5 % of our mock-transfected cells survived. PSPN supported the survival of the GFRα4-transfected cells so that about 50 % of these cells survived (**I**, Figure 4A). This survival supporting effect was almost as high as that of GDNF and GFRα1.

The capacity of PSPN-GFRα4 to mediate neuronal differentiation was tested in neurite outgrowth assays in PC6-3 cells. These cells express endogenous RET at low levels, enabling us to see some neurite outgrowth after PSPN stimulation of GFRα4-transfected cells and a quite high induction of neurite outgrowth after GDNF stimulation of GFRα1-transfected cells. However, after co-transfection with GFRα1/RET or GFRα4/RET, both GDNF and PSPN induced a high level of neurite outgrowth (**I**, Figures 4B and 4C).

In the study that suggested that recruitment of RET to lipid rafts could be a critical determinant of RET signalling efficiency (Tansey *et al.*, 2000), the authors used an artificial transmembrane (TM) form of GFRα1 that is not localised in lipid rafts, and a soluble GFRα1, to determine the function of the localisation of RET to the rafts. Both receptors mediated GDNF-induced phosphorylation, but did not recruit RET to lipid rafts, and the survival and differentiation promoting effect was decreased in comparison to the GPI-anchored receptor. In contrast, our results clearly show that although GFRα4 cannot recruit RET in the lipid rafts as efficiently as GFRα1, PSPN/GFRα4 can still contribute to neuronal differentiation and survival. However, in Tansey *et al.* (2000) the characterisation of GFRα1-TM was done in Neuro 2a cells, which were in our study found to express endogenous GFRα1. Therefore, it remains unclear if the GFRα1-TM construct was functional. In addition, Paratcha *et al.* (2001) have shown that soluble GFRα1-GDNF complex can actually recruit RET by an unknown mechanism to lipid rafts and potentiate downstream signalling, neuronal survival, and differentiation. This study also points out some technical problems in the setup of experiments in Tansey *et al.* (2000). For these reasons, more studies are required in order to assess the importance of RET recruitment to the lipid rafts.

In conclusion, we have found that GFRα4 has a significantly weaker capacity than GFRα1 to recruit RET to the lipid rafts. In spite of that, it can phosphorylate RET in the presence of PSPN and contribute to neuronal differentiation and survival. The localisation of RET when it gets activated by PSPN-GFRα4 remains unclear, but it seems clear that – in contrast to what has been previously suggested (Tansey *et al.*, 2000) – the recruitment of RET to the lipid rafts is not always crucial for the biological activity of a GFRα receptor. Further studies on the interactions between different GFRα

receptors and RET may shed light on the mechanism and significance of RET recruitment to the lipid rafts.

2. The role of domain 1 in the function of GFRα1 (II)

As mentioned earlier, the first cysteine-rich domain D1 present in GFRα1-3 receptors is not present in GFRα4 (see Review of the literature, Figure 1). This raises the question of the functional significance of this domain. The major ligand binding region has been found in the D2 of GFRα receptors, and the third domain has been suggested to interact with RET (Scott and Ibáñez 2001, Leppänen *et al*., 2004, Wang *et al*., 2006, **IV**), but no function for D1 had been shown before this study.

In this study, we used both full-length and truncated soluble and GPI-anchored GFRα1 molecules to study the functional role of D1 in GFRα1. Soluble GFRα1 variants were named GFRα1[20], GFRα1[114] and GFRα1[145], according to the first residue in the N-terminus. These variants were expressed in Sf9 insect cells and purified by Ni[2+]-affinity chromatography. The purity of the receptors was analysed by Western blotting and the proteins were quantified on an SDS-PAGE gel. To further characterise the GFRα1[20] and GFRα1[145] variants, these receptors were subjected to reverse-phase chromatography, N-terminal sequencing, MS-analysis, and gel filtration fractionation. The GFRα1 containing fractions obtained in the gel filtration were identified by assessing their GDNF binding capacity by scintillation proximity assay (SPA) and by Western blotting.

2.1 Characterisation of soluble and GPI-anchored GFRα1 receptors

In reverse-phase chromatography, both GFRα1[20] and GFRα1[145] eluted as a single peak, and in N-terminal sequencing both gave a sequence that matches with the known sequence of GFRα1. MALDI-TOF (matrix-assisted laser desorption/ionisation – time-of-flight mass spectrometer) MS-analysis gave molecular masses, which indicated that both proteins are post-translationally modified. The information about the sizes of the modifications is consistent with previous identification of putative N-glycosylation sites in GFRα1 (Jing *et al*., 1996). Also the results of the assays in which GPI-anchored GFRα1 variants GFRα1[WT] and GFRα1[122] (named according the first residue in the N-terminus) were expressed in the presence or absence of tunicamycin, and then analysed in Western blotting, showed corresponding differences in molecular masses (**II**, Figure 2). Thus, we concluded that, as expected, both GPI-anchored GFRα1 variants were N-glycosylated and the glycosylation seemed to correspond to the glycosylation of the soluble GFRα1s. Gel filtration chromatography, followed by Western blotting and the SPAs, showed that both soluble GFRα1 variants were monomeric (**II**, Figure 1). This analysis also showed that the purified proteins could actively bind GDNF.

2.2 Differences in GDNF binding and biological activity between truncated and full-length GFRα1

The cell-based binding assays of GDNF to both full-length and truncated GPI-anchored GFRα1 variants were carried out to study whether the D1 of GFRα1 has any impact on ligand binding capacity. An

IC_{50} of 1.12 ± 0.14 nM (mean $\pm$ S.E.M.; n = 4) was determined for the binding of [125]I-GDNF to $GFR\alpha1^{WT}$, and an IC_{50} of 2.06 ± 0.40 nM for the binding of [125]I-GDNF to the $GFR\alpha1^{122}$ (**II**, Figure 3A and Table 1). RET has been shown to stabilise the binding of GDNF to GFRα1 (Cik *et al.*, 2000, Leppänen *et al.*, 2004) and, to assess whether D1 has any effect on this, the binding assays were also done in the presence of RET. RET stabilised the binding of GDNF to $GFR\alpha1^{WT}$ by a factor of 2.3, and the binding of GDNF to $GFR\alpha1^{122}$ by a factor of 2.7 (**II**, Figure 3B and Table 1). Thus, D1 seems to be important for binding of GDNF, but not for the interaction with RET.

These results were confirmed with the soluble GFRα1 variants in a cell-free SPA. The results were consistent with the results from the binding assays with GPI-anchored GFRα1s: The IC_{50} of the binding of [125]I-GDNF to $GFR\alpha^{20}$ and $GFR\alpha^{145}$ were 0.75 ± 0.15 and 1.59 ± 0.18, respectively (**II**, Figure 3C and Table 2). Also the stabilising effect of RET was approximately as strong with the full-length and the truncated GFRα1 receptors (**II**, Figure 3D and Table 2). In addition, this cell-free binding assay confirmed that D1 stabilises the binding of GDNF, without any other components like Met receptor, heparin or NCAM that have been suggested to interact with GDNF-GFRα1 (Popsueva *et al.*, 2003, Rickard *et al.*, 2003, Paratcha *et al.*, 2003). Taken together, the results of the binding assays show that the presence of D1 stabilises the binding of GDNF to both the soluble and GPI-anchored receptor.

The ability of the soluble GFRα1 variants to mediate GDNF-induced neurite outgrowth was studied in neurite outgrowth assays in PC6-3 cells. The assays were repeated three times with three different batches of soluble GFRα1 proteins. In these assays full-length GFRα1 was more than 2-fold more active than either of the truncated receptors (**II**, Figure 4A). None of the receptors induced neurite outgrowth without GDNF, as was expected. The phosphorylation of endogenous RET seen in lysates of these GDNF-GFRα1^{WT}-induced cells confirmed that neurite outgrowth is linked with endogenous RET phosphorylation, induced by the soluble GFRα1 and GDNF (**II**, Figure 4B).

2.3 The effect of GFRα1 concentration on RET phosphorylation

Similar assays were also done to determine how the truncated GFRα1 variants can induce RET phosphorylation. Assays done with GPI-anchored GFRα1 receptors in MG87RET cells showed that GDNF alone does not induce any RET phosphorylation, but transfection of $GFR\alpha1^{WT}$ and $GFR\alpha1^{122}$ enable equally strong phosphorylation (**II**, Figure 5A). Thus, according to these results, D1 would not have any effect on RET phosphorylation.

Since the binding assays, as well as the neurite outgrowth assays done with soluble GFRα1 variants, however, show a clear difference between the full-length and the truncated receptor, we concluded that the high expression levels of GPI-anchored GFRα1 receptors could mask the impaired function of the truncated receptors. In this case, we might be able to see differences between the full-length and truncated GFRα1 variants when the number of GFRα1 receptors is the limiting factor in the GDNF-GFRα1-RET complex formation. We set up to study this hypothesis by performing the RET phosphorylation assays with increasing

concentrations of the soluble GFRα1 variants.

The results of these assays indeed showed that, at low concentrations, the full-length GFRα1[20] together with GDNF induced RET phosphorylation more efficiently than any of the truncated receptors: GFRα1[20] induced maximal RET phosphorylation already at a concentration of 100 ng/ml whereas GFRα1[114] and GFRα1[145] reached the maximal level only at concentrations of 500-1000 ng/ml (**II**, Figure 5B).

2.4 Domain 1 stabilises the GFRα1-GDNF interaction, affects RET phosphorylation and contributes to the biological activity in vitro

Our results show that RET stabilises the binding of GDNF to the truncated and full-length forms of GFRα1 equally well. Therefore, it can be concluded that D1 of GFRα1 does not participate in interactions with RET. However, according to our results D1 stabilises the GDNF/GFRα1 complex and thus affects the phosphorylation of RET. What the mechanism of this stabilisation is, is still unclear, because the published structures of GFRα1 (**IV**) and GFRα3 (Wang *et al.*, 2006) both lack the D1. However, in our gel filtration chromatography studies, both the full-length and the truncated soluble GFRα1s were eluted as monomers, which makes direct D1-D1 contacts unlikely. Hence an interaction between D1 and GDNF seems more likely. However, since the difference in GDNF-binding between the truncated and full-length GFRα1 proteins only accounts for much less than one hydrogen bond, it is possible that instead of having a direct contact with GDNF, D1 actually has an effect on the conformation of domains 2 and 3.

In their studies, Scott and Ibáñez (2001) had found D1 dispensable for both ligand binding specificity and RET phosphorylation. In their work, the truncated GFRα1 lacking D1 binds GDNF and there is no significant difference in RET phosphorylation in the presence of full-length GFRα1 and GFRα1 lacking D1. However, their phosphorylation assays were done in Neuro 2a cells and thus in the presence of endogenous GFRα1, which may have masked the difference. Moreover, our finding that the high expression levels of GPI-anchored GFRα1 receptors indeed mask the impaired function of the truncated receptors might also explain why this study failed to show differences for full-length and truncated GFRα1 receptors. We also speculate that, at high concentrations of GDNF, a similar effect might occur: most GFRα1 receptors will become saturated in spite of their slightly weaker capacity to bind GDNF.

Taken together, D1 has a small, but clear stabilising effect for the function of GFRα1. This effect may be important in physiological conditions, where the concentration of the ligand or the soluble GFRα1 receptor is low. The spreading of GDNF in tissues has been shown to be very limited and a major problem in therapeutic approaches (Hamilton *et al.*, 2001). Due to the poor spreading in tissues, the concentrations of GDNF, even at a short distance from the injection site, can be very low. Therefore, our findings may become useful in designing and considering the use of new therapeutic molecules.

It is possible that D1 has also other, unidentified functions. In our later study (**IV**), full-length GFRα1 was found to bind heparin more strongly than GFRα1 that lacks D1. This result needs to be confirmed, but it may suggest a role for D1

in the distribution of putatively released GFRα1, formation of the GFL/GFRα/RET complex or cell adhesion-related functions of GFRα receptors, proposed by Ledda *et al.* (2007). Results of further studies, such as complete structures of GFL/GFRα/RET complexes, will probably enlighten these possibilities.

3. Activity of RET$^{MEN\ 2B}$ in the endoplasmic reticulum (III)

As mentioned previously, in most cell types RET proteins are expressed as glycoproteins of 150 and 170 kDa, which differ in their subcellular localisation. The 170 kDa isoform of RET is present at the plasma membrane, whereas the incompletely processed 150 kDa form of RET is present in the ER (van Weering *et al.*, 1998). In this work, we characterised the activation of RET$^{MEN\ 2B}$ precursor in the endoplasmic reticulum. The RET$^{MEN\ 2B}$ mutant that was used has the kinase domain point mutation M918T, which is the most common mutation in MEN 2B (Mulligan and Ponder, 1995).

3.1 Technical concerns related to RET activation

Studying the intracellular activation of RET precursors has been technically challenging because mutations that impair the maturation of RET, and thus prevent its transportation to the cell surface, have initially been reported to decrease its kinase activity (Chappuis-Flament *et al.*, 1998). For this reason, we first used Brefeldin A to trap RET in the ER. Brefeldin A disrupts the function of the intermediate compartment and Golgi apparatus and thus jams the secretory proteins in the ER.

Another obstacle in studying any RET activation is that a long-term overexpression of RET leads to its auto-phosphorylation (**III**, Figure 1A). In many cases, this background phosphorylation is so strong that the detection of the ligand-induced phosphorylation can be very difficult, or even impossible. To overcome this problem, we transfected the cells transiently with RET/GFRα1 and kept the expression time short. By taking early time course samples, we could follow the production and phosphorylation of RET^{WT} and RET$^{MEN\ 2B}$ and see that the 150 kDa form of both RET variants is phosphorylated already at very early stages (**III**, Figure 1B). In addition, the background phosphorylation of RET is already at 8 h after the transfection so strong that monitoring ligand-induced phosphorylation is very difficult. Therefore, we used 8 h or shorter expression time in most experiments. However, in some experiments, we used phosphotyrosine specific antibodies that recognised only high levels of phosphorylated RET, and thus higher overexpression was necessary in those particular experiments.

3.2 Localisation and phosphorylation of RET precursor

We transfected transiently PC6-3 cells with different RET variants and then expressed these proteins in the presence of Brefeldin A. Only the 150 kDa form of all the kinase active RET variants can be seen in the Western blots made of these samples, which confirms that the 150 kDa form of RET is located in the ER (**III**, Figure 1C). In the case of RET^{WT}, RET$^{MEN\ 2A}$ and RET$^{MEN\ 2B}$, this 150 kDa precursor form is also phosphorylated. The kinase-dead MEN 2A form of RET

(double mutation C634R/E921K, Pelet *et al*. 1998) is, however, not phosphorylated, which indicates that the activation of the precursor is dependent on its own kinase domain. The activity is also not dependent on GFRα1 because GFRα1 was not present in this experiment.

With the help of antibodies that specifically recognise phosphorylated tyrosines of RET, it has been shown that residues 905, 1015, 1062 and 1096 get phosphorylated upon GDNF-stimulation in the mature 170 kDa form of RET (Tsui-Pierchala *et al*., 2002a). We used these same antibodies to study whether the phosphorylation pattern in the precursor form of RET[MEN 2B] is similar to that of the precursor and mature form of RET[WT]. Although the ligand-independent phosphorylation of RET was high due to the overexpression of RET, we could see that in the mature form of RET[WT] GDNF caused a clear phosphorylation of Tyr905, Tyr1062 and Tyr1096 (**III**, Figure 1D). The signal detected by the antibody against the phosphorylated Tyr1015 was so weak that we could not detect RET phosphorylation and therefore the data are not included. The precursor of RET[WT] was also phosphorylated at Tyr905, Tyr1062 and Tyr1096. However, in the precursor, as well as in the mature form of oncogenic RET[MEN 2B], the phosphorylation at these tyrosines was much stronger than in RET[WT].

3.3 Interactions between RET[MEN 2B] precursor and adapter proteins SHC and GRB2 in the ER

It has been shown that the docking of SHC to the phosphorylated Tyr1062 in RET is crucial for the transforming activity of activated oncogenic RET (Ishiguro *et al*., 1999) and important for

the pathogenic function of RET in MEN 2B (Salvatore *et al*., 2001). To study whether the precursor of RET[MEN 2B] can also recruit SHC to the ER, we used two kinds of co-immunoprecipitations: In one experiment, we immunoprecipitated SHC and then detected by Western blotting with RET antibodies whether the precursor forms of RET[WT] and RET[MEN 2B] were also precipitated. In another experiment, we expressed RET[WT] and RET[MEN 2B] in the presence of Brefeldin A to keep all RET in the precursor form. RET was then immunoprecipitated and the presence of SHC in the precipitate was detected by Western blotting with antibodies to SHC. The results of the first experiment showed that RET[MEN 2B] was more phosphorylated and its precursor recruited more SHC than the precursor of RET[WT] (**III**, Figure 2A). The results of the latter experiment confirmed that the precursor of RET[MEN 2B] and SHC were associated (**III**, Figure 2B). The latter assay was done in the absence of GFRα1, so the results also proved that the interaction of RET[MEN 2B] precursor and SHC does not depend on GFRα1.

Brefeldin A, which had been so far used to trap RET after its synthesis in the ER disrupts the function of the intermediate compartment and Golgi. Conditions where Brefeldin A is present are thus unnatural for the cell, and it can be speculated that the addition of Brefeldin A could affect some of the signalling pathways. Therefore, we wanted to use another approach to further characterise the binding of adapter proteins to RET[MEN 2B] precursors. In addition to the kinase-dead mutant (RET[E921K]), we made two other mutant forms of RET (characterised in Carlomagno *et al*., 1996). Of these mutants RET[MEN 2B/S32L] was previously reported to have a severely impaired transport to the cell surface, while the

RET[MEN 2B/F393L] mutant was reported to be less severely affected (Carlomagno *et al.*, 1996). However, in our experiments, the 170 kDa mature form of neither mutant was detectable. In contrast to the original report (Carlomagno *et al.*, 1996), the kinase activity of the 150 kDa form was comparable to that of RET[MEN 2B].

With these mutants, we set out to study the binding of GRB2 and found that the precursors of RET[WT] and RET[MEN 2B] are indeed associated with GRB2 (**III**, Figure 2D). In the same experiment, we also confirmed the SHC association with RET precursors (**III**, Figure 2C). Both GRB2 and SHC associations were shown to be dependent on the kinase activity of RET, since this association did not happen with the kinase-dead RET variant.

3.4 Downstream signalling mediated by RET[MEN 2B] precursor in the ER

To characterise the downstream signalling possibly caused by the bound adapter proteins, we chose to study the activation of three proteins participating in the signalling mediated by RET. The AKT pathway activation has been linked with strong phosphorylation in Tyr1062 (Salvatore *et al.*, 2001) and we had shown that Tyr905, Tyr1062 and Tyr1096 are phosphorylated already in the precursor of RET[MEN 2B]. In addition, we had shown that SHC that binds to phosphorylated Tyr 1062 and GRB2 that binds to phosphorylated Tyr1096 were phosphorylated by the precursor of RET[MEN 2B]. Because the activation of SHC and GRB2 leads to activation of PI3K/AKT, we wanted to know whether also AKT becomes activated.

In the AKT phosphorylation assay, we used a short transient transfection of RET.

However, our first results showed that the transfection reagent Lipofectamine 2000 (Invitrogen) induced a clear phosphorylation of AKT (**III**, Figure 3A), and therefore, we had to use a different transfection method based on a magnet assisted system, which did not cause AKT activation in our experiments. To trap RET in the ER, we used Brefeldin A. The results of this assay showed that the expression of the precursors of RET[WT] and RET[MEN 2B] leads to AKT phosphorylation, although the phosphorylation was somewhat stronger in the absence of Brefeldin A, when some of the RET synthesised matures into the 170 kDa form (**III**, Figure 3B).

For further characterisation of the AKT activation and the other two signalling proteins STAT3 and ERK, we used the ER-retaining mutants RET[MEN 2B/S32L] and RET[MEN 2B/F393L]. With transient transfection and expression of RET variants, we showed that in addition to AKT, also ERK and STAT3 become activated by the 150 kDa precursor forms of RET[MEN 2B] and that the activation is dependent on the RET kinase activity (**III**, Figure 4).

3.5 The precursor of RET[MEN 2B] is biologically active

In spite of the relatively good documentation of RET synthesis and maturation (van Weering *et al.*, 1998), very little has been known on the activity of RET precursors. Miyazaki *et al.* (1993) have demonstrated that *in vitro* RET precursor (150 kDa form) can get even more strongly phosphorylated than the mature RET (170 kDa form). However, in several cell line experiments (e.g. Asai *et al.*, 1995, Carlomagno *et al.*, 1996, Frêche

et al., 2005), the 170 kDa form is more phosphorylated than the 150 kDa form, although the results of Carlomagno *et al*. (1996) are not completely consistent, and can be interpreted also differently from the authors' conclusions. In Carlomagno *et al*. (1996), the abundance of RET 150 kDa and 170 kDa forms also clearly differs from our observations, which is probably due to different cell types and different transfection methods. Asai *et al*. (1995) could not detect dimerisation of the 150 kDa RET[MEN 2A] protein, and they found that the transport of the RET[MEN 2A] protein to the plasma membrane and thus the maturation of RET[MEN 2A] is required for its transforming activity.

Taken together, our results show that the precursor of RET[MEN 2B] associates with SHC and GRB2 and has the capacity to activate downstream signalling molecules AKT, ERK and STAT3 in the ER. This result is well consistent with the findings that the intracellularly located PTC form of RET can activate AKT (Miyagi at al., 2004), ERK (Knauf *et al*., 2003) and STAT3 (Hwang *et al*., 2003), although it never reaches the cell surface. It has also been shown that EGF receptors form dimers in the absence of a bound ligand and this dimerisation occurs probably already in the endoplasmic reticulum (Tao and Maruyama, 2008), but also contradictory results have been published (Ferguson *et al*., 2003). Either way, the activity of the preformed dimers in the ER was not studied by Tao and Maruyama.

However, some plasma membrane RTKs can signal from different cellular compartments. It has been reported that RET that has been internalised after the ligand-induced activation can activate of ERK1/2 (Richardson *et al*., 2006). EGFR cannot activate MAPK (mitogen-activated protein kinase) signalling until it has been internalised (Vieira *et al*., 1996). Moreover, internalised TrkA receptors induce activation of many signalling pathways, in particular ERK1/2 and PI3K pathways (Heerssen and Segal, 2002). A mutated FGFR3 (fibroblast growth factor receptor 3) has been found to accumulate in its immature and phosphorylated form in the ER, where it also signals (Lievens *et al*., 2004). A mutant form of FLT-3 (Fms-like tyrosine kinase receptor 3) has an impaired maturation and transport to the cell surface, which seems to be caused by its constitutive kinase activity. Inhibition of this activity promotes cell surface localisation and, on the other hand, general phosphatase inhibition impairs the maturation of wild-type FLT-3 (Schmidt-Arras *et al*., 2005). This kind of accumulation or delayed cell surface expression of oncogenic RET was not found in our experiments, but otherwise our results are consistent with these earlier findings.

Our finding that the oncogenic precursor of RET[MEN 2B] is active in the ER may be significant in the development for methods to inhibit the activity of oncogenic RET because it shows that the inhibition should reach not only the mature form of RET, which is located on the cell surface, but also the intracellular precursor form RET. Therefore, some of the various kinds of therapeutic approaches suggested for the treatment of RET-associated cancers (discussed in Kodama *et al*., 2005), for example using RET dimerisation inhibitors or blocking antibodies that affect RET only extracellularly, may not be sufficient alone, but need to be complemented by approaches that can affect the activity of intracellular RET.

4. Structure of the GDNF-GFRα1 complex (IV)

The structure of the GFRα1 domains 2 and 3 (GFRα1 D23C) in complex with GDNF was solved with X-ray crystallography by molecular replacement and refined to R_{work} 18.4 % (R_{free} 23.7) (**IV**, Table 1). The structure contains residues 150-349 of GFRα1 and residues 34-134 of GDNF.

4.1 GDNF-GFRα1-SOS complex

The structure of the domains 2 and 3 of GFRα1 resemble each other closely: both consist of five α helices of which three helices form a spiral (**IV**, Figure 2A). These domains pack so that D3 stabilises D2. Each GDNF monomer in the $GDNF_2$-$GFRα1_2$ structure binds GFRα1 D2 with its finger domains 1 and 2 (numbering starts from the N-terminal part). The binding site in GFRα1 is located in the triangular spiral formed by the helices α1, α2 and α5 of the domain 2. 14 residues from GDNF and 17 residues from GFRα1 form the interface (**IV**, Table 2; Review of the literature, Figure 1), which buries a total surface area of about 1600 $Å^2$. $N162^{GFRα1}$ stabilises a central ion triple $R171^{GFRα1}$ – $E61^{GDNF}$ – $R224^{GFRα1}$ by positioning $R171^{GFRα1}$ guanidine group and forming hydrogen bonds to $E61^{GDNF}$, $E62^{GDNF}$ and $S112^{GDNF}$ (**IV**, Figure 2C). Hydrophobic interactions are formed between $Y120^{GDNF}$ –$I175^{GFRα1}$ – $L114^{GDNF}$ and $I175^{GFRα1}$ – $I122^{GDNF}$ – $T176^{GFRα1}$. Two ionic interactions at the edge of the GDNF-GFRα1 interface are formed between $E62^{GDNF}$ – $K159^{GFRα1}$ and $K168^{GFRα1}$ – $D108^{GDNF}$– $D109^{GFRα1}$.

Previously, Eketjäll *et al.* (1999) had mapped GDNF residues that participate in GFRα1 binding by mutating GDNF residues and testing the mutants in GDNF-GFRα1 binding assays. In this study, they found several negatively charged and hydrophobic residues that are critical for GFRα1 binding. Our structure is well in line with their results: at least six GDNF residues that they suggested to be involved in GFRα1 binding are found in the core region of GDNF-GFRα1 interface. Of these, E61, E62 and I64 are located in the tip of finger 1 of GDNF whereas L114, Y120 and I122 are located in finger 2.

Two carbohydrates can be seen in the structure: one N-acetylglucosamine molecule is covalently linked to $N49^{GDNF}$ and a sucrose octasulfate molecule (SOS, a heparin analogue) binds to D2 and D3 of GFRα1 (**IV**, Figures 2A and 3A). According to the structure, the sulphate groups of the SOS molecule bind to five residues on GFRα1 D2 and three residues on D3. Through a crystal contact, the same SOS molecule also binds to the N-terminus of the GDNF of the neighbouring complex.

4.2 Interactions between GDNF, GFRα1, SOS and RET

To demonstrate that the residues found in the GDNF-GFRα1 and SOS-GFRα1 interfaces in the structure are really involved in GDNF and SOS binding, and to understand which residues in the GDNF-GFRα1 interface are important for specificity, a series of mutants was tested in RET phosphorylation assays. In addition, we wanted to study experimentally whether the conserved GFRα1 residues (D164, K202, R257, R259, E323 and E324, GFRα1 numbering) proposed by Wang *et al.* (2006) are really involved in RET binding. The mutants can be thus divided into three categories: 1) mutants in the GDNF-GFRα1 interface, 2) mutants in the SOS binding region and 3) mutants in the putative RET-binding region. The

latter two regions were expected to overlap at least partly. In addition, we tested two other mutants that had shown differences in GDNF-binding or RET phosphorylation in earlier studies (Leppänen *et al*., 2004)

The RET phosphorylation assays in MG87RET cells and subsequent pRET ELISAs showed that the mutations in N162, which stabilises the GDNF-GFRα1 interface central ion triple, and I175, which is part of the hydrophobic core, caused drastic reduction in RET phosphorylation (**IV**, Figure 1B). Mutations in more peripheral residues in this interface reduced RET phosphorylation less. The binding studies with SPA supported these results.

Mutations in the residues expected to be on the RET/SOS binding surface of GFRα1 all reduced the phosphorylation of RET about by a factor of three or more, except for D164A, which only lowered the amount of RET phosphorylation to about half of wild-type (**IV**, Figure 1B and table 3). Binding studies were also consistent with these results. Thus, all of these residues in GFRα1 are likely to participate in forming the RET interface.

4.3 Comparison of GDNF-GFRα1 to ARTN-GFRα3

Comparison of the structure of the GDNF-GFRα1 complex and the recently published (Wang *et al*., 2006) structure of ARTN-GFRα3 complex reveals similarities, as well as differences (**IV**, Figures 2A and 2B). The overall structures of the receptors are very similar (root mean square deviation of 0.89 Å for 166 Cα atoms). In addition, both GFLs reach their fingers to the triangular spiral of GFRα (formed by the helices α1, α2 and α5 of the domain 2) and the centre of this interface contains the above mentioned ion triple (**IV**, Figure 4A).

However, there are three important differences. The finger loops of the ARTN and GDNF are inclined differently in relation to the heel region, so that when the heel regions of GDNF and ARTN are superimposed, the angle between the finger loops is about 20 ° (**IV**, Figure 4C). The position of the ligand fingers in relation to the GFRα interface is also different: in comparison to GDNF, the finger loops of ARTN twist about their longitudinal axis and turn around a vertical axis in relation to the GFRα binding site so that the angle difference is about 20 ° (**IV**, Figure 4A).

The third significant difference is seen in the core region of the GFL-GFRα interface (**IV**, Figure 4B). N162, which buttresses the ion triple in GDNF-GFRα1 complex, does not have a counterpart in GFRα3: the equivalent GFRα3 T170 does not interact with the ion triple. The ARTN binding pocket in GFRα3 is also much wider and less deep than the GDNF binding pocket in GFRα1, since it contains G in comparison to GFRα1 I175 and A in comparison to GFRα1 V230. The W205 and M199 from ARTN fit thus in the GFRα3 pocket as well as the Y120 and L114 in the GFRα1 pocket.

These differences in the binding pocket are important for the ligand specificity, whereas the differences in the angles between the GFL finger loop and the heel region, and between the GFL and the GFRα ligand binding site affect the quaternary structures of the whole GFL-GFRα complex. The different bend angles can change the conformation of the intracellular part of RET, causing alterations in the adaptor protein docking surfaces. This could putatively lead to different signalling through RET by different GFLs, although overall RET

phosphorylation level would not be changed.

To investigate this hypothesis, we set up to study differences in downstream signalling of RET induced by GDNF-GFRα1 and ARTN-GFRα3. According to our results, GDNF causes stronger and faster MAPK pathway activation than ARTN (**IV**, Figure 4D). However, this does not give direct evidence of differences in RET conformation or phosphorylation of docking surfaces. Therefore, more studies are needed to confirm the hypothesis. To study the detailed phosphorylation pattern of RET after stimulation with different ligands, I have recently managed to set up a method to purify endogenous RET from mammalian cells after ligand stimulation. After digestion of RET with trypsin, the phosphorylated peptides will be subjected to mass-spectrometry analysis, as has recently been done with EGFR (Boeri Erba *et al.*, 2005). Results of these experiments are expected to shed light on the differences in phosphorylation and downstream signalling of RET induced by different GFLs.

4.4 Role of heparin in the GDNF-GFRα1-RET complex

Our work gives some insight into how heparin might inhibit RET phosphorylation, even when the heparin-binding region of GDNF has been removed. In our structure, we could see a SOS molecule (heparin analogue added prior to the purification) bound to GFRα1 (**IV**, Figure 3A). The region which binds SOS in our structure overlaps partially with the RET-binding region of GFRα1 (**IV**, Figures 3A and 3D). Since a heparin molecule could fit in the same position as SOS (**IV**, Figure 5), it seems that heparin could indeed bind

to this region and prevent the binding of RET.

Our model also gives a possible explanation to how GDNF-GFRα1 complex could work in adhesion and induce synapse formation, as suggested by Ledda *et al.* (2007). In our structure, SOS links a $GDNF_2$-$GFR\alpha1_2$ complex to a neighbouring complex. We can thus speculate that heparin might mediate this kind of dimerisation of the $GDNF_2$-$GFR\alpha1_2$ complexes as well as $NRTN_2$-$GFR\alpha2_2$ complexes.

However, although SOS can be seen in the structure, it can be speculated that binding is not specific; as a strongly negatively charged molecule SOS might bind to a positively charged surface of GFRα1 unspecifically. In our experiments where heparin binding of GFRα1 was studied, the full-length GFRα1 eluted from the heparin column at a very high (>1 M) NaCl concentration, which indicates strong binding. The GFRα1 D23C that was used in the crystallisation eluted at about half of this concentration, which suggests reduced but still strong binding. Yet Alfano *et al.* (2007) have found that GFRα1 itself does not bind heparin. They postulate that the heparin binding of the commercial recombinant chimeric GFRα1 protein (R&D Systems Europe) can be caused by the His-tag (Lacy and Sanderson, 2002). Since we also had a His-tag in our GFRα1 construct used for heparin-binding studies, we cannot exclude the possibility that the observed binding was unspecific. Although the GFRα1 used in the crystal structure was untagged, the interaction of SOS and GFRα1 in the crystal may not be specific. Therefore, the results of SOS/heparin binding to GFRα1 in this study should be considered preliminary.

CONCLUSIONS

The aim of this thesis was to study the structure of GFRα1 and the localisation and site of activation of both GFRα4 and RET. We found that PSPN/GFRα4 is not associated with lipid rafts as tightly as GDNF/GFRα1, and does not recruit RET to lipid rafts as efficiently as GDNF/GFRα1 (**I**). This might be due to the lack of domain 1 in GFRα4 as domain 1 in GFRα1 seems to stabilise the binding of GDNF to GFRα1 and thus to strengthen the GDNF-GFRα1-RET complex (**II**). In addition to the stabilisation of GDNF binding to GFRα1, we found that domain 1 may contribute to the heparin-binding of GFRα1 (**IV**). As our heparin-binding studies give only preliminary information, further studies are needed to confirm this function for GFRα1 domain1.

In addition to studying in which compartment of the cell membrane RET^WT is activated, we studied in which cellular compartment the oncogenic RET^MEN 2B gets activated. We found that RET^MEN 2B gets activated and signals in the ER (**III**). This is consistent with our conclusion that localisation on the lipid rafts is not necessary for the signalling and biological activity of RET (**I**). Further studies on the interactions between different GFRα receptors and RET may shed light on the mechanism and significance of RET recruitment to the lipid rafts. More detailed studies on RET signalling in different compartments of the cell may also reveal interesting information of the significance of the localisation of the signalling complex.

The main conclusions are:

I. The mouse GFRα4 is less tightly associated with the Triton X-100 insoluble fraction than GFRα1. It also recruits RET upon ligand stimulation to lipid rafts more weakly than GFRα1. However, mouse GFRα4 can mediate PSPN-dependent differentiation and survival of neurons.

II. The first cysteine-rich domain of GFRα1 stabilises the binding of GDNF to GFRα1. It affects the level of RET phosphorylation at low concentrations of GFRα1 and contributes to the biological activity.

III. The precursor of oncogenic RET^MEN 2B is active in the endoplasmic reticulum. It associates with adaptor proteins SHC and GRB2 and has the capacity to activate downstream signalling molecules AKT, ERK and STAT3 in the endoplasmic reticulum.

IV. The crystallographic structure of GFRα1 domains 2 and 3 in the complex with GDNF differs from ARTN-GFRα3 structure in three significant ways: 1) The finger loops of ARTN and GDNF are inclined differently in relation to the heel region. 2) The position of the ligand fingers in relation to the GFRα interface is different. 3) The residues in the GDNF-GFRα1 interface form interactions different from the residues in the ARTN-GFRα3 interface.

VI. ACKNOWLEDGEMENTS

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