GDNF Receptors:
Veterans and Novices

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Academic dissertation

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<tbody>
<tr>
<td>ARTN</td>
<td>Artemin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CLD</td>
<td>Cadherin-like domain</td>
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<td>E</td>
<td>Embryonic day</td>
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<tr>
<td>ECD</td>
<td>Extracellular domain</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>ENS</td>
<td>Enteric nervous system</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>FMTC</td>
<td>Familial medullary thyroid carcinoma</td>
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<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
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<tr>
<td>GF</td>
<td>Growth factor</td>
</tr>
<tr>
<td>GFL</td>
<td>GDNF family ligand</td>
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<tr>
<td>GFRα</td>
<td>GDNF family receptor α</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HB-GAM</td>
<td>Heparin-binding growth associated molecule</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfates</td>
</tr>
<tr>
<td>HSCR</td>
<td>Hirschsprung’s disease</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>K_d</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MEN2</td>
<td>Multiple endocrine neoplasia type 2</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
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<tr>
<td>NF</td>
<td>Neurotrophic factor</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NRTN</td>
<td>Neurturin</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PI-PLC</td>
<td>Phosphoinositide-specific phospholipase C</td>
</tr>
<tr>
<td>PSPN</td>
<td>Persephin</td>
</tr>
<tr>
<td>RET</td>
<td>Rearranged during transfection</td>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SKF</td>
<td>Src family kinase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
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<tr>
<td>TOF</td>
<td>Time of flight</td>
</tr>
<tr>
<td>Trk</td>
<td>Tropomyosin-related kinase</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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LIST OF ORIGINAL PUBLICATIONS

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


ADDITIONAL PUBLICATIONS

The published papers that are not included in the thesis. They are referred in the text in the same style as all other publications.


Neurotrophic factors play an essential role in the development and functioning of the nervous system and other organs. Glial cell line-Derived Neurotrophic Factor (GDNF) family ligands (GFLs) are of particular interest because they promote the survival of dopaminergic neurons in vitro, in Parkinson’s disease animal models and in patients. GDNF is also a potent survival factor for the central motoneurons and thus is considered as a potential lead for the treatment of amyotrophic lateral sclerosis. The survival promoting receptor complex for GFLs consists of a ligand-specific co-receptor, GFRα and a signal transducing module, receptor tyrosine kinase RET. At least GDNF and persephin, a GFL, have established functions outside central nervous system. GDNF is crucial for enteric nervous system and kidney development as well as for spermatogenesis. Persephin controls calcitonin secretion.

Communication between cells often occurs in the extracellular matrix (ECM), a meshwork, which is secreted and deposited by the cells and is mainly composed of fibrillar proteins and polymerized sugars. We evaluated the relationship between GFLs and extracellular matrix components and demonstrated that three GFLs - GDNF, neurturin and artemin bind heparan sulfates with nanomolar affinities. The fourth member of the family - persephin binds these polysaccharides thousand times less tightly. GDNF, neurturin and artemin also bind with high affinity to heparan sulfate proteoglycan (HSPG) isolated from the nervous system, syndecan-3.

GDNF signals through HSPGs, evoking Src family kinase activation. This signaling induces cell spreading, hippocampal neurite outgrowth in vitro and cellular migration. Specifically, GDNF signaling through syndecan-3 is important for embryonic cortical neuron migration. Syndecan-3-deficient mice, similarly to mice lacking GDNF, have less GABAergic neurons in their cortex, as compared to the wild-type mice. This fact provides indirect evidence that GDNF interaction with syndecan-3 is important for cortical brain development. Noteworthy, in non-neuronal tissues GFLs may signal via other syndecans.

We also present the structural model for a GDNF co-receptor, GFRα1. The X-ray structure of the GFRα1 domain 3 was solved with 1.8 Å resolution, revealing a new protein fold. Later we also solved the structure of the truncated GFRα1 in the complex with GDNF and this model was confirmed by site-directed mutagenesis.

In summary, our work contributed to the structural characterization of GFRα-based receptor complex and revealed a new receptor for GDNF, neurturin and artemin – the HSPG syndecan-3. This information is critically important for the development of GFRα/RET agonists for the treatment of neurodegenerative diseases.
1. LITERATURE REVIEW

1.1. Growth factors and neurotrophic factors

In October 1986, the Italian developmental biologist Rita Levi-Montalcini and the American biochemist Stanley Cohen were awarded the Nobel Prize in Physiology or Medicine for “their discovery of growth factors: nerve growth factor (NGF) and epidermal growth factor (EGF), respectively”. The discovery of NGF was made while working in Viktor Hamburger’s laboratory in the USA. First, Levi-Montalcini demonstrated that when tumors from mice were transplanted to chick embryos they promoted a potent growth of chick embryo nervous system, specifically of the sensory and sympathetic nerves. When Stanley Cohen joined the laboratory they showed that snake venom promoted growth of the neurites in chicken sympathetic ganglia explants even more potently than tumor extracts. NGF from snake venom was purified and characterized. It was also the major nerve growth-promoting factor in tumor extracts (Cohen and Levi-Montalcini, 1956). Later, Cohen observed an unexpected acceleration of development when he injected salivary gland extract to newborn mice - it stimulated the proliferation of epithelial cells in skin and cornea. Cohen termed this substance epidermal growth factor (The Nobel Assembly at the Karolinska Institute, Press release, 1986).

NGF was the first of many growth-regulating signal substances to be discovered and characterized. These substances are collectively known as growth factors (GFs): “any of various chemicals, particularly polypeptides, that have variety of roles in the stimulation of new cell growth and cell maintenance” (Oxford Dictionary of Science, Oxford University Press, 2003).

Ironically, the first growth factor NGF does not fall precisely into this definition. NGF promotes axonal outgrowth but generally not a cell proliferation (with the exception of certain neuroblasts and lymphocytes). Accounting for this, it may be more appropriate to use the following definition of the growth factor: Small secreted protein that promotes cell growth, propagation, differentiation, survival, migration and process formation in the cells expressing cognate receptors for a given GF.

Growth factors are synthesized first as precursors, followed by maturation through proteolytic removal of the “Pro” region (Fig. 1A). Proteolytic cleavage occurs either in the Golgi or in the extracellular space by the extracellular matrix proenzyme convertases (e.g. metalloproteases) or plasmin upon release of the GF (Chang and Werb, 2001). The biologically active form of most GFs is a homodimer, which is formed immediately after synthesis in the endoplasmic reticulum. GFs exert their biological effects often through receptor tyrosine or serine-threonine kinases, the large single-pass transmembrane proteins. Receptor kinases are often activated by dimerization induced by GF binding to their extracellular domains. Upon dimerization of the receptor, transphosphorylation of certain amino acid residues in the catalytic intracellular domain (ICD) of the receptor takes place. This leads to the recruitment of a number of adaptor proteins or enzymes that assist activation of the signaling cascades, leading to a variety of biological effects.
Fig. 1. Structures of GDNF family ligands and neurotrophins. A. Gross structure of GDNF and NGF pre-pro polypeptides. B. Representation of X-ray structures of GDNF, ARTN, NGF and BDNF/NT3 (Eigenbrot and Gerber, 1997; Wang et al., 2006; McDonald et al., 1991; Robinson et al., 1995) and homology models of NRTN, and PSPN. Homology models were generated using I-TASSER server (Zhang, 2008) and visualized with PyMol (DeLano Scientific).
The first isolated GF is also the very first neurotrophic factor (NF). NGF was initially demonstrated to promote neurite outgrowth of sensory and sympathetic neurons (Levi-Montalcini and Cohen, 1956). Later, NGF was also demonstrated to promote survival of these neurons (Levi-Montalcini and Booker, 1960; Levi-Montalcini and Angeletti, 1963). Therefore, NGF is the prototype protein for the family of neurotrophins. Strictly speaking NFs are growth factors that support the survival of neurons by activating specific cell-surface receptors. However, many other GFs that were traditionally thought to have functions elsewhere in the organism were shown to have a survival promoting effect on a number of neuronal populations (Barde, 1989; Digicaylioglu and Lipton, 2001; Jin et al., 2005; Mendez et al., 2005). The opposite is true as well. Outside the nervous system, NFs stimulate processes generally attributed to other GFs (Meng et al., 2000; Vega et al., 2003). This observation highlights the remarkable similarity in the signaling of the receptor kinases in different tissues and raises the question about the specificity of their downstream signaling, induced by different GFs.

Current classification defines four major families of NFs: neurotrophins, neurokines, GDNF family ligands and CDNF/MANF neurotrophic factors (Lindholm et al., 2007).

NGF is a founding member of the neurotrophin family of proteins. They play many roles in the development of the organism as well as in the adulthood. Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT3) and Neurotrophin-4 (NT4) are the other members of this family. The most profound function of these factors is to keep neurons alive. The importance of the neurotrophins for the organism becomes obvious when analyzing respective knockout mice. NGF-deficient mice, for example, have a massive loss (95%) of superior cervical ganglion (SCG) sympathetic neurons (Crowley et al., 1994). Mice lacking bdnf gene suffer from a 80% loss of sensory neurons in vestibular ganglion (Ernfors et al., 1994). Survival promoting effects of neurotrophins are mediated by receptor tyrosine kinases (RTK) named tropomyosin-related kinase (Trk). NGF preferentially activates TrkA receptor, BDNF and NT4 share TrkB receptor, whereas NT3 activates TrkC.

The study of the neurotrophins led to the concept aiming to explain the regulatory mechanisms that control the neuronal number by the functionality of their connections. The concept is known as the “target field hypothesis” (Korsching, 1993; Lewin and Barde, 1996) and is based on the fact that early in the development peripheral nervous systems (PNS) neurons are overproduced. Roughly half of the neurons, those that fail to establish functional connection with the target tissue (for example to innervate the muscle), will die via apoptosis. The selection of the neurons occurs on the basis of accessibility of the trophic factors, which are produced at the limited amounts by the target tissue. Only those neurons that establish functional connections with the target tissue receive enough trophic support to survive. Importantly, this concept underlines the neurotrophic factor-regulated mechanism ensuring apoptotic elimination of the neurons that fail in communication. The findings that NF overexpression in muscles leads to increased innervation of neuromuscular junction (Keller-Peck et al., 2001; Nguyen et al., 1998) and that NF-deficiency results
in decreased innervation and number of neurons (Huang and Reichardt, 2003) advocate for the target field hypothesis. However, in the central nervous system (CNS) this concept is not yet well established (Kramer et al., 2007).

In the CNS, neurotrophins act as neuromodulators for synaptic transmission and plasticity. Upon activity-regulated release of neurotrophins into the synaptic cleft they in turn induce immediate changes in synaptic efficacy and morphology. This leads to long-term functional and structural modifications of the synapses (reviewed by Poo, 2001; Arancio and Chao, 2007). Therefore, neurotrophins were considered for the treatment of CNS disorders. NGF and BDNF are trophic factors for cholinergic neurons, the primary neuronal population in the brain that degenerates in Alzheimer’s disease (AD).

For example, BDNF was tested in Parkinson’s disease (PD), AD and amyotrophic lateral sclerosis (ALS). NGF-blocking antibodies are in clinical trial against chronic pain (Dray, 2008). Another clinical trial, which took advantage of NGF-overexpressing cells transplantation, demonstrated the benefit for the patients suffering from AD (Tuszynski et al., 2005).

Neurotrophins (and pro-neurotrophins) also bind low affinity neurotrophin receptor p75, a non-tyrosine kinase receptor of the tumor necrosis factor receptor superfamily (TNFR-SF). p75 interacts with neurotrophins either directly or modulates the affinity and specificity of the Trk receptors for neurotrophins (Huang and Reichardt, 2003). The data on the role of p75 is to some extent controversial not the least because p75 can be influenced by co-receptors NgR/Lingo1 (Mi et al., 2004; Vilar et al., 2009) and sortilin (Nykjaer et al., 2004). ProNGF triggers cell death via p75/sortilin and mediates axonal growth inhibition via NgR/Lingo1. In the absence of Trk, p75 activation leads to apoptosis of certain neurons, however, in other neuronal populations solely expressing p75 activation leads to survival (Dechant and Barde, 2002; Rabizadeh and Bredesen, 2003).

Neurotrophins have several functions outside the nervous system. For example, NGF is known to regulate the function of the immune system (Vega et al., 2003). BDNF has a function in vascular system (Donovan et al., 2000) and hair follicle development (Botchkarev et al., 2004).

Another group of neurotrophic factors – the neurokines, consist of ciliary neurotrophic factor (CNTF), interleukin-6 and -11 (IL-6 & IL-11), leukemia inhibitory factor (LIF, CDL), oncostatin M, cardiortrophin-1 (CT-1), CT-like cytokine and neuropoietin (Halvorsen and Kaur, 2006) These NFs are cytokines, which are produced by immune cells, Schwann cells, fibroblasts and sensory neurons. Similarly to neurotrophins, neurokines act as mediators between nervous system and immune system by inducing response to injury and stress. Several neurokines like CNTF, IL-6 and CT-1 have profound effects on motoneurons, supporting their survival (Oppenheim, 1996). All neurokines exert their action via a 2- or 3-component receptor system. They all share non-tyrosine kinase receptor gp130 (Taga and Kishimoto, 1997). However, CNTF binding to gp130 requires a cell surface glycosyl-phosphatidylinositol (GPI)-anchored co-receptor CNTFRα (Davis et al., 1991) and LIFRβ (Halvorsen and Kaur, 2006). Likewise, binding of IL-6 to gp130 is mediated by a transmembrane co-receptor IL-6Rα (Taga et al., 1989).
1.2. GDNF family ligands

**GFLs structure and receptor complexes**

The third group of neurotrophic factors is GDNF family ligands (GFLs), which consist of GDNF and the homologous neurotrophic factors neurturin (NRTN), artemin (ARTN) and persephin (PSPN). GFLs are structurally and evolutionarily different from other NFs (Fig. 1). They are distant relatives of the TGF-β superfamily. They contain seven cysteine residues with the similar relative spacing characteristic for TGF-β. Six cysteines form three intermolecular disulphide bonds (cystines) and the seventh cysteine is involved in the homodimer formation. GFLs and TGF-βs (and neurotrophins) share the structural motif known as cystine-knot (Eigenbrot and Gerber, 1997), in which a covalent ring formed by two cystines and a third cystine passing through it (McDonald et al., 1991). The cystine knot forms intracellularly and prevents the formation of a globular structure commonly found in other extracellular polypeptides (Vitt et al., 2001). All known structures for TGF-β superfamily members share the following structural features: The β-sheets comprising two “fingers” and the α-helix connected to cysteine knot via flexible loops. GDNF poses the unique 38 amino-acids-long protrusion, which is not visible in the crystal structure due to high flexibility or disordered structure (Eigenbrot and Gerber, 1997). The 92RRLTSDK sequence in the loop connecting C–terminus of the α-helix with a cystine knot is also missing from their X-ray structure.

The resolved and modeled structures of other GFLs resemble the structure of GDNF (Fig.1B). ARTN has a different angle between α-helix and fingers (Wang et al., 2006). The amino acid determinants of the GFLs interaction with their receptors are on the GFLs fingers (Eketjäll et al., 1999). These amino acids contribute to electrostatic and hydrophobic components of the free energy of the interaction. According to the presented models of the GFLs, the tertiary structure of this region does not vary significantly among GLFs and several amino acids important for the interaction are either identical or complementary.

Most TGF-β’s use transmembrane receptor serine-threonine kinases to transduce their signal. GFLs use different receptors for signal transduction. Their receptor complex consists of the signaling component RET, a receptor tyrosine kinase that is commonly shared by the four GFLs. However, GFLs do not bind directly to RET. To present the corresponding GFL to RET, the ligand-specific surface-bound GDNF family co-receptor alpha (GFRα) is required. GFRαs are GPI-anchored proteins as the co-receptor (CNTFRα) in CNTF receptor system (Davis et al., 1991; Jing et al., 1996).

GDNF preferentially binds to GFRα1, NRTN to GFRα2, ARTN to GFRα3 and PSPN to GFRα4 (Fig. 2), followed by GFRα/RET receptor complex oligomerization (or complex stabilization) and transphosphorylation (Airaksinen and Saarma, 2002; Sariola and Saarma, 2003).

Paratcha et al. (2003) found that the neural cell adhesion molecule (NCAM) in the complex with GFRα functions as an alternative signaling receptor for GFLs. NCAM-mediated GDNF signaling leads to activation of cytoplasmic Src-family kinases (SFK) and subsequently to enhanced neuronal migration and neurite outgrowth. Although the vast majority of GFL signaling utilizes GFRα/RET or GFRα/NCAM receptor systems, the data indicated that additional, still unidentified
GFL signaling pathways may exist. In that study, GDNF promoted differentiation and tangential migration of cortical GABAergic neurons in RET- and NCAM-independent manner (Canty et al., 2009; Pozas and Ibáñez, 2005).

**Biological effects and therapeutic potential of GFLs**

GFLs have all been shown to act as potent neuronal survival factors, but at least GDNF and PSPN have several functions outside the nervous system. Gene ablation shows that GDNF is crucially important for the development of the enteric nervous system (ENS) (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996) and for sensory neurons (Erickson et al., 2001).

**Fig. 2.** GDNF family ligands (GFLs) and their receptors. GFLs cannot bind the signal-transducing receptor RET directly but require glycosylphosphatidylinositol (GPI)-anchored co-receptor GFRα. GDNF preferentially binds to GFRα1, neurturin (NRTN) to GFRα2, artemin (ARTN) to GFRα3 and persephin (PSPN) to GFRα1. They all rely on a common receptor tyrosine kinase RET to transduce their signal. RET consists of four cadherin-like domains (CLDs), the cysteine-rich domain (CRD), the transmembrane domain (TM) and an intracellular part with the tyrosine kinase domain. RET CLDs and CRD were modeled using EC-cadherin as a template and laminin gamma 1 chain, respectively, by HHpred program (Söding et al., 2005). GFRα2 and GFRα4 homology models were generated by I-TASSER server. Calcium (yellow circle) is bound between CLD2 and CLD3 of RET (Anders, 2001). All proteins were visualized by ViewerLite (Accelrys) program and are represented as solid ribbons.
Mice lacking GDNF or its receptors have a loss of motor neurons (Garces et al., 2000; Oppenheim et al., 2000) and defects of parasympathetic nervous system (Enomoto et al., 2000). GDNF is absolutely required for kidney formation (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996) and play a pivotal role in the regulation of spermatogenesis (Meng et al., 2000).

GDNF was initially purified from glial cell line supernatant and shown to support the survival of the midbrain dopaminergic neurons in vitro (Lin et al., 1993). In animal models of Parkinson’s disease GDNF efficiently rescues dopamine neurons and it has shown promising results in two clinical trials (Gill et al., 2003; Patel et al., 2005; Slevin et al., 2005). Another study failed to demonstrate a clear clinical benefit of GDNF (Lang et al., 2006), which may be explained by the configuration of the catheter used to infuse GDNF (Salvatore et al., 2006). GDNF is also a potent survival factor for central motoneurons and, therefore, it may have clinical efficacy in the treatment of ALS (Henderson et al., 1994; Oppenheim et al., 1995). Current treatment of these diseases is symptomatic. Hence, GFLs represent a novel possibility for the treatment of Parkinson’s and ALS patients, as they are the most efficient molecules available that, in animal models, prevent death and repair neuronal populations affected in these diseases. Additional results highlight the importance of GDNF as a new target for the treatment of drug addiction (Airavaara et al., 2004; Messer et al., 2000) and alcoholism treatment (Carnicella et al., 2009; He et al., 2005). Although the original paper by Messer and co-workers is considered controversial, the phenomenon deserves further investigation.

NRTN also supports the survival of the dopaminergic neurons (Kotzbauer et al., 1996). Unlike GDNF, NRTN fails to induce neurite sprouting in vivo or the neuronal hypertrophy (Åkerud et al., 1999). Nevertheless, NRTN holds a promise for Parkinson’s disease therapy (Marks et al., 2008; Ceregene Inc., Press release, 2009) and for epilepsy (Nanobashvili et al., 2000). In addition, NRTN promotes the survival of basal forebrain cholinergic neurons (Golden et al., 2003) and spinal motor neurons (Garces et al., 2000). Thus, NRTN has potential in treatment of Alzheimer’s disease and ALS. NRTN is important for the development and maintenance of the parasympathetic neurons (Rossi et al., 1999). In addition, similarly to GDNF, NRTN and GFRα2 mediate innervation of the gut by enteric neurons as demonstrated by gene ablation (Heuckeroth et al., 1999; Rossi et al., 1999).

ARTN acts as a chemoattractant for sympathetic neurons and gut haematopoietic cells (Veiga-Fernandes et al., 2007). In the absence of ATRN signaling SCG neurons are misguided because they lack guidance clues to reach their target. ARTN also supports the survival of sensory neurons and has a therapeutic potential for treatment of neuropathy (Gardell et al., 2003; Wang et al., 2008). ARTN is currently in Phase I clinical trial by Biogen Idec/NsGene for chronic pain (NsGene’s Press release, 2009)

PSPN promotes the survival of mouse embryonic basal forebrain cholinergic neurons in vitro (Golden et al., 2003). Hence, PSPN may be useful for the treatment of Alzheimer’s disease. In vivo, PSPN regulates calcitonin production by thyroid C-cells (Lindfors et al., 2006). PSPN can promote the survival of dopaminergic neurons (Milbrandt et al., 1998; Åkerud et al., 2002; Zühlmann et
In addition, PSPN may have clinical applications in the treatment of stroke (Tomac et al., 2002).

1.3. GFLs receptors: GFRα/RET receptor complex

The RET oncogene was originally cloned by Masahide Takahashi and coworkers (1985) as a novel gene with transforming activity. The oncogenic activity appeared as the result of RET-encoded tyrosine kinase domain recombination with N-terminal domain bearing the dimerization motif of another gene.

RET proto-oncogene encodes for the type I transmembrane RTK, which consist of four cadherin-like domains, membrane-proximal cysteine-rich domain and intracellular tyrosine kinase domain (Anders et al., 2001) (Fig. 2).

Mechanism of RET activation

The classic hypothesis implies RTK activation occurs when a dimeric ligand binds simultaneously to two receptor molecules, inducing their dimerization. Receptor dimerization is further stabilized by additional receptor-receptor interactions (Schlessinger, 2000). Dimerization of the receptors leads to the association of tyrosine kinase domains and subsequent transphosphorylation of the activating loops of the kinases. This in turn renders a conformational change in the kinase domain structure unleashing the full kinase activity of the enzyme (Hubbard, 2004).

However, there are several exceptions from this concept. Insulin receptor family are covalently-linked heterotetramers (Van Obberghen, 1994). They are activated by conformational change induced by the ligand (Hubbard, 2004). Another exception is EGF receptor (EGFR). Its extracellular domain (ECD) assumes a folded configuration in the absence of ligand, which efficiently prevents spontaneous dimerization and activation of the receptor (Ferguson et al., 2003). Upon ligand binding a conformational change occurs that is likely to involve rotation of the transmembrane α-helices and concomitant realignment of the cytoplasmic domains. Realigned kinase domains of the EGFR are more likely to transphosphorylate each other (Moriki et al., 2001).

Erythropoietin (Epo) is a cytokine, which has neurotrophic factors activities (Digicaylioglu and Lipton, 2001). Epo receptor (EpoR) is a non-tyrosine kinase receptor associated constitutively with the tyrosine kinase JAK2. Unliganded ECD of the EpoR exists as a non-covalent dimer in a X-shaped conformation with wide gap between the membrane proximal domains, which are oriented at an angle of 135° and transmembrane domains are separated by 73Å (Livnah et al., 1999). This separates JAK2 kinases making the transphosphorylation unlikely. When the ligand binds to the receptor, the X-shaped conformation of the ECD changes to the Y-shape and the gap closes (30Å). Thus, JAK2 kinases are placed in proximity of each other and become fully active (Remy et al., 1999). Hence, while dimerization is required, it is not always sufficient for receptor activation.

Unliganded RTKs always have a basic level of activity, as auto-phosphorylation of RTK can be enhanced by tyrosine phosphatase inhibitors or by receptor overexpression even in the absence of ligand binding (Runenberg-Roos et al., 2007; Schlessinger, 2000). It is logical to assume that the receptor monomers are in the equilibrium with the receptor dimers (inactive dimer). Inactive dimers are in turn in the equilibrium with a population of the receptor dimers existing in a configuration of their ECD and TK that
are compatible with transphosphorylation (active dimer) (Fig. 3). Hence, unlike the wide-spread “all-or-nothing” mechanism, the activation of the RTK by the ligand is “all-or-something”.

The extracellular domain of RET is very different from other RTKs. It lacks leucine zipper, immunoglobulin or fibronectin-like domains, which are the characteristics of most RTK’s ECD. RET ECD harbors four cadherin-like domains and is similar to the cadherin family of cell adhesion molecules, sharing up to 40% of homology (Anders et al., 2001). Cadherin’s functioning depends on calcium. Ca\(^{2+}\) binds in the cleft between domains thus rigidifying and linearizing the molecule. According to the RET model by Anders and colleagues (2001), Ca\(^{2+}\) binds in-between cadherin-like domain two and three and is required for GDNF-induced RET activation. Ca\(^{2+}\) binding to this position would make connection between second and third domains rather rigid but

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**Fig. 3.** Activation modes of RTKs. Free receptor monomers are in a dynamic equilibrium with the dimerized receptors. Some of the dimers are active while the others are inactive. In the presence of the specific ligand the balance shifts towards active dimers and results in biologically significant signaling (after Schlessinger, 2000).
would permit other domains flexibility. Another study suggests that Ca\textsuperscript{2+} binding to cadherins promotes dimerization of cadherin domains (Nagar et al., 1996) and may be important for the stabilization of RET dimerization upon ligand binding.

Activation of RET, similarly to the activation of other RTKs, requires dimerization. Evidence that dimerization alone is sufficient for activation comes from the oncogenic form of RET. Germline missense mutations in the RET gene cause multiple endocrine neoplasia type 2A (MEN2A) by producing constitutively active disulfide-bonded dimer (Mulligan et al., 1994). All mutations known to cause MEN2A are in the extracellular cysteine-rich domain of RET. MEN2A mutations involve cysteine substitutions in positions 609, 611, 618, 620, 630 and the most common mutation for MEN2A - cysteine 634 substitution by arginine (Marx, 2005). This mutation eliminates one of the cysteines, which normally makes intramolecular disulfide bond with another cysteine. This allows the unpaired cysteine to participate in the intermolecular reaction to generate an active oncogenic dimer. RET overexpression also leads to its activation in the absence of ligand apparently due to spontaneous dimerization (Liu et al., 1996; Trupp et al., 1998; Runeberg-Roos et al., 2007).

One of the plausible mechanisms that cells utilize to diminish possible adverse effects of the spontaneous unliganded RET activation may be its localization in the microenvironment incompetent for signaling. In the absence of the ligand, RET is located outside lipid rafts, which are small dynamic plasma membrane-located “signalosomes”. Upon ligand binding RET is recruited to lipid rafts where it interacts with a variety of signaling molecules (Tansey et al., 2000).

The MEN2A form of RET is further activated by the administration of the ligand (Mograbi et al., 2001). This suggests that covalently-linked dimer exists with a suboptimal quaternary structure and that the ligand triggers a conformational change, realigning kinase domains for optimal transactivation.

The most membrane-distant RET domain (first cadherin like domain; CLD1), is crucial for GDNF-induced activation (Kjær and Ibáñez, 2003). GDNF binds GFRα1 close to the membrane, whereas CLD1 domain of RET is perceived to be distant from the membrane. Therefore, this result suggests that RET bending towards plasma membrane is necessary for RET activation by the ligand. However, the study that used chemical cross-linking of purified receptor-ligand complexes followed by mass spectrometry fingerprinting revealed that the third cadherin-like and cysteine-rich domains of RET are in direct contact with GFRα1 (Amoresano et al., 2005). The first cadherin-like domain of RET is separated from the second one by 10 amino acids and has profound solvent accessible surfaces with negative and positive charges (Anders et al, 2001). This fact hints that CLD1 may interact intramolecularly with CLD2 or CLD3 (and possibly have autoinhibitory function for unliganded RET as domain 1 of FGFR). Alternatively, CLD1 may interact with another RET protomer (Fig. 10) or with other molecules like heparin. Alternatively, the first three cadherin-like domains may have stabilization function for the ligand-receptor complex or create a tertiary structure permissive for ligand-induced dimerization and activation.

A question remains whether RET dimerization is sufficient for its full activation. This information is crucial for the design of RET agonists for clinical
applications. Therefore, structural information on the RET in complex with GFRα/GFL would be important to obtain.

Similarly to muscle-specific-kinase (MuSK) and unlike other RTKs, RET forms a multi-component signaling complex (Hubbard and Miller, 2007). The stoichiometry of the signaling complex is not known, but based on the fact that GFLs are covalently-linked dimers it was proposed by Jing et al. (1996) to be (GFL)₂:(GFRα)₂:(RET)₂. Currently, there is no agreement whether the true ligand for RET is the GFRα/GFL complex or whether GFL is a ligand for the preformed GFRα/RET receptor complex (Schlee et al., 2006). This question is essential when considering the GFLs mimetics for the treatment of the neurodegenerative diseases. If the preformed GFRα/RET complex exists in biologically significant concentration, the possible repertoire of targets for GFL-based drug design broadens. Bivalent molecules binding two monomeric GFRαs or small molecules that allosterically alter GFRα/RET structure inducing transphosphorylation may be clinically useful.

Supportive of the pre-formed complex hypothesis are the facts that GFRα1/RET –complex provides additional binding sites for GDNF compared to GFRα1 alone (Eketjäll et al., 1999). In the presence of RET GDNF binds to GFRα1 10 times more tightly (Cik et al., 2000). An alternative explanation of these facts is that GFRα1 in complex with RET might undergo a conformational change becoming more affine to GDNF.

Another evidence of this hypothesis was a finding that small molecules selected by competition with GDNF for GFRα1 binding site can act as RET agonists (Tokugawa et al., 2003).

**RET kinase domain**

RET tyrosine kinase domain shares up to 55% homology with other kinases and the crystal structure of inactive RET kinase domain represents a typical kinase fold (Knowles et al., 2006) (Fig. 4A, 4B). The structure also revealed an activation loop, which is a key structural element in the regulation of catalytic activity in many kinases. When unphosphorylated, the activation loop is usually not positioned optimally for phosphorylation (reviewed by Hubbard and Till, 2000). The basal activity of the unphosphorylated kinase domain is low, but it is sufficient for transphosphorylation to occur within the ligand-stabilized dimer (Hubbard, 2004). Transphosphorylation of the activation loop is accompanied by a conformational change that secures the domain in catalytically competent configuration (Johnson et al., 1996). This happens in most kinases but not in RET.

The activation loop of RET contains two tyrosine residues at positions 900 and 905 (Knowles et al., 2006). Both tyrosines were found to be phosphorylated in vitro and Tyr-905 was proposed to stabilize the active conformation of the kinase (Iwashita et al., 1996; Pandey et al., 1995). However, the more recent biochemical data revealed only a moderate increase (3-4 times) of the kinase activity after phosphorylation of tyrosines in the activation loop (Knowles et al., 2006). It is, however, possible that tyrosine residues, which are used by downstream adaptors to relay the signal, are only effectively phosphorylated in trans. Thus, moderate increase in the activity of phospho-Tyr-905-RET may not fully reflect the consequences for the downstream signaling. Another important feature of RET revealed by the crystal structure of its kinase domain posing RET in unique position was that no significant
**Fig. 4.** RET kinase domain structure.

A. Unphosphorylated “inactive” RET kinase.

B. Phosphorylated “active” RET kinase domain. Adenosine monophosphate (AMP) is shown in the binding pocket. Phosphotyrosine 905 in RET “activation loop” and methionine-918 are shown as sticks.

C. Substrate binding pocket and catalytic center of the active RET-MEN2B kinase. Threonine in position 918 (instead of wild-type methionine, M918T) may stabilize bound substrate (orange ribbon with amino acids represented as sticks).
difference between phosphorylated (active) and unphosphorylated forms was observed. These observations leave a question on the molecular mechanisms of RET kinase activation open.

**Downstream signaling**

GDNF binding to GFRα1/RET activates several pathways typical for RTK signaling. The cytoplasmic domain of the long isoform of RET contains 18 tyrosine residues, twelve of which are phosphorylated upon receptor activation (Kawamoto et al., 2004; Liu et al., 1996).

Phosphorylated residues Tyr905, Tyr981, Tyr1015, and Tyr1096 are the docking sites for Grb7/Grb10, Src, phospholipase C-γ (PLC-γ), and Grb2, respectively (Alberti et al., 1998; Borrello et al., 1996; Encinas et al., 2004; Pandey et al., 1995; Pandey et al., 1996). Tyr1062 has a broad spectrum of engaged effector proteins: IRS1/2, Dok1, Dok4/5, Dok6, Enigma, FRS2, and PKC-α, She and ShcC (reviewed by Santoro, 2004). There is evidence that also Tyr752 and 928 can be phosphorylated and may activate STAT3 (Schuringa et al., 2001). The role of phosphorylation of Tyr806, 809, 826, 900, 1029 and 1090 remains to be shown.

The recruitment of these adaptor proteins activates a number of signaling pathways. For example this triggers Ras/RAF pathway, which leads to the activation of the mitogen-activated protein kinases ERK1 and ERK2, ERK5, p38MAPK, phosphatidylinositol 3-kinase (PI3K) and subsequent activation of the Akt-kinase, Jun NH2-terminal protein kinase (JNK) and PLC-γ. These pathways lead to profound biological effects: cell survival, differentiation, growth and migration, among others.

Tyr1062 appears to be the most important docking site for RET functioning. Knock-in mice with Tyr1062Phe substitution revealed the phenotype similar to full RET knockout (Jijiwa et al., 2004; Wong et al., 2005). Tyr1015 also appears to be crucial as mice with Tyr1015Phe substitution have severe kidney defects (Jain et al., 2006) providing insights into the pathogenesis of congenital anomalies of kidneys and the lower urinary tract (CAKUT).

In response to an elevated level of cyclic AMP (cAMP) the receptor tyrosine kinase RET can be phosphorylated on serine 697 (or 696 in human) . This molecular event is protein kinase A (PKA)-dependent and may be important to GDNF-induced lamellipodia formation through Rac activation (Fukuda et al., 2002). Serine/threonine phosphorylation by protein kinase C in the juxtamembrane regions of EGFR and Met suppresses tyrosine phosphorylation (Gandino et al., 1990; Hunter et al., 1984). However, it is unlikely that the juxtamembrane region of RET plays an autoinhibitory role (Hubbard, 2004; Knowles et al., 2006).

RET-deficient mice, similarly to GDNF- and GFRα-deficient mice (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996), die at birth due to the renal agenesis and severe hypodysplasia (Schuchardt et al., 1994). Enteric nervous system is also severely impaired in mice lacking RET - neurons fail to innervate the gut below stomach (Taraviras et al., 1999). The conditional ablation of GFRα1 in enteric neurons also leads to the defects in enteric innervation (Uesaka et al., 2007). The defects were also found in the sympathetic and parasympathetic neurons in the absence of GDNF signaling via RET (Enomoto et al., 2001; Enomoto et al., 2000). In the CNS, loss of motoneurons was observed when RET was deleted (Oppenheim et al., 2000).
A study by Kramer and co-authors (2007) on conditional RET knockout in the CNS and in dopaminergic neurons suggest age-dependent loss of these neurons. The effect was significant in one year old mice and further manifested itself during aging. An earlier study performed in younger animals that also lack RET gene in dopamine neurons reported no phenotype (Jain et al., 2006b). A recent study has also indicated the significance of GDNF for the maintenance of dopaminergic neurons (Pascual et al., 2008).

**Lipid rafts and their role in GDNF signaling**

As GFRα1 is a GPI-anchored protein, it is located in lipid rafts. The definition of lipid rafts is as follows: “Small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes at the cell membrane. Small rafts can sometimes be stabilized to form large platforms through protein-protein and protein-lipid interactions” (Pike, 2006). Although the methods to study lipid rafts and the concept itself were heavily criticized (Munro, 2003; Shaw, 2006) it remains important for understanding of many biological phenomena (Simons and Toomre, 2000).

Lipid rafts contain many signaling molecules. Some of them, for example Src kinases or adaptor protein FRS2, are covalently linked to the cytoplasmic leaf of the raft, which is enriched in phospholipids. There is increasing evidence that lipid microenvironment is crucial for GFLs signaling (reviewed by Saarma, 2001; Paratcha and Ibáñez, 2002). Unliganded RET is located outside lipid rafts. GFRα1-mediated RET activation by GDNF brings RET to lipid rafts where signaling occurs (Tansey et al., 2000). It is possible that unliganded RET localization outside the lipid rafts is one of the mechanisms to prevent signaling of the spontaneous RET dimers.

Oligomerization of other GPI-anchored proteins by antibodies or ligands leads to Src family kinases activation, resulting in the transmembrane-receptor-independent signaling (Stefanova et al., 1991). A plausible mechanism is protein oligomerization-driven clusterization of distinct lipids rafts followed by trans-activation of Src kinases. A similar process might contribute to RET-independent GDNF-induced Src activation through GFRα1 (Trupp et al., 1999; Poteryaev et al., 1999).

GDNF binding to soluble GFRα1 also triggers RET recruitment to lipid rafts. The exact mechanism of this phenomenon is not fully understood but it requires RET tyrosine kinase activity (Paratcha et al., 2001). Paratcha and coworkers (2001) also demonstrated that GFRα1 can be released by Schwann cells and neurons. They showed that sciatic nerve lesion leads to release of GFRα1.

These findings may help to rationalize the fact that GFRα1 is often expressed in the absence of RET (Trupp et al., 1997; Yu et al., 1998). According to models proposed by Paratcha et al. the free GDNF and GFRα1 can diffuse over long distances to RET-expressing cells. Whereas, GPI-anchored GFRα1 can present GDNF only to neighboring RET expressing cells (this transactivation needs a direct contact between cells).

In vitro, soluble and extracellular matrix (ECM)-associated GFRα1 potentiates intracellular signaling, neurite outgrowth and neuronal survival. In vivo, the relevance of this type of signaling maybe restricted to CNS (Enomoto et al., 2004; Canty et al., 2009).
**RET as a dependence receptor**

An emerging theme in the growth factor receptors biology is the concept of “dependence receptors”. Activation of the dependence receptor by its ligand promotes cell survival while death may be actively promoted by the same receptor in the absence of ligand (Tauszig-Delamasure et al., 2007). Several receptors including deleted in colorectal cancer (DCC), Netrin-1 receptor UNC5H2 and TrkC act as dependence receptors (Forcet et al., 2001; Llambi et al., 2005; Tauszig-Delamasure et al., 2007). When unliganded, RET also triggers cell death when overexpressed in some cell lines, and this effect is blocked by GDNF (Bordeaux et al., 2000). The mechanism is likely to include caspase-mediated cleavage of a cytoplasmic fragment of RET. This fragment appears to have pro-apoptotic activity because it alone is sufficient to trigger apoptosis (Bordeaux et al., 2000).

The hypothesis of RET as a dependence receptor is supported by the analysis of somatotrophs from pituitary of RET knockout animals and cell cultures (Cañibano et al., 2007). This study revealed that in RET-deficient mice the adenopituitary was larger than normal with somatotroph hyperplasia. In vivo transduction of RET into pituitary reduced the manifestation of this phenotype.

In GDNF-deprived sympathetic and dopaminergic neurons, RET is likely to induce cell death via a novel non-mitochondrial pathway (Yu et al., 2003; Yu et al., 2008). Overexpression of the pro-apoptotic RET fragment did not cause death of sympathetic neurons. Yu and colleagues, therefore, reasoned that RET, similarly to DCC receptor (Forcet et al., 2001), may be able to recruit and activate caspases directly. However, to reveal the physiological relevance of this concept the development of knock-in mouse models with mutated putative caspase cleavage sites would be required.

**1.4. RET in disease**

Chromosomal aberrations and point-mutations in RET cause a large portion (up to 61%) of papillary thyroid cancers (Thomas et al., 1999), the majority of medullary thyroid carcinomas and all multiple endocrine neoplasia type 2 (Hofstra et al., 1994). Abnormalities in RET gene are especially common in patients with pediatric thyroid cancers (Nikiforov et al., 1997) but are also responsible for up to 43 % of adult cases (Lam et al., 1998).

Somatic rearrangements of RET gene can occur as a result of ionizing radiation and because thyroid follicular cells accumulate $^{131}$-iodine (Santoro et al., 2000), which is the first stable isotope of uranium decay. Up to 57% of children who suffered from thyroid cancer as a result of Chernobyl disaster had a papillary thyroid carcinoma (PTC). In these cases RET tyrosine kinase domain was fused with 5'-terminal regions of heterologous genes (Fugazzola et al., 1995; Ito et al., 1994), generating chimeric oncogene designated as RET/PTC. RET normally is not expressed by thyroid follicular cells but in the case of RET/PTC the expression is driven by promoter of the fused gene. The fusion partner, which may be considered as an activating element, usually lacks signal peptide to deliver the RET/PTC chimera to the plasma membrane but the activating element contains a dimerization motif (Santoro et al., 2002). Thus, this fusion protein induces ligand-independent RET kinase activation in the cytoplasm.

Gain-of-function germline missense mutations in RET cause MEN2A and MEN2B syndrome and familial
medullary thyroid carcinoma (FMTC). Molecular signature of MEN2A syndrome has already been discussed earlier in this text. MEN2B-RET is activated independently of ligand and does not require dimerization. MEN2B syndrome is characterized by the most aggressive disease progression, the earliest onset and the worst prognosis among the patients with RET mutations. Majority of MEN2B cases are caused by single substitution of methionine-918 with threonine in the kinase domain of RET (Carlson et al., 1994) (Fig. 4C). Substitution of alanine 883 to phenylalanine also causes MEN2B (Smith et al., 1997). Double mutations Val804Met/Val804Met and Val804Met/Ser904Cys are involved in MEN2B as well (Menko et al., 2002; Miyauchi et al., 1999).

The residue corresponding to methionine 918 is highly conserved in all receptor tyrosine kinases, whereas cytoplasmic protein tyrosine kinases have a threonine in this position. This residue is predicted to alter the substrate selection, as it was mapped to the substrate binding pocket (Pandit et al., 1996; Songyang et al., 1995). Indeed, substitution of bulky methionine with smaller and polar threonine may result in tighter substrate binding and increased catalytic activity (Fig. 4C).

However, the model of RET inhibition in trans (Knowles et al., 2006) suggests that Met918 may be a hot-spot of inhibitory dimer formation, which efficiently blocks ATP- and substrate-binding pockets. The substitution with threonine in position 918 would result in kinase dimer destabilization and thus kinase activation.

Similarly, two transforming mutations, P766S and E768D/A919P (Iwashita et al., 2000; Santoro et al., 2004), are at the dimer interface (Knowles et al., 2006).

The MEN2B mutations lead to a qualitatively distinct from wild type RET signaling, as revealed by phosphopeptide mapping analysis (Liu et al., 1996). The pattern of phosphorylated intracellular proteins is also different (Murakami et al., 1999; Santoro et al., 1995).

FMTC-causing mutations in the kinase domain of RET include Glu768Asp, Leu790Phe, Tyr791Phe, Val804Leu/Met, Ser891Ala and a double mutation Val778Ile/Val804Cys (reviewed by Ichihara et al., 2004). Some somatic RET mutations may cause sporadic medullary carcinoma. The molecular mechanisms underlying RET activation by these mutations are poorly understood.

In addition to gain-of-function mutations, loss-of-function mutations of RET exist. RET is abundantly expressed in neuronal progenitors of the vagal, trunk and sacral neural crest cells (Pachnis et al., 1993), which give rise to ENS and are important to the formation of ENS. Loss-of-function mutations in RET cause Hirschsprung’s disease (HSCR), which is characterized by megacolon with aganglionosis (Parisi and Kapur, 2000). The management of the disease currently requires operational removal of aganglionic segment of colon.

HSCR mutations in RET are divided into four classes. Class I mutations are in the ECD. These mutations appear to interfere with RET maturation and inhibit its translocation to the plasma membrane (Carломagno et al., 1996). Class II mutations cause the substitution of the cysteine residues in the cysteine-rich domain (Cys609, 611, 618, and 620). Paradoxically, these mutations are also responsible for MEN2A and FMTC, which involve constitutively active forms of RET.
Furthermore, in some individuals MEN2A/FMTC coincides with HSCR (Takahashi et al., 1999). This could be explained by the fact that the HSCR-RET residing in the endoplasmic reticulum cannot respond to GDNF and guide neurons along GDNF gradient, but its oncogenic potential is nevertheless realized in the cytoplasm (Runeberg-Roos et al., 2007). Class III mutations affect the tyrosine kinase domain and suppress its catalytic activity. Class IV mutations specifically interfere with the binding of the adaptor proteins to activated RET (Iwashita et al., 2001).

One may envisage that pharmaceuticals enhancing protein folding (i.e. chemical chaperones) may help patients with HSCR type I and type II mutations. Conversely, tyrosine kinase inhibitors like Gleevec inhibit cell growth and RET kinase activity in vitro (Cohen et al., 2002). The pyrazolo-pyrimidine PP1 blocks tumorigenesis induced by RET/PTC (Carlomagno et al., 2002a). Another pyrazolo-pyrimidine PP2 and the 4-anilinoquinazoline are inhibitory to oncogenic RET kinases (Carlomagno et al., 2002b; 2003). These two pyrazolo-pyrimidines are, however, not clinically relevant because of unspecificity.

One phase II clinical trial was recently completed with Sorafenib (multi-kinase inhibitor targeting RET, PDGFR, VEGFR and Raf) demonstrating its effectiveness in advanced thyroid cancer (Gupta-Abramson et al., 2008). Another drug XL184 is under intense clinical study and ZD6474 (Zactima) may also result in an improvement of advanced thyroid cancer management (www.ClinicalTrials.gov). In addition, a novel class of inhibitory macromolecules has been developed. These molecules are known as aptamers (specific oligonucleotide ligands) that were selected by in vitro evolution technology (Cerchia et al., 2005). The neutralizing aptamers obtained in this study interfere with the MEN2A mutation-induced RET dimerization and thus prevent its activation.

1.5. RET-independent GFLs signaling

Evidence for RET-independent GFLs signaling

The widespread expression of GFRα1 in the absence of RET suggests that alternative GFLs receptor may exist. In the brain GFRα1 is expressed in certain RET-lacking areas like forebrain, cortex and hippocampus (Trupp et al., 1997; Yu et al., 1998). GFRα1 is expressed also in the inner ear, where RET is not (Ylikoski et al., 1998). Being an extracellular GPI-anchored protein, GFRα1 may evoke intracellular signaling directly (Stefanova et al., 1991; Ledda et al., 2007) but, in most cases, GFRα1 requires a transmembrane receptor to mediate GDNF-induced signal.

RET-independent signaling was discovered in cell lines and neurons deficient in RET (Trupp et al., 1999; Poteryaev et al., 1999). GDNF can rapidly induce Src-family kinases (SFK) activation in GFRα1-dependent manner. GDNF also robustly activates MAPK and PLCγ leading to CREB phosphorylation. Furthermore, in kidney explants from RET-deficient mice GDNF increased branching morphogenesis through Src kinase-dependent activation of the receptor tyrosine kinase MET. Activation of MET by GDNF is likely to occur via intracellular signaling circuit, for direct interaction between GDNF and MET was not detected (Popsueva et al., 2003). In addition, GDNF activates rat peritoneal macrophages in a RET-independent manner (Hashimoto et al., 2005).
GDNF can, in the absence of RET, induce homophilic interaction between two GFRα1 molecules located on opposed synaptic termini (Ledda et al., 2007). This trans-interaction leads to precise spatial presynaptic differentiation and was termed ligand-induced cell adhesion.

Chao and colleagues (2003) demonstrated that GDNF can upregulate the expression of NCAM and αv integrin subunit in rat midbrain dopaminergic neurons. Function-blocking antibodies against NCAM and αv integrin inhibited GDNF-induced survival and neurite outgrowth. Authors therefore proposed that αv integrin and NCAM mediate effects of GDNF. Another report suggested that GDNF can signal via β1 integrin (Cao et al., 2008).

**Neural cell adhesion molecule is an alternative GFL receptor**

The study highlighting the role of NCAM in GDNF-induced signaling was carried out by Paratcha and coworkers (2003). NCAM is a cell adhesion molecule in the nervous system (Rutishauser et al., 1988) that promotes cell adhesion and migration, neurite outgrowth, synaptogenesis and regulates synaptic plasticity (Fields and Itoh, 1996; Schachner, 1997). NCAM is highly expressed in many developing tissues and, in particular, it localizes in the nerve growth cone (van den Pol et al., 1986). Hence, NCAM is thought to play a role in the development of nervous system.

The regulation of these developmental processes is mediated mostly by homophilic interaction of NCAM. It belongs to immunoglobulin superfamily with its five Ig-like extracellular domains. Homophilic interaction occurs between NCAM Ig-domain 3 on opposing cells (Rao et al., 1993). This interaction may result in the activation of cytoplasmic signaling molecules associated with NCAM (Maness et al., 1996).

Isoforms of 180, 140 and 120 kD result from alternative mRNA splicing. Isoforms 180 and 140 are single-pass transmembrane proteins, whereas 120 kD isoform is a GPI-anchored protein lacking cytoplasmic domain. By virtue of its GPI anchor isoform, 120 is located in the lipid rafts. Other NCAM isoforms are also likely to be located in the lipid rafts due to palmitoylation, which is crucial for NCAM-induced signaling. A negatively charged glycan, the polysialic acid (PSA), is associated with NCAM (Finne et al., 1983). In vitro studies on purified PSA-NCAM demonstrated that PSA decreases homophilic NCAM-mediated interaction. It is therefore considered to be the anti-adhesive component of NCAM molecule that promotes migration (Kleene and Schachner, 2004).

The cytoplasmic domain of NCAM is constitutively associated with a SFK Fyn. Fyn becomes rapidly and transiently activated upon NCAM homophilic oligomerization (Beggs et al., 1997). This leads to the cytoskeletal rearrangements including neurite outgrowth and cell migration. NCAM can also transactivate the fibroblast growth factor (FGF) receptor (Saffell et al., 1997). NCAM contains a heparin binding pocket and therefore interacts with heparan sulfate proteoglycans (HSPGs) (Storms and Rutishauser, 1998), the family of proteins that includes transmembrane receptors modulating various cellular processes (see below). In addition, PSA-NCAM modulates the activity of neurotrophic factors. BDNF is accumulated by PSA-NCAM and presented to the BDNF receptor TrkB, thus enhancing biological activity of BDNF for survival and
differentiation of neurons, presumably by increasing local concentration of BDNF in the vicinity of TrkB (Vutskits et al., 2001).

NCAM-deficient mice are viable and fertile (Cremer et al., 1994). The reported phenotypes include deficits in neuronal migration and synaptic organization, as well as marked impairment in synaptic plasticity, spatial learning and long-term potentiation (LTP). The most distinctive abnormality in the CNS of these mice is a reduction of the size of the olfactory bulbs (Cremer et al., 1994). This is caused by migratory deficits of neuronal precursors in the rostral migratory stream (RMS). This pathway directs cell migration from subventricular zone to the olfactory bulb. PSA-NCAM is expressed along entire RMS and is important for neuronal migration towards olfactory bulb. In the absence of NCAM cells accumulate in the RMS thus widening it (Paratcha et al., 2003).

GDNF binds to NCAM in the presence of GFRα1 with high affinity ($K_d \sim 1nM$). GDNF also interacts with NCAM alone, although with lower affinity ($K_d \sim 5nM$) (Paratcha et al., 2003). Downstream signaling pathways associated with NCAM could be activated in non-homophilic manner. GDNF was demonstrated to potently activate Fyn through GFRα1/NCAM receptor complex. Cell-cell adhesion mediated by homophilic interaction of NCAM was impaired in the presence of GFRα1, further supporting the hypothesis of functional complex formation between NCAM and GFRα1. Interestingly, the inhibition of cell-cell adhesion was impaired even in the absence of ligand when both NCAM and GFRα1 were overexpressed. This suggests that, similarly to GFRα1/RET, functional preformed GFRα1/NCAM complex possibly exists. The impairment of cell-cell adhesion hinted that cellular migration, especially when cells use other cells as a substrate to move (known as chain migration), may be enhanced when NCAM-NCAM homophilic interaction is inhibited. Indeed, the migration of Schwann cells, expressing both GFRα1 and NCAM, from explants of rat sciatic nerve was greatly enhanced when explants were grown on the fibroblasts overexpressing GDNF. Other biological effects elicited by GDNF-NCAM signaling pathway include axonal growth of cortical and hippocampal neurons, when plated on immobilized GDNF. Importantly, soluble GDNF is ineffective inducer of neurite outgrowth. These findings suggest distinct signaling pathways to modulate short- and long-range (via RET) intercellular communication (Sariola and Saarma, 2003; Paratcha et al., 2006a)

Migration-promoting (or cell-cell adhesion-inhibiting) effects of GFRα1/NCAM complex have implications in vivo. In GFRα1-deficient mice, there is a characteristic widening of RMS similarly to NCAM-deficient mice.

In addition, adult heterozygous GDNF-deficient mice have impaired spatial learning similar to the NCAM mutants (Cremer et al., 1994; Gerlai et al., 2001). These results indicate that GDNF and GFRα1 can regulate neuronal synaptic plasticity through NCAM in the adult brain. The cross-talk between NCAM and RET may result from GDNF stimulation of the cell where both receptors are present. For example, NCAM function-blocking antibodies inhibited GDNF survival promoting effect in midbrain dopaminergic neurons, in which RET and GFRα1 are also present (Chao et al., 2003).

As NCAM and RET are not expressed in the developing brain cortex,
the finding that GDNF promotes migration of cortical GABAergic neuronal precursors (Pozas and Ibáñez, 2005) suggests the notion that additional receptors for GDNF exist. Another study further confirmed this, as mice manipulated to express RET and GFRA1 under the same promoter (Enomoto et al., 2004) exhibit a loss of GABAergic cells in the cortex (Canty et al., 2009).

1.6. Heparan sulfate proteoglycans

As communication between cells is of crucial importance for multicellular organisms, the environment for this interaction, i.e. the ECM, must be considered. The long range communication between cells requires the messenger molecules to be released, diffused and docked to the receptors on the target cell. The ECM can modulate all of these steps (Geiger et al., 2001; Hynes, 1999; Juliano, 2002).

ECM mostly consists of fibrillar proteins, polymerized sugars and proteins that present them. ECM provides spatial clues and a scaffold for the cellular adhesion and migration (Couchman, 2003). Although, these processes generally rely on the major ECM components, for many fine-tuned biological processes matrix-associated proteins are required. These proteins have an affinity to major ECM components and are expressed only transiently and/or locally (Kinnunen et al., 1996; Taipale et al., 1994).

Sugar components of the ECM are mostly glycosaminoglycans (GAGs). They are long, repeating, linear polymers of disaccharides. Usually, one sugar is a glucuronic acid or its epimer iduronic acid, and the other is either N-acetylg glucosamine or N-acetylgalactosamine (Bishop et al., 2007). GAGs are negatively charged carbohydrates due to their sulfate component. Therefore, GAGs may serve as a binding site for many matrix-associated proteins.

Proteoglycans that carry GAGs show considerable diversity, with variations in the protein core identity and structure. Nevertheless, the biochemical and biological properties of proteoglycans are dominated by their sugar components. Despite of the seemingly low complexity of GAGs’ constituents, the diversity created by the differences in epimerization and sulfation of these carbohydrate polymers can be huge (Bernfield et al., 1999). Notably, the epimerisation and sulfation signature of the GAGs can be unique for certain cells and can vary in the same cells during development.

There are several major classes of GAGs in the ECM: heparan sulfates (presented by ECD of syndecans, glypicans, perlecan and agrin), chondroitin sulfates (by syndecans, decorin and aggrecan), dermatan sulfates (by betaglycan) and keratan sulfates (by aggrecan) (Alberts, 2002).

Many of the matrix-associated proteins are growth factors that bind to one of the key ECM components, heparan sulfates (HS) (Bernfield et al., 1999; Esko and Selleck, 2002). This interaction modulates growth factors’ diffusion and binding to their receptors. Binding of some proteins to HS results in direct activation of signaling pathways induced by receptors bearing HS (Bass et al., 2007; Rauvala et al., 2000).

HS are presented by HSPGs. HSPGs can reside in ECM (perlecan, agrin and others) or be associated with cell plasma membrane (syndecans, glypicans, betaglycan, CD44v3) (Bishop et al., 2007). Glypicans are GPI-anchored proteins that lack transmembrane and intracellular domains and are unable to relay GF
signaling. Syndecans represent the type I class of transmembrane proteins (Fig. 5).

HSPGs were first brought into light by the discovery that HS chains are necessary for FGF signaling (Rapraeger et al., 1991; Yayon et al., 1991). FGFs are unable to activate their receptors (FGFR) in the absence of HS. Disruption of this interaction leads to drastic developmental consequences (García-García and Anderson, 2003).

Soon after the discovery of the HS requirement for FGFR activation, evidence from *Drosophila melanogaster* genetic screens suggested the involvement of HSPGs in the regulation of Wnt and Indian Hedgehog (Hh) pathways. Phenotypes of the mutations that affect embryonic segment polarity are strikingly similar to the phenotypes of those with mutations in the components of Wnt and Hh signaling pathways. Five genes were identified: *sugarless* (*sgl*), *sulfateless* (*sfl*), *tout velu* (*ttv*), *fringe connection* (*frc*) and *slalom* (*sll*). All of these genes were subsequently found to encode enzymes or nucleotide sugar transporters that are involved in GAG biosynthesis (reviewed by Häcker et al., 2005).

HSPGs are present on the surface of every eukaryotic cell (Sasisekharan et al., 2002), except certain lymphoblastoma cell lines (Lebakken and Rapraeger, 1996). HSPGs are generally more abundant at the cell surface than most receptors (Bernfield et al., 1999). Despite of high abundance, the interaction between GF and HS chains of syndecan can be very specific, in “lock-and-key” fashion, and tight (Bourin and Lindahl, 1993). Interactions are

**Fig. 5.** Homology model of syndecan-3. Heparan sulfate (HS) chains are shown as stick models. HS are attached to the serines in the Ser-Gly/Ala dipeptide sequences. Extracellular domain of syndecan was modeled by HHpred using invasin structure as a template. Intracellular domain was modeled on the basis of syndecan-4 cytoplasmic domain NMR structure.
characterized by relatively low $K_d$ values, ranging from 1nM to 100nM (Bernfield et al., 1999). Remarkably, interaction of certain GFLs with their high-affinity receptors also falls into this range (Cik et al., 2000).

The tissue-specific expression of individual proteoglycan core proteins will obviously determine when and where HS are expressed. It is important to stress that ligand binding by proteoglycans depends on the structure of their HS chains. Different extent of sulfation of disaccharides and epimerization of glucuronic acid can theoretically yield as many as 48 different disaccharides. However, only 23 disaccharides have been identified (Esko and Selleck, 2002), which is still a greater number than 20 naturally occurring amino acids. The structure of the HS chains ultimately depends on the regulated activity of multiple glycosyltransferases, sulfotransferases and an epimerase (Esko and Lindahl, 2001). These enzymes are arrayed in the lumen of the Golgi apparatus. A series of cytoplasmic enzymes are needed to catalyze the nucleotide-sugar and nucleotide-sulfate formation, which are actual substrates for the transferases. Surprisingly, the general organization of HS rather than their fine structure appears to determine the specificity of HS-protein interactions (Kreuger et al., 2006).

The biosynthesis of GAGs chains starts with the addition of xylose to serine residues in the core protein and proceeds by stepwise addition of two galactose residues and one glucuronic acid (Bishop et al., 2007). Alternating addition of N-acetylglucosamine and glucuronic acid leads to formation of the HS. The polymerization is accompanied by limited epimerization of glucuronic acid to iduronic acid (at C-5 position of the sugar) and by sulfation of the disaccharides at different positions (Fig. 6). Sulfation can occur at 2-O position in glucuronic or iduronic acid. Acetyl in N-acetylgalactosamnine can be substituted with sulfate. In addition, sulfation at 2-O, 3-O and 6-O positions of N-acetylgalactosamine can take place (Esko and Selleck, 2002).

Defects in the HS biosynthesis are devastating to the developing organism. The mutation in one of the crucial enzymes for HS biosynthesis in mice, UDP-glucose dehydrogenase, results in embryos arrest during gastrulation because of the failure of mesodermal migration (García-Garcia and Anderson, 2003). FGF8 knockout mice have very similar phenotype (Sun et al., 1999). Dysfunction of copolymerase EXT1, the enzyme that catalyzes HS polymerization, leads to the disruption of gastrulation and embryonic lethality before embryonic day 8.5 (E8.5). In EXT1−/− embryos Indian Hedgehog (Hh) fails to bind to appropriate target cells (Lin et al., 2000). Neural-specific conditional knockout of EXT1 showed that EXT1 is crucial for brain patterning and axon scaffold formation in the forebrain (Inatani et al., 2003). The mutation in enzyme 2-O-sulfotransferase, which is important for disaccharides’ sulfation, leads to renal agenesis in mice (Bullock et al., 1998). This defect in HS synthesis may affect many signaling pathways including Wnt, Hh and GDNF signaling. Furthermore, targeted disruption of a murine glucuronyl C5-epimerase gene results in kidney agenesis, lung and skeletal defects (Li et al., 2003). Similarly, mice deficient in N-deacetylase/N-sulfotransferase isoform 1 show immature lungs and severe skeletal abnormalities (Ringvall et al., 2000). These mice also exhibit forebrain and eye defects, cerebral hypoplasia and the
olfactory bulbs are absent (Grobe et al., 2005).

Mice deficient in the 6-O-sulfotransferases SULF1 and SULF2 have defects in the esophagus innervation (Ai et al., 2007).

**Syndecans**

The founding member of syndecan family of transmembrane HSPGs – syndecan-1 was discovered by Rapraeger and Bernfield (1983) in mammary epithelial cells. To date four syndecans have been characterized in vertebrates. The syndecan family includes: syndecan-1, syndecan-2 (also known as fibroglycan) (Marynen et al., 1989), syndecan-3 (N-syndecan) (Carey et al., 1992; Gould et al., 1992) and syndecan-4 (ryudocan or amphiglycan) (David et al., 1992; Kojima et al., 1992). Syndecan-1 is mostly expressed by epithelia. Syndecan-2 is abundant in mesenchymal tissues, liver and neuronal cells. Syndecan-3 is mostly neuronal. Syndecan-4 is ubiquitously expressed (Bernfield et al., 1999). Most cells express at least one member of the syndecan family (Lebakken and Rapraeger, 1996). However, many cells express two or more syndecans, suggesting that each syndecan may have specific functions at the cell surface (Kim et al., 1994).

The unifying features of the syndecan family, in addition to the presence of HS chains, are their highly conserved transmembrane and cytoplasmic domains. Intracellular domains of syndecans interact with many adaptor proteins that have an effect on actin dynamics thus driving cell adhesion and migration, neurite outgrowth and synapse formation (Couchman, 2003). In contrast, their ECDs are very divergent with the exception of HS chains attachment.

Syndecans are shown to “function as membrane receptors for a bewildering array of ligands” (Couchman, 2003). The growing list of proteins interacting with syndecans includes growth factors (TGFβ1&2; FGFs; VEGF; IGF-II, PDGF-AA; EGF family; among others), GF binding proteins (TGFβ-BP; follistatin; IGF-BP), morphogens (activin; BMPs; chordin; Dpp; Hh; Sprouty and Wnts), cytokines (ILs, GM-CSF; Interferon-γ; TNF-α), ECM components (Heparin-binding growth associated molecule (HB-GAM/pleiotrophin), fibronectin, laminins, tenascin, thrombospondin, vitronectin and collagen), cell adhesion molecules (NCAM, L-selectin, PECAM-1) and many others (Bernfield et al., 1999; Bishop et al., 2007).

**Fig. 6.** The structure of a major heparan sulfates building block, the disaccharide glucuronic acid (GlcA)/N-acetylglucosamine (GlcNAc). Epimerisation at position C-5 (5) of GlcA results in iduronic acid. N-sulfation occurs when acetyl in GlcNAc is substituted with sulfate. 2-O-sulfatation occurs in both GlcA and GlcNAc. However, 3-O-sulfatation and sulfatation in 6-O-position was observed only in GlcNAc.
Role of HSPGs in GDNF-signaling?

GDNF was originally purified by heparin-affinity chromatography (Lin et al., 1993) and later its interaction with heparan sulfates was documented (Rickard et al., 2003). Heparan sulfates appear to be required for the GDNF signaling via GFRα1 and RET (Sainio et al., 1997; Tanaka et al., 2002; Barnett et al., 2002). Thus, syndecans may act as co-receptors for GFRα1/RET receptor complex.

Barnett and colleagues (2002) demonstrated that in HS-deficient cells GDNF fails to efficiently induce RET phosphorylation. This results in the reduced biological activity of GDNF leading to impaired GDNF-induced neurite outgrowth from dorsal root ganglion neurons and tubulogenesis failure by epithelial kidney cells.

However, the molecular identity of GDNF binding ECM components was not revealed in these studies. Later, the HSPG agrin located in ECM was suggested to interact with GDNF (Iwase et al., 2005). Interaction with HS leads to obstructed diffusion of the exogenous GFLs from the injection site in the brain. This could exert adverse effects in Parkinson’s disease therapies utilizing GDNF or NRTN. Coinfusion of GFLs with heparin drastically increases GDNF, NRTN and ARTN biodistribution (Hamilton et al., 2001). In addition, mutated GDNF lacking its heparin-binding determinants on N-terminus (Alfano et al., 2007) diffuses more efficiently than its wild-type counterpart (Piltonen et al., 2009). This may have implications in the development of new GFL-based protein drugs against neurodegenerative diseases.

Syndecans mainly serve as low-affinity but high-capacity co-receptors for a variety of signal-transducing receptors. Binding to syndecan leads to ligand immobilization on the plasma membrane, which facilitates ligand binding to its primary receptor (Derksen et al., 2002). Localization of syndecans to specific membrane compartments, such as focal adhesions, sequesters ligands in the vicinity of their high-affinity receptors that are localized to the same structures. The distribution of syndecans on the cell surface is dictated in part by association with the cytoskeleton via their cytoplasmic domains (Carey, 1997).

As discussed above, syndecans being a HSPG provide HS chains for the stabilization of the FGF-FGFR complex. In addition, syndecans interact with NCAM (Storms and Rutishauser, 1998), a cell adhesion molecule and an alternative receptor for GDNF (Paratcha et al., 2003). ECD of NCAM contains a heparin-binding site in its second Ig-like domain (Cole and Glaser, 1986; Cole et al., 1986). It may interact with HSPG in the ECM, and with syndecans expressed on the same or on apposing cells. Therefore, syndecans may modulate the NCAM homophilic interaction or NCAM/GFRα1-mediated GDNF signaling.

Syndecans are also thought to modulate the activity of the axon guidance receptors: Robo, DCC and Eph. The modulation is likely to occur via interaction of the ECD of these receptors and HS chains of the syndecans (reviewed by Lee and Chien, 2005).

Syndecans also modulate the activity of integrins (Morgan et al., 2007). Cell attachment and spreading appears to be integrin-mediated, whereas later cytoskeletal rearrangement required for full spreading is likely to be HSPG-mediated (Gallagher, 1997). Syndecans cooperatively with integrins bind to the same ECM substrate but to discrete structural domains of the protein. For
instance, fibronectin contains a heparin-binding motif in one domain and the Arg-Gly-Asp (RGD) sequence, which is known to activate integrins, in another domain. It was also proposed that syndecans, via their HS chains, may directly modulate the integrin interaction with its ligand (Carey, 1997). However, whether the effect is promoted by HS chains or by syndecan core protein remains open (Beauvais et al., 2004). Affinity modulation of some integrins is complex and involves conformational changes within its ECD (Beglova et al., 2002). This process is regulated by “inside-out” signaling that affects integrin’s cytoplasmic domain either by proteolysis, phosphorylation or binding of intracellular proteins such as talin (reviewed by Hynes, 2002). These intracellular events lead to the exposure of the ligand-binding epitopes in integrin’s ECD.

As syndecans activate Src family kinases (Kinnunen et al., 1998) and protein kinase C alpha (PKCα) (Lebakken and Rapraeger, 1996; Oh et al., 1998) syndecan signaling may consequently activate integrins in an inside-out manner. Similar “inside-out” signaling may trigger syndecan ectodomain shedding (Bass et al., 2009).

**Syndecans signaling**

Syndecans lack intrinsic kinase or phosphatase activity but all syndecans induce a number of biological effects by activating several intracellular signaling pathways. The exact mechanism of how syndecans activate downstream signaling pathways is not known. Some evidence advocates that syndecan clustering and/or oligomerization occurs in several ligand-triggered cellular events occurs, including the syndecan-4 signaling in focal adhesions or HB-GAM-induced syndecan-3 activation (Rauvala et al., 2000).

Molecular mechanisms underlying spreading, migration and axon guidance involve changes in actin dynamics, and intracellular domains of syndecans can interact with many cytoskeletal proteins. Indeed, syndecan-2 interacts with actin-binding protein ezrin that regulates many aspects of actin assembly (Granés et al., 2000). Syndecan-3 activation by its ligands leads to Src-kinase activation and phosphorylation of cortactin, the protein that regulates branching of actin filaments (Kinnunen et al., 1998). Syndecan-4 is found in focal adhesions (Woods and Couchman, 1994), interacts with PKCα (Oh et al., 1998) and actin-bundling protein α-actinin (Greene et al., 2003). Syndecan-4 also modulates FAK phosphorylation (Wilcox-Adelman et al., 2002). Syndecan-1 has not been reported to interact with cytoskeletal proteins yet. However, Syndecan-1 overexpression renders floating lymphoblastoid cell line adhesive (Lebakken and Rapraeger, 1996). Taken together, these facts strongly suggest that syndecans can commute extracellular signals to the cytoskeleton, playing a pivotal role in many ECM-induced cellular processes.

Syndecan-3 is the only syndecan shown to signal independently via SFK activation without assistance from other receptors (Raulo et al., 1994; Kinnunen et al., 1998). The immobilized (but not the free) HB-GAM induces via syndecan-3 neurite outgrowth in rat embryonic hippocampal neurons and spreading of many cell types (Raulo et al., 1994; Kinnunen et al., 1998)

HB-GAM is a non-covalent homodimeric protein. The thrombospondin type I repeats in HB-GAM are responsible for heparan sulfate binding (Rauvala et al., 2000).
This protein structure, containing two or more heparin binding sites, provides a scaffold for syndecan-3 ligation. Indeed, it is easy to imagine that a protein with several valences for HS chains can induce clustering of syndecan while being bound to ECM. The syndecan-3 cytoplasmic domain is thought to be associated directly or via adaptor proteins with SFK. Oligomerized syndecan-3 thus may locally increase SFK concentration leading to transactivation of SFK. Alternatively, SFK may bind with higher affinity to the oligomerized receptor.

Furthermore, syndecan-4 clustering induces receptor translocation to lipid rafts (Tkachenko and Simons, 2002). This finding may be extrapolated to other syndecans because the translocation is likely to be mediated by the EFYA motif in the cytoplasmic domain, which is absolutely conserved in all syndecans (Bernfield et al., 1999). The fact that syndecans undergo translocation to the lipid rafts upon activation may shed light on the mechanisms of HB-GAM-induced SFK activation. Syndecans function as co-receptors for RTK may also require their localization in the rafts, since FGF2 signaling through FGFR requires HSPG-mediated FGFR translocation to lipid rafts (Chu et al., 2004).

Syndecan-3-deficient mice are viable and fertile. However, they exhibit an enhanced level of long-term potentiation (LTP) in CA1 region of the hippocampus (Kaksonen et al., 2002). Conversely, in HB-GAM-deficient mice, a lowered threshold for induction of LTP was observed, which was restored to the wild-type level by application of recombinant HB-GAM (Amet et al., 2001). Consistently, HB-GAM overexpressing mice show attenuated LTP. The changes in LTP, are accompanied by behavioral alterations. These findings pose HB-GAM as the only known extracellular macromolecule that inhibits LTP and suggest that syndecan-3 influences synaptic plasticity and hippocampus-dependent memory.

**Syndecans in disease**

In mammals, feeding behavior is controlled by several hypothalamic peptides. Antisatiety peptides (e.g. agouti-related protein, Agrp) enhance feeding when introduced into the brain, whereas satiety peptides, such as α-melanocyte stimulating hormone (α-MSH), reduce feeding. Agrp is an antagonist competing with α-MSH for binding to the common G-protein-coupled receptor (GPCR) (Woods et al., 1998). Syndecan-3 is expressed in hypothalamic feeding centers and modulates feeding behavior (Reizes et al., 2001). Syndecan-3 via its HS chains acts as a co-receptor for Agrp, thus increasing affinity of this antisatiety peptide to GPCR. Upon regulated cleavage of syndecan-3 ECD from the cell-surface, Agrp cannot interact with the receptor any longer and α-MSH can bind to GPCR, thus reducing feeding. These findings allow suggesting that mutations of syndecan-3 cleavage site, syndecan-3 overexpression or misexpression of other syndecans in hypothalamus as well as defects in proteases responsible for syndecan-3 ECD shedding would result in changes in feeding behavior and subsequently obesity. Defects in syndecan-3 gene or HB-GAM signaling may also lead to neurological (e.g. memory) disorders (Kaksonen et al., 2002; Pavlov et al., 2002) and developmental defects due to impaired neuronal migration (Hienola et al., 2006).

**Syndecan-1- and syndecan-4-null mice** do not have obvious developmental defects, and are fertile. However, when
challenged by wounding, their skin regeneration is slow (Alexopoulou et al., 2007). In syndecan-1-deficient mice the defect may arise from an impaired cell adhesion or GF binding (Stepp et al., 2002). Syndecan-4-deficient mice demonstrate reduced migration rate of fibroblasts and possibly impaired angiogenesis (Echtermeyer et al., 2001). RhoA and Rac1 are key proteins in cellular migration (Bar-Sagi and Hall, 2000) and were shown to be activated by syndecan-4 (Saoncella et al., 1999; Bass et al., 2007).

Because syndecan-1 is mostly epithelial, the question arose, whether there is a relationship between epithelial phenotype and syndecan-1 expression. As demonstrated by Kato et al. in 1995, the loss of syndecan-1 causes epithelia to transform into anchorage-independent mesenchyme-like cells. Mesenchymal cells have much higher migratory and invasive potential than epithelial cells. This prompted studies of syndecans in tumorigenesis, in which epithelial-mesenchymal transition occurs (Couchman, 2003). Indeed, there are several correlations between the loss of syndecan-1 and poor prognosis for patients with some head and neck carcinomas (Anttonen et al., 1999), as well as severity of the tumor grade in invasive cervical carcinoma (Rintala et al., 1999). Intriguingly, the loss of syndecan-1 is correlated with the loss of E-cadherin – a homophilic cell-cell adhesion receptor with certain tumor suppressor properties (Leppä et al., 1996).
2. AIMS OF THE STUDY

The aims of this study were to structurally and functionally characterize the known and novel GDNF receptors.

The specific aims were:
- Structural characterization of the GDNF co-receptor GFRα1 and their complex
- Search for novel GDNF receptors
# 3. MATERIALS AND METHODS

The selected methods used in this study are presented in the table below:

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heparin binding site usually consists of a stretch of basic amino acids (Lys, Arg or His). The canonical motifs were described by Cardin and Weintraub (1989) as B-B-X-B-B or B-B-B-X-X-B-B, where B is a basic amino acid and X is any residue. However, there is a number of deviations from this motif. For instance, in collagen V the positively charged residues that constitute heparin-binding site are separated by two to five amino acids (K905R909R912R918R921R924) (Delacoux et al., 1998).

Likewise, the GDNF’s secondary putative heparin-binding site consists of basic amino acids (R88K84K81) that are separated by 3 or 2 other residues. Nevertheless, this site can bind heparin because these residues are located on the same side of the α-helix, with the side chains pointing to the same direction (Fig. 7). It appears essential for heparin binding that the side chains of the basic residues are lined up. This is important because both heparin and HS are linear polymers with very limited winding capabilities. In addition, the spacing between lined basic residues defines the preferential binding of the protein to certain HS. The sulfation pattern of disaccharides constituting HS should comply with spatial arrangement of these basic amino acids for an effective interaction. This “lock-and-key” concept (Bourin and Lindahl, 1993; Kreuger et al., 2006) could help to define the structural determinants of protein-HS interaction.

By three independent methods – heparin-sepharose column chromatography, surface plasmon resonance (SPR) and scintillation proximity assay, we demonstrated that GDNF, NRTN and ARTN bind at high affinity (10-50 nM) to
heparin, whereas PSPN binds with a three orders of magnitude lower affinity.

The presence of 2-O-sulfation was previously found crucial for the interaction between GDNF and heparin/HS (Rickard et al., 2003; Rider, 2003), with additional involvement of other sulfated groups. We confirmed this finding and further demonstrated that 6-O-sulfation and N-sulfation are also important structural determinants of GDNF interaction with HS (I).

In most tissues the HS molecules are less sulfated and more complex glycosaminoglycans than heparin. However, in the brain HS are highly sulfated. To test whether the heparin-binding GFLs bind to natural HSPGs, we assayed by SPR the direct interaction between immobilized GFLs and the ECD of syndecan-3 purified from rat brain and carrying natural HS side chains. The interaction occurs specifically via HS chains, because the heparinase III treatment strongly inhibited the interaction (data not shown).

The dissociation constants derived for GDNF, NRTN and ARTN were in the 10-50 nM range, demonstrating high affinity for syndecan-3 and supporting the notion that these GFLs could also interact with other HSPGs, including those in the brain.

Fig. 7. Putative heparin binding sites in GFLs. GDNF protomer contains two secondary putative heparin binding sites: in the α-helix and in the finger 2. Basic amino acids constituting the sites are colored in light yellow. Manually docked heparin is surrounded by the surface of a calculated electrostatic potential. (transparent palettes were set as red and blue, corresponding to positive and negative charges). In NRTN and ARTN, basic residues constituting the putative heparin binding site are colored in light yellow and shown as ball-and-stick models.
ECM (Iwase et al., 2005) and possibly in other tissues. PSPN binds syndecan-3 at much lower affinity than the other GFLs. This also suggests that PSPN would lack affinity to HSPGs in the ECM, thus raising an intriguing possibility that, unlike other GFLs, PSPN diffuses long distances in tissues ultimately reaching body fluids. Conceivably, it may act as hormone-type growth factor, which would explain the fact that PSPN and its specific co-receptor GFRα4 are expressed in different tissues (Lindahl et al., 2000; Lindahl et al., 2001; Lindfors et al., 2006).

GDNF interacts with syndecan-3 also on the cell surface as demonstrated by chemical crosslink of iodinated GDNF followed by immunoprecipitation with syndecan-3 antibodies. In addition, GDNF induces oligomerization of syndecan-3 in cells expressing CFP- and YFP-tagged receptors, as revealed by Förster resonance energy transfer (FRET) studies (I).

The development and maintenance of an organism is regulated by concerted action of ECM-immobilized (matrix-bound) and diffusible (free) growth factors. To mimic the state in which matrix-bound proteins exist in vivo, we used surfaces coated with GFLs. When immobilized in this way, GDNF, NRTN and ARTN induce adhesion and spreading of cells in HSPG-dependent manner. Previously, soluble GDNF was demonstrated to induce robust Src-kinase and PLCγ activation in SHEP cells (Poteryaev et al., 1999). This cell line expresses GFRα1 and GFRα2 but lacks RET. Surprisingly, phosphatidylinositol-specific phospholipase C (PI-PLC) treatment, which specifically cleaves GPI-anchor of the proteins, failed to decrease the spreading of SHEP cells on immobilized GFLs. In addition, preincubation of GDNF-coated surface with GDNF function-blocking antibodies (G90, Amgen Inc.) also did not inhibit spreading. These antibodies disrupt the GDNF interaction with GFRα1/RET by binding to the central part of GDNF (Xu et al., 1998), whereas the primary heparin-binding site is located on the unique N-terminal protrusion of GDNF (I and Alfano et al., 2007) and, therefore, remains uncovered by the antibodies. Furthermore, the immobilized heparin-binding-deficient mutant that lacks 38 N-terminal amino acids (ΔN-GDNF) is unable to induce adherence of cells. This mutant fails to bind GFRα1 (Eketjäll et al., 1999) in the absence of RET but exerts full biological activity, promoting GFRα1-mediated RET activation (I and Eketjäll et al., 1999; Piltonen et al., 2009) in the same cells. Taken together, these facts support the hypothesis that matrix-bound GDNF, NRTN and ARTN can act directly through HSPG.

Immobilized GFLs are likely to induce the aforementioned effects by SFK activation. SFK activation in SHEP cells is also HS-dependent, since it is abolished by the heparinase III treatment and does not occur on immobilized ΔN-GDNF. In addition, chemical SFK inhibitor (SU6656) (Paveliev et al., 2004) and an adenoviral Src dominant-negative construct inhibit GDNF-induced spreading but did not significantly affect adhesion.

Cell adhesion and spreading are important initial steps in many biological processes, such as cell migration and neurite outgrowth. Free GFLs are known to induce neurite outgrowth in many types of neurons (Airaksinen and Saarma, 2002). Neurite outgrowth is important for long-term memory formation (Dityatev and Schachner, 2003), which is thought to occur in the hippocampus. Immobilized HB-GAM and GDNF both promote neurite outgrowth via syndecan-3 in...
these neurons (Raulo et al., 1994 and I). Interestingly, syndecan-3 localizes in varicosities and growth cones as revealed by Raulo and colleagues (1994), and thus may participate in axon guidance.

Adult murine hippocampus expresses RET (www.brain-map.org) but embryonic hippocampal neurons express little or no RET (Trupp et al., 1997). Despite this fact, we observed that immobilized GDNF stimulates robust neurite outgrowth in rat E17 hippocampal neurons. Quantitative analysis demonstrated that the neurite outgrowth was neither affected by PI-PLC treatment nor by NCAM function-blocking antibodies, which suggests lack of involvement of conventional GDNF receptors. The number of neurites was significantly lower in cells treated with heparinase III, and neurons failed to form processes on the following non-heparin-binding substrates, ΔN-GDNF, PSPN, and BSA, confirming role of HS chains in the interaction. Correspondingly, cultures from syndecan-3-null mice had significantly less neurons on immobilized GDNF. Likewise, the SFK inhibitor SU6656 prevented neurite outgrowth. It is important to note that, unlike Paratcha and co-authors (2003) who observed inhibition of GDNF-induced neurite outgrowth by NCAM function-blocking antibodies, we studied the initiation of neurite outgrowth (counting the number of neurites) and not their progression (i.e. neurite length). These are different processes governed by distinct mechanisms (da Silva and Dotti, 2002). We conclude that the interaction between syndecan-3 and the immobilized, but not the free GDNF induces neurite formation in rat embryonic hippocampal neurons via SFK activation.

GFLs were shown to act as chemotactic guidance molecules (Paratcha et al., 2006b). Soluble GDNF binding to GFRα1 and subsequent RET activation induce neurite outgrowth and neuronal migration. GDNF is well known to chemotactically promote migration (i.e. guide migration along concentration gradient) of enteric neuroblasts in the developing gastrointestinal tract (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996) and of olfactory bulb interneuron precursors (Paratcha et al., 2006b). ARTN-, GFRα3- and RET-deficient mice have abnormalities in the migration and axonal projection pattern of the entire sympathetic nervous system. Indeed, soluble ARTN exerts its chemoattractive action by stimulating, through GFRα3/RET, the migration of sympathetic neuroblasts (Nishino et al 1999; Honma et al., 2002). Our observation that the immobilized, but not the soluble GDNF induces hippocampal neurite outgrowth in a RET-independent manner suggests that GDNF also serves as a haptotactic (immobilized, short-range acting) ECM-associated guidance molecule.

Notably, behavioral tests performed on the syndecan-3-deficient mice and GDNF heterozygous mice revealed impaired performance in tasks assessing hippocampal function (Gerlai et al., 2001; Kaksonen et al., 2002).

Brain cortex development is a complex process that involves migration of neuronal precursors from subventricular zones along tangential migration route to the outer brain areas. Murine embryonic cortical neurons lack both RET and NCAM (Pozas and Ibáñez, 2005). In the cell migration assays, GDNF showed prominent induction of haptotactic migration in embryonic neural cells, suggesting that GDNF signals through a migration-associated receptor system (I). Binding of GDNF to this receptor is mediated by heparan sulfate chains, since
ΔN-GDNF did not induce migration in this assay. Strong evidence of GDNF binding to the syndecan-3 ectodomain led to the assumption that syndecan-3 would be the migration-inducing receptor for GDNF.

We tested this hypothesis in syndecan-3-deficient embryonic cortical cells and observed reduced haptotactic migration in embryonic cortical neurons of the syndecan-3-knockout. The neurons from these animals and wild type neurons were equally well migrating when stimulated in this assay by laminin, suggesting that general migratory systems are intact in the knockout neurons (Hienola and Rauvala, unpublished). Interestingly, the highest concentration of GDNF could also stimulate migration of the knockout neurons, indicating the potential involvement of GFRα1 receptor (Pozas and Ibáñez, 2005). The finding that migration of cortical neurons depends on syndecan-3 further supports our view of syndecan-3 as a novel receptor for GDNF in neural cells.

The migration of embryonic GABAergic cortical neurons in vivo depends on GDNF and GFRα1. GDNF promotes the migration of GABAergic interneurons along the tangential migration route from medial ganglionic eminence (MGE) to developing cortex in mice (Pozas and Ibáñez, 2005). The finding that migration of cortical neurons depends on syndecan-3 further supports our view of syndecan-3 as a novel receptor for GDNF in neural cells.

An intriguing possibility exists that, in addition to its possible signaling role, syndecan-3 acts as a co-receptor for GFRα1 in GDNF-induced migration of GABAergic neurons in embryonic cortex.

In summary, this work highlights a new receptor for immobilized GDNF, NRTN and ARTN, which induces distinct biological effects and is fundamentally different from the Ret/GFRα or NCAM/GFRα signaling systems utilized by free GFLs and (Fig. 8). Our results clearly show that binding of immobilized GDNF to syndecan-3 alone triggers specific intracellular signaling leading to the activation of SFKs, cell spreading, neurite outgrowth and migration of embryonic cortical neurons. Thus GDNF, NRTN and ARTN are the first growth factors to signal
via HSPGs. This finding is conceptually new because it implies distinct cellular responses to the protein in different physical state (free vs. immobilized).

In addition, GFLs may be sequestered by HSPGs in the ECM and by syndecan-3 on neuronal cell surfaces. Other syndecans may play a similar role in other tissues. For example, syndecan-1 is the likely GDNF binding receptor in developing kidney.

This model also supports the idea that interaction of GFLs with HS concentrates GFLs in the vicinity of its conventional GFRα/RET receptors. Since syndecan-3 has several HS side chains and each of them contains multiple GFL binding sites, we expect that a single syndecan-3 receptor can bind many GFL molecules. Therefore, compared to high-affinity and low capacity GFRα/RET receptor complex, syndecan-3

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**Fig. 8.** Hypothetical model of the syndecan-3 role in GFLs signaling. GDNF, ARTN and NRTN interacting with syndecan’s heparan-sulfates (HS) are sequestered from GFRα/RET. A plausible heparanse-mediated cleavage of HS would release GDNF allowing it to interact with GFRα/RET as a free/diffusible protein. Syndecans may also serve as co-receptors for GFRα/RET by concentrating and presenting GFLs to the signaling complex. When immobilized in extracellular matrix (ECM) GDNF, NRTN and ARTN may induce signaling directly via syndecan-3 affecting actin dynamics.
is a low affinity and high capacity GFLs receptor. Our model also predicts a new regulation step in GFL signaling - cleavage of HS or syndecan-3 ectodomain (Reizes et al., 2001) to regulate the release of soluble GFL that is required for GFRα/RET receptor activation. However, the full extent of HSPG roles in GFL signaling remains to be elucidated.

4.2 The structure of GDNF-GFRα1 complex
The rat GFRα1 gene encodes for a cysteine-rich protein with 468 amino acids including secretory and glycosylphosphatidylinositol (GPI)-anchoring signals. The conserved internally homologous cysteine pattern (e.g. 30 cysteines are conserved between GFRα1 and GFRα2) led to the proposal that GFRα1 contained three homologous cysteine-rich domains (Suvanto, 1997; Airaksinen et al, 1999). In this model, the N-terminal domain 1 is linked by a hinge region to a core of domains 2 (D2) and 3 (D3), which is followed by a C-terminal extension.

As demonstrated by Scott and Ibáñez (2001), GDNF binds to the central domain of the GFRα1, which corresponds to joint D2 and D3 in Suvanto’s model (Suvanto, 1997). Specifically, hydrophobic site (213MLF) and arginine triplet (224RRR) were shown to be crucial for GDNF binding to GFRα1. Interestingly, these GFRα1 mutants were fully active in mediating RET activation by GDNF. This finding, together with previous work from the Ibáñez laboratory (Eketjäll et al., 1999), argues in favor for the existence of a substantial fraction of preformed GFRα1/RET complex on the cell surface. This complex may provide additional binding sites for GDNF either by rendering conformational change to GFRα1, revealing cryptic binding sites, or by direct GDNF-RET interaction. Our mutagenesis data (IV) also support this hypothesis.

Furthermore, we observed a substantial increase in the binding of radiolabelled GFRα1 (in the absence of GDNF) to the surface of the MDCK cells expressing RET (Popsueva et al., 2003) compared to cells lacking RET (unpublished data). This finding also supports the direct interaction between GFRα1 and RET in the absence of GDNF.

The question whether GFRα1/RET complex is pre-formed has a therapeutic significance. Small molecule XIB4035 can bind to GFRα1 and activate RET (Tokugawa et al., 2003). This molecule is a quinoline that has earlier been used to treat Protozoan infections. As XIB4035 weighs less than 500 Da, it is unlikely that it activates GFRα1/RET in the same manner as GDNF, which “crosslinks” two GFRα1 molecules. We, therefore, suggested that the mechanism of XIB4035 action is by binding to a “hot-spot” on the preformed GFRα1/RET complex triggering a conformational change (V).

As RET in the crystal adopts an “active” conformation regardless of its phosphorylation status (Knowles et al., 2006), the signaling may be initiated by trans-phosphorylation of the residues that are distant from the catalytic site (e.g. Y1062, Y1096). This is in accordance with the classic model of RTK signaling (Schlessinger, 2000). It is conceivable that XIB4035 binding to a “hot-spot” in GFRα1/RET complex would change its conformation and align the apposing
kinase domains into positions suitable for trans-phosphorylation of distal tyrosine residues.

Expression and crystallization of the full-length GFRα1 poses a significant challenge. Therefore, we expressed and tested for binding ability a number of protein variants (12 constructs). Initially, only the third domain produced well-diffracting crystals. Crystal structure at 1.8Å resolution was reported (IV), revealing a new protein fold. It is a five-helix bundle with five disulfide bridges.

The helix α1 is connected at 80º to α2, followed by the loop 1. Helix α3 is hydrophobic and antiparallel to α2, packing against it. The fourth helix is connected to α3 by the long loop (loop 2) that could only be partially visualized in the initial structure. This region contains many hydrophobic amino acids and, together with α3, was later demonstrated to be an interface for D2, assuming antiparallel β-strands structure (II). The helix α4 is at 70 º to both α2 and α3. The helix α5 is an extension of α4, which stabilizes positions of α2 and α3.

This helical arrangement resembles a triangular spiral, with 5 disulfide bridges locking the “corners” of the triangle. The network of disulfide bridges and the hydrophobic residues buried inside the helical bundle, suggest that D3 folds independently from other domains.

The structural data on D3 allowed us to model the homologues D2. Earlier analysis (Eketjäll et al., 1999; Scott and Ibáñez, 2001) and our crystallographic and mutagenesis data lead us to construct the first structural model for GDNF-GFRα1 interaction. This model suggested that GDNF binds at the interface between the second and third GFRα1 domains. The mutagenesis appeared to confirm this model as Arg224, Arg225 and Ile229 were thought to mediate GDNF-GFRα1 interaction and their substitution resulted in impaired GDNF binding. However, as we learned, this model of interaction turned out to be wrong.

Our later study (II) and the structure of GFRα3-ARTN (Wang et al., 2006) revealed symmetrical (GDNF)2(GFRα1)2 complex (Fig. 9) with GDNF and ARTN binding to a center of the D2 “triangular helix spiral”. We showed that at the center of the interface an ion triplet is formed: Arg171GFRα1-Glu61GDNF-Arg224GFRα1, which is critical for the interaction. This triplet is conserved among GFLs and GFRαs, allowing all four ligands to signal via GFRα1/RET receptor complex (Sidorova et al., unpublished). Another critical residue is Ile175, which is present in GFRα1 and GFRα2. In GFRα3 Ile175 is substituted with glycine, which precludes GDNF-GFRα3 interaction when RET is absent.

The structure of D2 is similar to D3. The length and the relative positions of the first three helices are almost identical. Instead of beta-strands in loop 2, a short helix α4 is present in D2. Helices α5 and α6 are analogous to α4 and α5 in D3. The angle of these helices relative to α2 and α3 is smaller in D2 and, as its α6 is rudimentary, the whole domain is more compact than D3.

Residues of helix α5 interact simultaneously with GDNF and with loop 2 of D3. Helices α3, α4 and α6 also contribute to the interface with D3.

The sucrose octosulfate (SOS), a disaccharide heparin mimic, binds to GFRα1/GDNF and co-purifies with the complex. In the crystal structure SOS can be clearly seen in the highly positively charged region on GFRα1 surface between the second and third domains, suggesting a role of HS in GDNF signaling.
SOS binds a stretch of positive amino acids in α3 of D2 (R189-K202) and R257/R259 in α2 of D3. The side-directed mutagenesis of GFRα1 (R190A/R197A) reduced GDNF-induced RET phosphorylation by 3 folds. This suggests that either heparin-binding is critically important for GDNF-signaling or/and that these GFRα1 residues participate in interaction with RET.

The exact role of HS in GFRα1/RET activation by GDNF is debated. Barnett at al. (2002) demonstrated that HS on the cellular surface are essential for GDNF-signaling and biological effects. As exogenous heparin inhibited RET phosphorylation, they concluded that the role of HS is to concentrate GDNF in GFRα1/RET vicinity. The free heparin competed with HS on the plasma membrane. Two other studies reported neurite inhibition by exogenous heparin (Ai et al., 2007; Alfano et al., 2007).

Our structural data suggest an alternative mechanism of GDNF signaling inhibition by the free heparin. It may compete with RET for the same binding site on GFRα1 (II).

However, Tanaka et al. (2002) and our recent cellular data indicate that HS may stimulate GDNF signaling in vitro (Piltonen et al., 2009).

These contradictory facts need to be critically examined. For example, neurite outgrowth inhibition by the free heparin (Ai et al., 2007; Alfano et al., 2007) might have been a manifestation of heparin (or heparin preparation impurities) toxicity. The researches used heparin of different molecular weights, which further complicates the analysis of the results. On the other hand, Piltonen et al. (2009) have demonstrated that ΔNGDNF, which interacts with GFRα1 much weaker than wild type GDNF (Eketjäll et al., 1999), signals more efficiently at

Fig. 9. GDNF complex with GFRα1. GDNF interacts with the central part of GFRα1 domain two. The critical interaction is formed by an ion triplet GFRα1^{Arg-171}(R171)-GDNF\textsuperscript{Glu-61}(E61)-GFRα1^{Arg-224}(R224). Helices of domain 2 of GFRα1 are designated α1-α6. The heparin mimetic sucrose octosulfate (SOS), represented as sticks, binds to the interface of domains two and three, and this area is suggested to interact with RET.
sub-optimal concentrations than wild type GDNF. In addition, the effect of HS on GDNF-signaling may depend on the source of GDNF. We have observed significantly lower signaling enhancement by HS when we used glycosylated GDNF produced in insect cells compared to E. coli-produced protein (Sidorova et al., unpublished). These two facts suggest an unspecific interaction between GDNF and HSPG on the cell surface (or binding to plastic of the labware), resulting in sequestering of GDNF from the solution, thus decreasing its effective concentration. Co-administration of heparin and GDNF results in shielding GDNF’s excessive positive charge by negative heparin molecules, preventing GDNF from unspecific interactions.

Another interesting crystallographic observation was that three N-terminal residues of GDNF from one GDNF-GFRα1 heterodimer interact with SOS bound to another heterodimer. GFRα1 is a ligand-induced cell adhesion molecule (LICAM) (Ledda et al., 2007), which plays a role in synapse formation. The crystallographic interaction between two GDNF/GFRα1 heterodimers (II) suggests that on the plasma membrane GDNF/ GFRα1 oligomerization is mediated by HS. As HS are long molecules, the intercellular cross-linking of heterodimers into larger aggregates is feasible.

The comparative structural analysis of GDNF and ARTN and their complexes with corresponding GFRαs suggests distinct bend angle between the “heel” and the “fingers”. (Eigenbrot and Gerber, 1997; Silvian et al., 2006). The larger angle in GDNF results in shorter distance between two GFRα1 (Wang et al., 2006 and II). GDNF can adopt several conformations in the crystal and, thus, is likely to be more flexible than ARTN (Parkash and Goldman, 2009).

The structural differences between GDNF and ARTN lead to quantitative differences in their signaling. GDNF activates MAPK signaling more rapidly and robustly than ARTN (II). The levels of RET phosphorylation induced by GDNF and ARTN are comparable but minor differences in dynamics of RET phosphorylation were observed (Coulplier et al., 2002). The downstream amplification of the signal leads to quantitative differences in gene expression.

### 4.3. The putative structure of GDNF-GFRα1-RET complex

Although the molecular architecture of GFL-GFRα complexes became apparent, the structure of the whole GFL-GFRα-RET receptor complex remains to be elucidated. Our biochemical data suggested that the interface between RET and GFRα1 is located close to the SOS binding site. The positively charged Arg190, Lys194, Arg197, Lys202, Arg257 and Arg259 (Fig. 9) may interact with the cysteine-rich domain (CRD) of RET, which is negatively charged.

Using chemical cross-linking followed by MALDI mass spectrometry (MS), Amoresano and colleagues (2005) demonstrated that the C-terminal fragment of GDNF interacts with the \(^{199}\)RCHKALR fragment of GFRα1. They also suggested the interaction between GDNF N-terminal protrusion and the N-terminal domain of GFRα1. However, the suggested high flexibility of N-terminal region of GDNF (Eigenbrot and Gerber, 1997) might result in unspecific association between these regions. The detected interaction between the first domain of GFRα1 and RET-CRD may also be attributed to the
Results and Discussion

The homology between domains 1, 2 and 3 allows the construction of the model for the domain 1 (D1). The resulting model

Fig. 10. The model of the GFRα1/RET signaling complex.
A. RET ectodomains (shown in tinges of violet) interact homotypically as well as with the central part of GDNF/GFRα1 complex, assuming “saddled” position (McDonald, 2008, Conference presentation). GDNF in complex with GFRα1, represented by domains 2 and 3, is shown as electrostatic potential surfaces (blue – positive and red – negative charge). TM – transmembrane domain.
B. As in Fig. 10, rotated 90°
Results and Discussion

has a similar to D2 fold, and it is an all-α four-helix bundle with 3 cystines (Fig. 2).

The first domain of GFRα1 is important for GDNF binding (III). GFRα1 lacking first domain binds GDNF two times less tightly than the full-length protein. An impaired binding has biological consequences - GDNF-stimulated PC6 cells produce significantly less neurites when transfected with truncated GFRα1. D1 may interact with GDNF directly (Amoresano et al., 2005).

An alternative role that D1 may play is in protein sorting (Scott and Ibáñez, 2001). It was suggested that N-glycans participate in apical sorting of GPI-anchored proteins (Benting et al., 1999). Jing et al. (1996) predicted that GFRα1 contains three N-glycosylation sites, including one at N-terminus. MALDI-TOF mass spectrometry (MS) of the full-length and of the truncated receptor showed that both variants weigh more than predicted. The modification of the full-length GFRα1 is larger, suggesting that D1 is also post-translationally modified. In electro-spray MS the truncated receptor was ionized and a set of masses around 37 kD was detected. The mass heterogeneity corresponds to masses of N-acetylglucosamine and hexose (III).

Another possible role of D1 is an interaction with HS. D1 is positively charged – its calculated isoelectric point is 9.21 and it may interact with HSPG on the plasma membrane. Full-length GFRα1 elutes from a heparin column at higher ionic strength than N-terminally truncated receptor (II). The physiological relevance of this interaction remains to be established.

RET cadherin-like domains (CLDs) were modeled by homology with N-cadherin (Anders et al., 2001). However, modeling of the cysteine-rich domain of RET poses a problem because it lacks profound secondary structural features and the pattern of cysteines is not conserved. Nevertheless, the RET-CRD shares 18% identity with laminin gamma 1 chain. An alignment of cysteines allows the generation of the gross model of RET-CRD (Fig. 2). The model reveals two loops protruding in the same direction. Earlier, a similar type of loops in CRD domain 2 and 4 of EGFR were shown to be important in autoinhibition and dimerization. However, in the fully assembled RET signaling complex these loops would rather interact with GFRα1/RET than homotypically. The possibility exists that these loops can still promote RET dimerization when dissociated from GFRα1. Indeed, as demonstrated by Trupp et al. (1997) overexpressed RET has a high propensity for activation in the absence of GFRα1. Transmembrane domains of RET may also contribute to self-association (Kjær et al., 2006).

Our current structural understanding of the extracellular part of the RET signaling complex is summarized in the Fig. 10. The model represents a complex with a \((\text{GDNF})_2:(\text{GFRα1})_2:(\text{RET})_2\) stoichiometry (domain 1 of GFRα1 is omitted). According to the model, RET is bent towards GDNF/RET and to plasma membrane. The RET-CLDs are suggested to participate in homophilic interaction and to engage in direct interaction with GFRα1 and/or GDNF. This model is compatible with the pre-formed GFRα/RET complex hypothesis (V) and may be helpful in the development of clinically useful GFL-mimetics.
5. SUMMARY AND CONCLUSIONS

1. We identified neuronal heparan sulfate proteoglycan syndecan-3 as a novel receptor for GDNF, NRTN and ARTN. Syndecan-3 may transduce the GFL signal directly into the cells or serve as a co-receptor for either GFRα/RET or GFRα/NCAM or for an as-yet unidentified receptor. The physiological relevance of this signaling is to promote the migration of GABAergic interneuron precursors towards the developing cortical layers.

2. We characterized GDNF interaction with its co-receptor GFRα1 using biochemical and cell biological methods and solved the crystal structure of their complex.

3. We proposed a model for the fully assembled GDNF signaling complex and hypothesized on how small molecules mimicking GDNF can activate the receptor.
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