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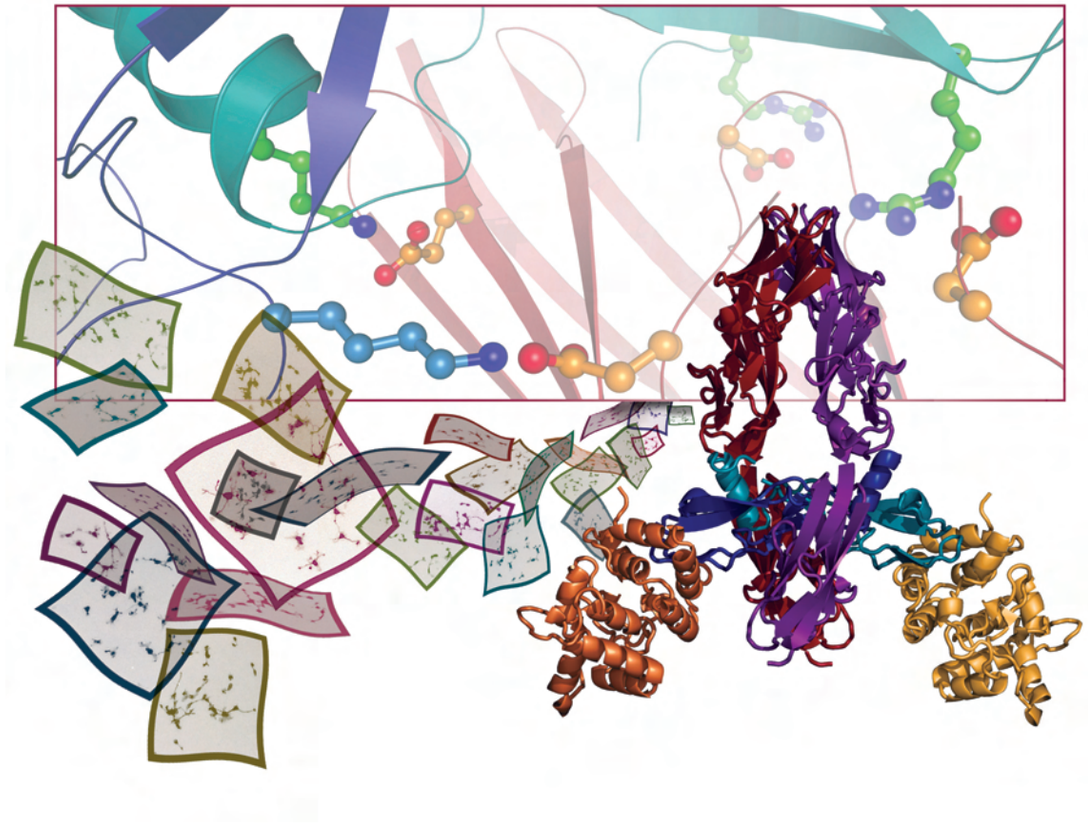

Dan Sjöstrand

Karolinska Institutet

Karolinska Institutet

From the Department of Neuroscience

Karolinska Institutet, Stockholm, Sweden

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Abstract

Neurotrophic factors are small, secreted proteins that are important players in the development and maintenance of the nervous system. Their functions span from neuronal survival and migration to neurite outgrowth and axon guidance, and in some cases even programmed cell death. Glial cell line-derived neurotrophic factor (GDNF) is the founding member of a family of four neurotrophic factors; the GDNF family of ligands. GDNF and its fellow family members Neurturin, Artemin and Persephin signal by forming a tripartite complex with one GPI-anchored receptor (GFRα1, -2, -3 or -4, respectively) and one transmembrane receptor, the latter being responsible for transmitting the signal through the plasma membrane into the cell. The transmembrane receptor can be either one of two molecules: The receptor tyrosine kinase Ret or the Neural Cell Adhesion Molecule (NCAM).

The interaction determinants of the Ret-GDNF-GFRα1 complex have been thoroughly analyzed, as well as the functional importance of its structural features. The more recently discovered NCAM-GDNF-GFRα1 complex, however, is much less studied. In paper I we mapped the GDNF binding domain in NCAM to the third immunoglobulin-like domain by analysing the ligand-binding properties of a large set of deletion mutants. Molecular modeling of the interaction interface revealed four charged contacts, and mutation of the NCAM residues involved in these contacts created a receptor unable to bind GDNF while keeping its cell adhesion properties, thus separating these two functions of NCAM.

In paper II we used a similar approach to map the NCAM-GFRα1 binding site. The major GFRα1-binding site in NCAM was mapped to the fourth Ig-like domain, and we found that the primary NCAM-binding site resides in the N-terminal domain of GFRα1. Deletion of the latter domain did not affect the ability of GFRα1 to potentiate GDNF-NCAM binding, implying that GFRα1 facilitates signaling by a ”ligand presentation” mechanism rather than by allosterically changing the GDNF-binding properties of NCAM. We also showed that deletion of the NCAM-binding N-terminal domain severely impaired the ability of GFRα1 to interfere with NCAM-mediated cell adhesion. This suggests that the strong GFRα1-NCAM interactions mediated by the N-terminal domain of GFRα1 are required for efficient modulation of NCAM-mediated cell adhesion.

Although the GDNF family of ligands appears to be a vertebrate-specific invention, orthologs of the receptors are present in other phyla. In paper III we examined the *Drosophila* Ret ortholog dRet, and showed that it did not bind GDNF, nor did it display any cell adhesion promoting properties despite its cadherin-like domain structural features. Interestingly, we found that a chimeric mammalian Ret molecule that included the dRet kinase domain was capable of inducing PC12 cell differentiation upon stimulation with GDNF and soluble GFRα1, suggesting that this functional feature of the intracellular domain is evolutionarily conserved.

GDNF and GFRα1 promote the migration and differentiation of cortical interneurons from the medial ganglionic eminence (MGE), a process in which neither RET nor NCAM appear to be involved, suggesting the possible existence of a third transmembrane receptor. In paper IV we examined two potential receptor candidates: The HGF receptor Met and the Neuregulin-1 receptor ErbB4. None of the two receptors were phosphorylated by GDNF in our biochemical assays, and neither HGF nor Neuregulin had any significant effects of their own on neurite outgrowth in our cultures. Intriguingly, two different Met inhibitors displayed outgrowth-promoting effects on their own. However, neither Met nor ErbB4 inhibitors impaired GDNF-induced neurite outgrowth, showing that these receptors are not involved in GDNF signaling in MGE neurons.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their roman numerals:

I. **Dan Sjöstrand**, Jonas Carlsson, Gustavo Paratcha, Bengt Persson and Carlos F. Ibàñez

Disruption of the GDNF Binding Site in NCAM Dissociates Ligand Binding and Homophilic Cell Adhesion

J Biol Chem, 2007, 282, 12734-12740

II. **Dan Sjöstrand** and Carlos F. Ibàñez

Insights into GFRα1 regulation of NCAM function from structure-function analysis of the NCAM/GFRα1 receptor complex

J Biol Chem, 2008, In Press, Epub March 19

III. Chiara Abrescia, **Dan Sjöstrand**, Svend Kjaer and Carlos F. Ibàñez

Drosophila RET contains an active tyrosine kinase and elicits neurotrophic activities in mammalian cells

FEBS Lett, 2005, 579, 3789-3796

IV. **Dan Sjöstrand**, Annalena Moliner and Carlos F. Ibàñez

GDNF signaling in GABAergic neurons of the medial ganglionic eminence: Roles of Met and ErbB4

Submitted manuscript

Table of contents

List of abbreviations

ALS	Amyotropic lateral sclerosis
ART	Artemin
BDNF	Brain-derived neurotrophic factor
Cdk5	Cyclin dependent kinase 5
CNS	Central nervous system
CREB	Cyclic AMP response element binding
ECD	Extracellular domain
EDC	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ENS	Enteric nervous system
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
Frs2	Fibroblast growth factor receptor substrate 2
GABA	Gamma-aminobutyric acid
GAP-43	Growth associated protein 43
Gas1	Growth arrest specific 1
GDNF	Glial cell-line derived neurotrophic factor
GFLs	GDNF family ligands
GFRα	GDNF family ligand receptor alpha
GPI	Glycosylphosphatidylinositol
GRAL	GDNF receptor alpha-like
Grb	Growth factor receptor-bound protein
HA	Hemagglutinin
HGF	Hepatocyte growth factor
ICD	Intracellular domain
Ig	Immunoglobulin
MAPK	Mitogen activated protein kinase
Met	Mesenchymal epithelial transition factor
NCAM	Neural cell adhesion molecule
NF-κB	Nuclear factor kappa B
NTN	Neurturin
PDGF	Platelet-derived growth factor
PI3	Phosphoinositide 3
PLCγ	Phospholipase C gamma
PNGaseF	Peptide N-glycosidase F
PNS	Peripheral nervous system
PSA	Polysialic acid
PSP	Persephin
Ret	Rearranged during transfection
RMS	Rostral migratory stream
RPTPα	Receptor-type protein tyrosine phosphatase alpha
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Shc	Src homology 2 domain containing transforming protein 1
S-NHS	*N*-hydroxysulfosuccinimide
STAT3	Signal transducer and activator of transcription 3
TGF-β	Tumor growth factor beta

Introduction

GDNF family ligands and their receptors

Glial cell line-derived neurotrophic factor (GDNF) is a small secreted protein and a distant member of the TGF-β superfamily of ligands. GDNF and its three paralogs Neurturin (NTN), Artemin (ART) and Persephin (PSP) together make up the GDNF family of ligands (GFLs), and each of the four ligands binds to one of the four members of the GDNF receptor α (GFRα) family (Airaksinen 2002). GDNF itself binds primarily to GFRα1, while the other members of the GDNF family have other preferential GFRα receptors (Fig. 1), although there is a certain amount of promiscuity in ligand binding (Scott 2001, Airaksinen 1999). Whereas most membrane proteins have a transmembrane region, connecting the extracellular and intracellular domains of the protein and allowing for the transmission of signals over the plasma membrane, the GFRα receptors lack such a feature. Instead, the membrane-proximal end of the protein is covalently bound to glycosylphosphatidylinositol (GPI), that anchors the protein in the outer monolayer of the plasma membrane by hydrophobic interactions with membrane lipids. This feature of the GFRα receptors makes them dependent on other, membrane-spanning, receptors for transmission of the signal into the cell. Two such receptors are currently known; the receptor tyrosine kinase Ret (Trupp 1996) and the neural cell adhesion molecule NCAM (Paratcha 2003). Ret binds only to a preformed ligand-GFRα complex, and although NCAM on its own can bind GDNF, GFRα1 strongly potentiates binding and is required for activation of NCAM signaling (Fig. 2).

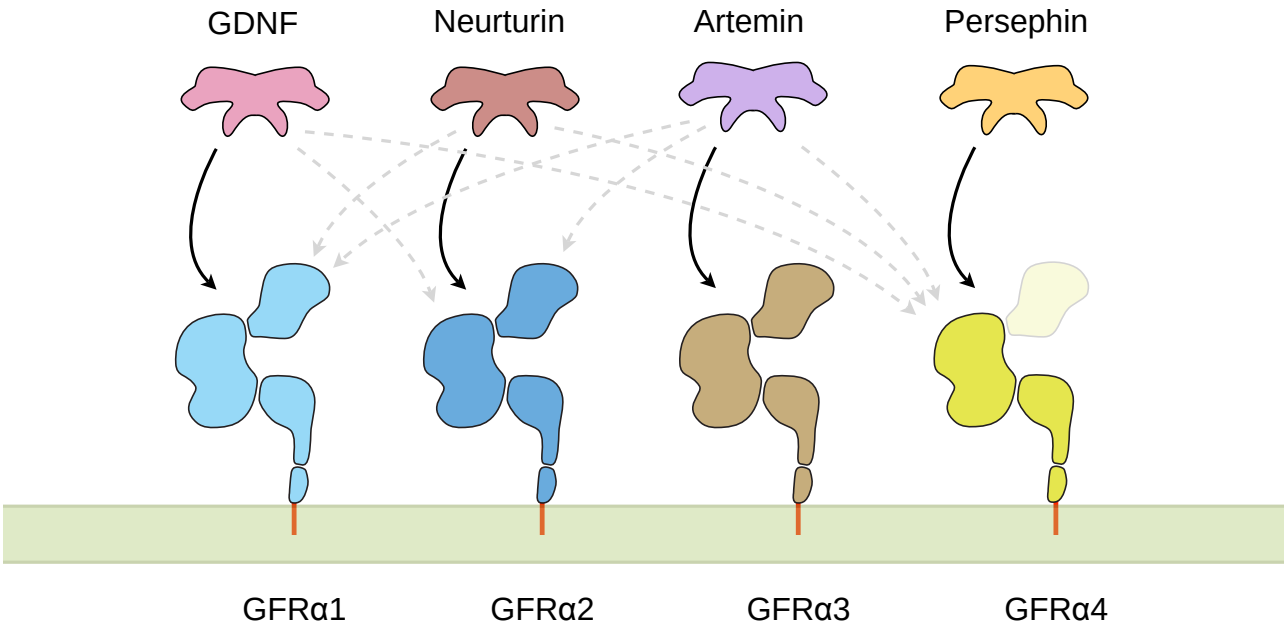

Figure 1. The GDNF family ligands and their preferred GFRα receptors. Note that there is a certain amount of cross-reactivity, represented in the figure by grey, dashed arrows. The N-terminal part of GFRα4 is lacking in mammals. It is however present in chicken, and this domain is therefore indicated (semi-transparent) in the figure.

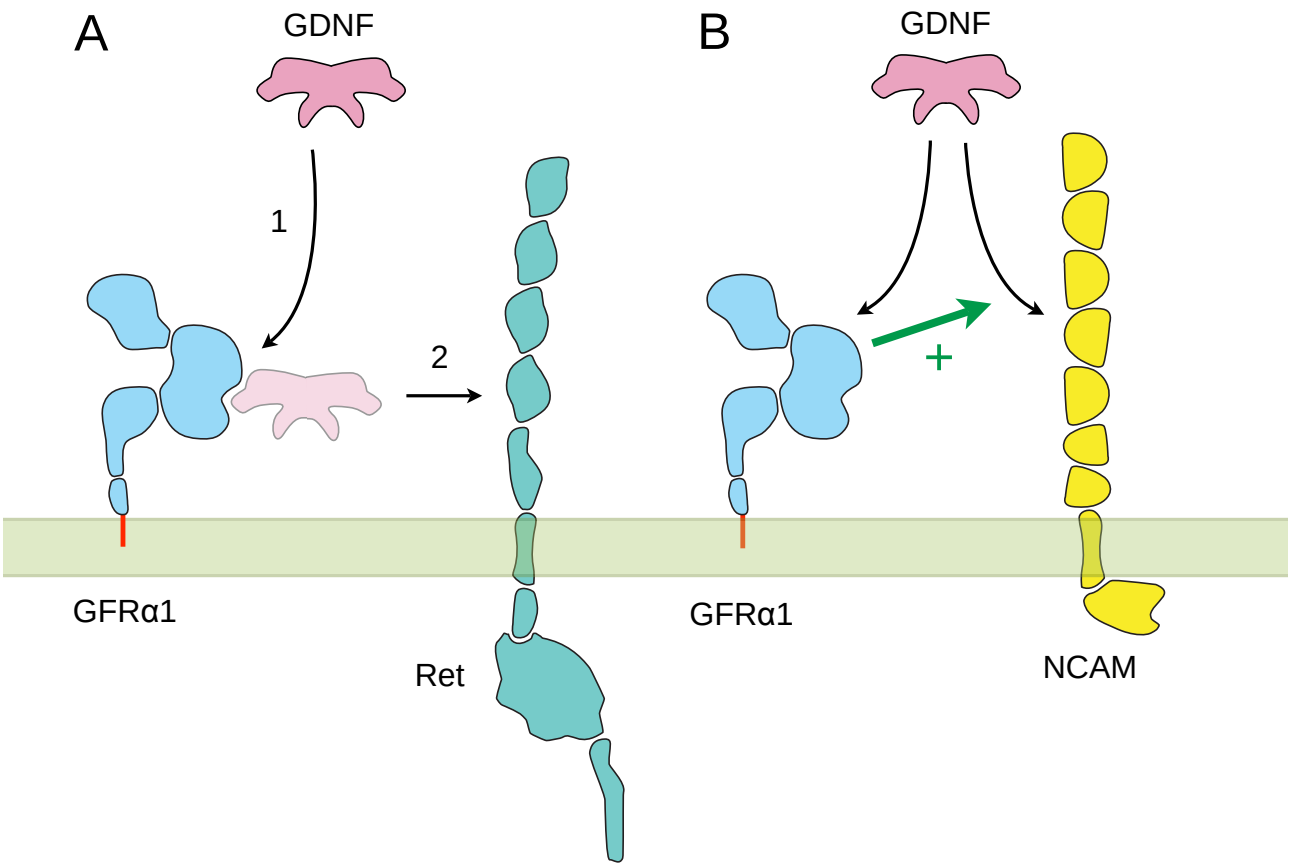

Figure 2. The transmembrane receptors of GDNF. (A): Ret binds to a preformed GDNF-GFRα1 complex. (B): NCAM binds GDNF on its own. Binding is however potentiated by GFRα1, which is also required for signaling.

GDNF and its receptors in development and disease

Embryonic development

During kidney development, GDNF induces ureteric budding from the wolffian duct, promotes ureteric bud tip cell proliferation and contributes to ureteric branching, all this in collaboration with a number of other factors (Costantini 2006). The importance of Ret, GDNF and GFRα1 for renal development is clearly displayed in null knockout mice of either molecule. These mice die soon after birth from kidney dysplasia and intestinal aganglionosis (Schuchardt 1994, Sánchez 1996, Cacalano 1998). The latter defect is linked to another developmental process intimately connected with GDNF signaling, namely the development of the enteric nervous system (ENS). During development of the gut, Ret-expressing neuronal precursors originally derived from the neural crest cells migrate along a a GDNF gradient, colonizing the wall of the gastrointestinal tract. Ret knockout mice have no enteric ganglia posterior to the stomach, and a recent report shows that Ret is also required for the development of Peyer's Patches, key structures of the intestinal lymphoid system. (Veiga-Fernandes 2007). GDNF and its receptors also play a role in development of the autonomic nervous system. Knockout mice of GDNF family ligands, Ret or GFRα receptors display deficien-

cies in the migration and axonal growth of sympathetic and parasympathetic neuronal precursors, and in accordance with this there is a reduction in cell numbers in sympathetic and parasympathetic ganglia (Sariola 2003, Airaksinen 2002). GDNF also promotes the survival of subsets of spinal motor neurons, diminishing programmed cell death during their development (Zhao 2004, Bohn 2004). GDNF has also been shown to induce Schwann cell migration and to increase myelination in cocultures with neurons, exerting its effects via a Ret-independent, NCAM-mediated mechanism (Paratcha 2003, Iwase 2005), and GDNF signaling via Ret has been described to play a role in spermatogenesis, being essential for the maintenance of spermatogonial stem cells (Oatley 2007, Jijiwa 2008).

GDNF and its receptors in brain development

GDNF family ligands and their receptors are also important for neuronal precursor migration and differentiation during development of the brain. GDNF is produced in the olfactory bulb and has been demonstrated to promote tangential migration of neuronal precursors from the rostral migratory stream to the olfactory bulb in an NCAM-dependent manner (Fig. 3A) (Paratcha 2006). GDNF signaling through NCAM has also been shown to promote neurite outgrowth in hippocampal neurons (Paratcha 2003), and recently GDNF and GFRα1 were demonstrated to promote synaptic formation in hippocampal neurons through a novel mechanism that involves GDNF-induced trans-dimerization of GFRα1 (Ledda 2007). Although GDNF signaling has been suggested to play a role in the development of dopaminergic neurons of the substantia nigra, the mechanism for this is not clear, and a recent report shows that Ret, although critical for long-term maintenance of the nigrostriatal system, is not needed for its earlier maintenance and initial establishment (Kramer 2007, Jain 2006). Interestingly, NCAM has been suggested to play a role in GDNF signaling in dopaminergic neurons (Chao 2003), but it is not known if it plays any role in their development. At early stages of embryogenesis NCAM is post-translationally modified with the unusual carbohydrate polysialic acid (PSA), which is believed to modulate NCAM function, possibly by masking the sites for homophilic interaction. Polysialylation decreases during development, and in adults it is only detectable in regions of active neurogenesis (Hildebrandt 2007). NCAM knockout mice have an unexpectedly mild phenotype (Cremer 1994). The most prominent features are a reduction in olfactory bulb size, concordant with its role in GDNF-mediated migration of RMS neuronal precursors, and disturbances in the mossy fibers of hippocampal area CA3 neurons (Cremer 1997). Possibly related to the latter deficiency, NCAM knockouts display impaired learning behaviour, suggesting a role of NCAM for memory formation in the adult (Conboy 2008).

The development of cortical interneurons

GDNF has also been shown to promote the tangential migration and differentiation of cortical GABAergic neurons from the medial ganglionic eminence. These functions of GDNF require the co-receptor GFRα1 but are independent of both Ret and NCAM, suggesting the existence of additional transmembrane GDNF receptors (Pozas 2005). In the mouse, GABAergic interneurons are generated in the medial, lateral and caudal

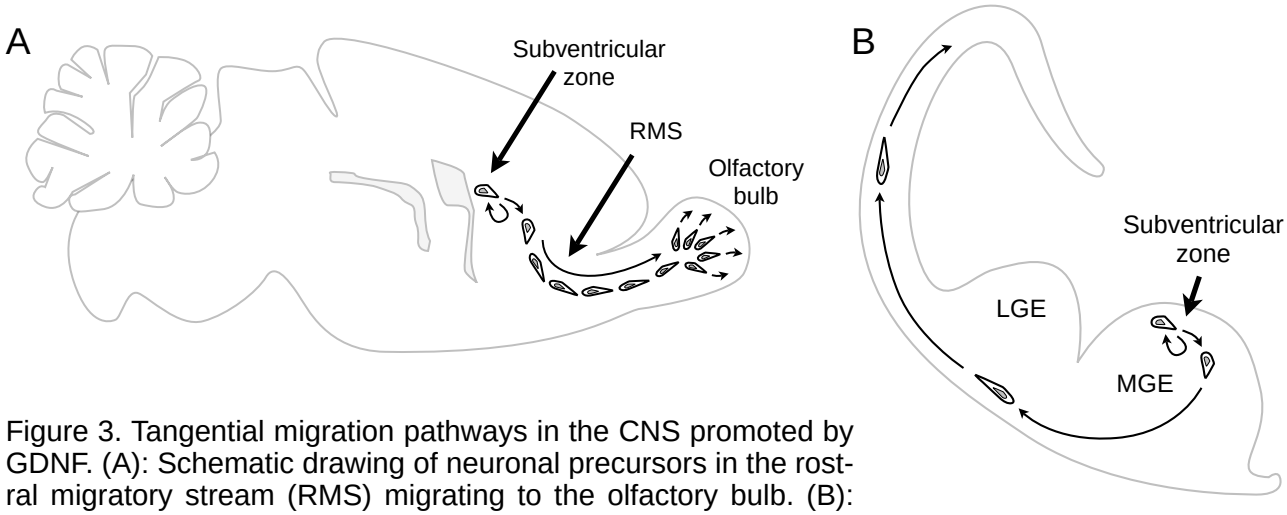

Figure 3. Tangential migration pathways in the CNS promoted by GDNF. (A): Schematic drawing of neuronal precursors in the rostral migratory stream (RMS) migrating to the olfactory bulb. (B): Schematic drawing of interneuron precursors migrating from the MGE to populate the cortex.

ganglionic eminences of the subpallium. These cells then migrate tangentially towards the olfactory bulb, hippocampus and the cerebral cortex (Fig. 3B) (Corbin 2001, Marin 2003). Several molecules have been shown to play a role in this process, including semaphorins, neurotrophins, Hepatocyte Growth Factor (HGF), Neuregulin-1 and according to a recent report also the dopamine receptor D1 (Marin 2001, Polleux 2002, Powell 2001, Flames 2004, Crandall 2007). How interneuron diversity is generated, and how interneurons are specifically targeted to their proper location, is however not fully understood. Neuregulin-1, signaling through the EGF receptor family member ErbB4, promotes the migration of interneuronal precursors in a similar way to GDNF. This similarity between the functions of GDNF and Neuregulin-1 indicate that ErbB4 could be a potential candidate for mediating the effects of GDNF on MGE neurons. The HGF receptor Met is another possible transmembrane receptor partner of GFRα in these cells. Met has been postulated to mediate GDNF signaling via an indirect mechanism that involves the activation of Src-family kinases (Popsueva 2003). In addition to this, HGF signaling via Met has been shown to promote tangential migration of neuronal precursors. Moreover, Met signaling in neuronal migration has been shown to involve the activation of Akt and ERK1/2 (Segarra 2006), and interestingly these two intracellular effectors were also phosphorylated upon GDNF stimulation of MGE cultures (Pozas 2005).

GDNF and its receptors in disease

The GDNF family of ligands and their receptors are involved in a number of human disorders. Ret is strongly associated with cancers, including multiple endocrine neoplasias and medullary thyroid carcinomas (Mulligan 1993, Donis-Keller 1993). In these conditions, mutations in the Ret molecule are believed to render its tyrosine kinase constitutively active either by causing the formation of active Ret dimers in the absence of ligand, or by changing the functional properties of the kinase domain itself (Asai 1995, Santoro 1995). In addition, a number of, mostly inactivating, mutations in the Ret gene have severe effects on development of the ENS, causing Hirschsprung's dise-

ase (Amiel 2008). A few mutations in GDNF have also been connected to this disorder, but usually in combination with Ret mutations (Angrist 1996, Salomon 1996, Ivanchuk 1996, Doray 1998). GDNF signaling also plays a role for the survival of dopaminergic cells projecting from the substantia nigra to basal ganglia, the functions of which are impaired in Parkinson's disease (Grondin 1998, Hurelbrink 2001). The mechanism behind this effect is not entirely clear, but recent reports suggest that Ret is not needed for the establishment of the nigrostriatal pathways, but is required for their long term maintenace, most likely by a mechanism where ligand-induced Ret activity has positive effects on the maintenance of axon terminals (Kramer 2007, Kowsky 2007). GDNF family ligands are in clinical trials for treatment of this disorder, and have given some promising results, although lately the safety of this treatment has been the subject for discussion (Evans 2008). Very recently, mutations in Ret and GDNF have also been associated with renal aplasia in stillborn infants, concordant with the role of GDNF and its receptors in kidney development (Skinner 2008). Furthermore, GDNF is being considered for treatment of the muscular disorder Amyotropic lateral sclerosis (ALS). ALS is characterized by degradation of motor neurons, and in mouse models of ALS GDNF has a protective effect on these motor neurons (Mohajeri 1999, Wang 2002, Suzuki 2007). In addition to this, GDNF family ligands are being examined for their role in neuropathic pain (Sah 2005, Bespalov 2007) and, interestingly, a mechanism for pain modulation involving GDNF signaling via NCAM has recently been proposed (Sakai 2007). In the brain, NCAM has been linked to schizophrenia, depression, anxiety disorders, bipolar disease and Alzheimer's disease (Brennaman 2008), although these disorders are very complex and the causal relationship is less clear. The contribution of GDNF-NCAM signaling to these disorders is not known.

GDNF signaling

Ret signaling

Ligand binding induces the formation of active Ret dimers, a mechanism of receptor activation that is the paradigm among Receptor Tyrosine Kinases (RTKs). An unusual feature of Ret compared to other RTKs, however, is the requirement of an additional receptor. Since Ret does not efficiently bind GDNF family ligands on its own, the ligand binding co-receptor GFRα is essential for its activation. In fact, Ret does not bind to GFRα in the absence of ligand, but only to a preformed ligand-GFRα complex (Jing 1996, Airaksinen 2002). A recent report suggests that Ret may be pre-dimerized, and that a conformational change upon ligand-coreceptor binding renders the dimer active (Knowles 2006). Another report, however, postulates that the components assemble in a stepwise fashion, with the GDNF dimer binding to one Ret and one GFRα molecule in an intermediate step, followed by the formation of the 2:2:2 complex (Schlee 2006). Upon ligand-coreceptor binding, Ret is trans-autophosphorylated by means of its intracellular tyrosine kinase domain activity. Intriguingly, some GDNF mutants defi-

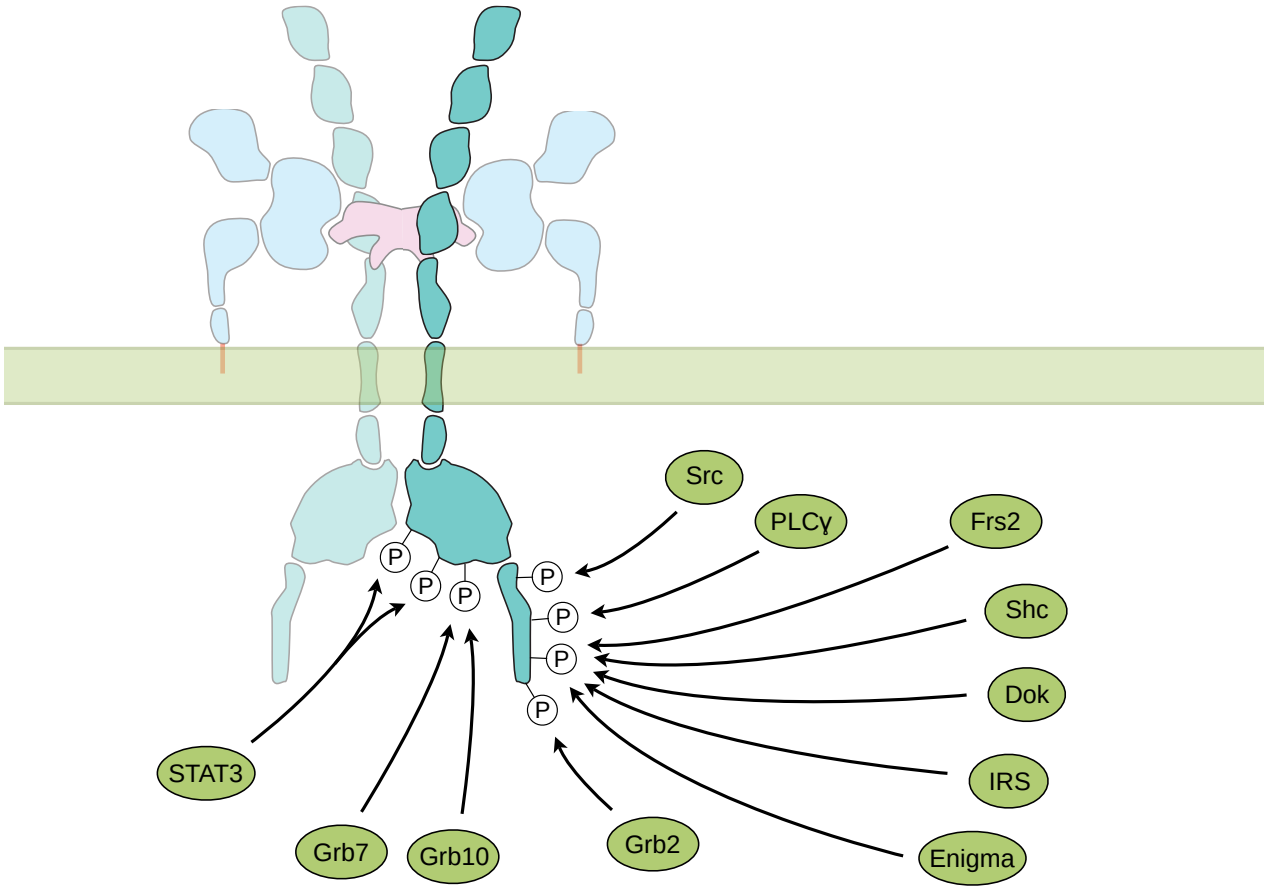

Figure 4. Upon autophosphorylation, Ret transduces the signal by the recruitment of a number of intracellular adaptor molecules that bind to the phosphorylated tyrosines.

cient in GFRα1 binding have been found to still activate Ret autophosphorylation (Eketjäll 2002). A number of intracellular adaptor proteins bind to the phosphorylated tyrosines, in turn modulating downstream intracellular pathways. Some of these adaptors even compete for binding to the same phosphotyrosine (Fig. 4). The Ret transcript is spliced into three different major isoforms, Ret 9, Ret43 and Ret 51, differing in the length of their C-terminal tail (Carter 2001). Of these, Ret9 and Ret51 are the most conserved between species and hence the most studied. Interestingly, monoisoformic mice expressing Ret9 but lacking Ret51 appear wild-type-like with respect to the enteric nervous system, suggesting that Ret 9 is the critical isoform for development of the ENS (de Graaff 2001). Moreover, the results also suggest that the same Ret9 isoform is required for renal development. The results of a similar, recent study (Jain 2006), however, in part contradict the results of the former paper, showing that the Ret51 isoform alone can drive normal renal development. Out of the residues known to interact with adaptors upon phosphorylation, tyrosines 752, 826 and 905 reside in the kinase domain itself, while tyrosines 981, 1015, 1062 and 1096 are found in the C-terminal tail. Tyrosine 752 and 826 bind to the transcription factor STAT3, and Tyrosine 905 recruits Grb7 and Grb10 (Schuringa 2001, Pandey 1996). Src docks to tyrosine 981, tyrosine 1015 binds PLCγ and at least five adaptors compete for binding to tyrosine 1062, including Frs2, Shc, Dok, IRS and Enigma (Encinas 2004, Kurokawa

2001, Airaksinen 2002, Besset 2000). Ret 51 also contains the additional tyrosine 1096, which has been demonstrated to interact with Grb2 and via the ubiquitin ligase Cbl affect Ret stability in an isoform-specific manner (Besset 2000, Scott 2005). These adaptor proteins in turn modulate a number of intracellular signaling pathways, including PI3 kinase/Akt signaling, MAP kinase pathways and Calcium-dependent signaling, as well as specific transcriptional responses (Airaksinen 2002).

NCAM signaling

The NCAM transcript is differentially spliced, resulting in the membrane-spanning protein isoforms NCAM-140 and NCAM-180, the latter having a larger intracellular domain than the former, and the GPI-anchored NCAM-120. In addition to these three major isoforms, there are also a number of cell-type specific splice variants. NCAM-120, -140 and -180 exhibit different expression patterns. NCAM-140 is expressed in glial cells and axons and migratory growth cones of developing neurons, while the 180kD isoform appears later during development, and is enriched in postsynaptic densities of mature neurons. NCAM-120, on the other hand, is expressed primarily in glial cells (Maness 2007, Persohn 1989). GDNF-NCAM signaling is so far not as thoroughly studied as GDNF signaling via Ret, but GDNF has been shown to stimulate phosphorylation of the Src-family kinase Fyn with subsequent activation of the focal adhesion kinase FAK (Fig. 5A) (Paratcha 2003). Although GDNF can bind to NCAM on its own, the interaction is strongly potentiated by the presence of the co-receptor GFRα1, and the latter is required for the activation of Fyn. In Schwann cells, GDNF-NCAM signaling has been proposed to activate cyclic AMP-dependent protein kinases A and C and the cAMP-responsive element CREB. The heparan sulphate proteoglycan Agrin has also been suggested to facilitate assembly of the receptor complex (Fig. 5A). (Iwase 2005). In the rostral migratory stream, the effects of GDNF on neuronal precursors migrating to the olfactory bulb were shown to be NCAM-dependent and to involve activation of the cyclin-dependent kinase Cdk5 (Paratcha 2006). The role of NCAM-180 in GDNF signaling is hitherto unknown. Since the activation of Fyn mimics the mechanism proposed for signaling mediated by homophilic NCAM interactions, it is however possible that these two mechanisms of NCAM signaling share intracellular downstream pathways (Fig. 5A-B). Work performed using homophilic NCAM interactions and antibody crosslinking of NCAM molecules has shown that activation of the focal adhesion kinase FAK is followed by activation of c-Ras1 with subsequent activation of the MAP kinase pathway and CREB phosphorylation (Fig. 5B), with downstream neurite outgrowth-promoting effects. NCAM has also been shown to signal through the FGF receptor, a mechanism that involves PLCγ and subsequent calcium channel activation (Maness 2007, Ditlevsen 2008). It has been postulated that separate pools of NCAM molecules activate Fyn and FGF receptor signaling, the Fyn pathway specifically being activated by NCAM molecules residing in lipid raft compartments (Niethammer 2002). The activation of Fyn by NCAM is intriguing, as the NCAM ICD itself appears to lack enzymatic activity, and the well-known Fyn activator RPTPα has recently been shown to be required for NCAM-mediated Fyn activation (Bodrikov 2005). The complex was also demonstrated to include the cytoskeleton-

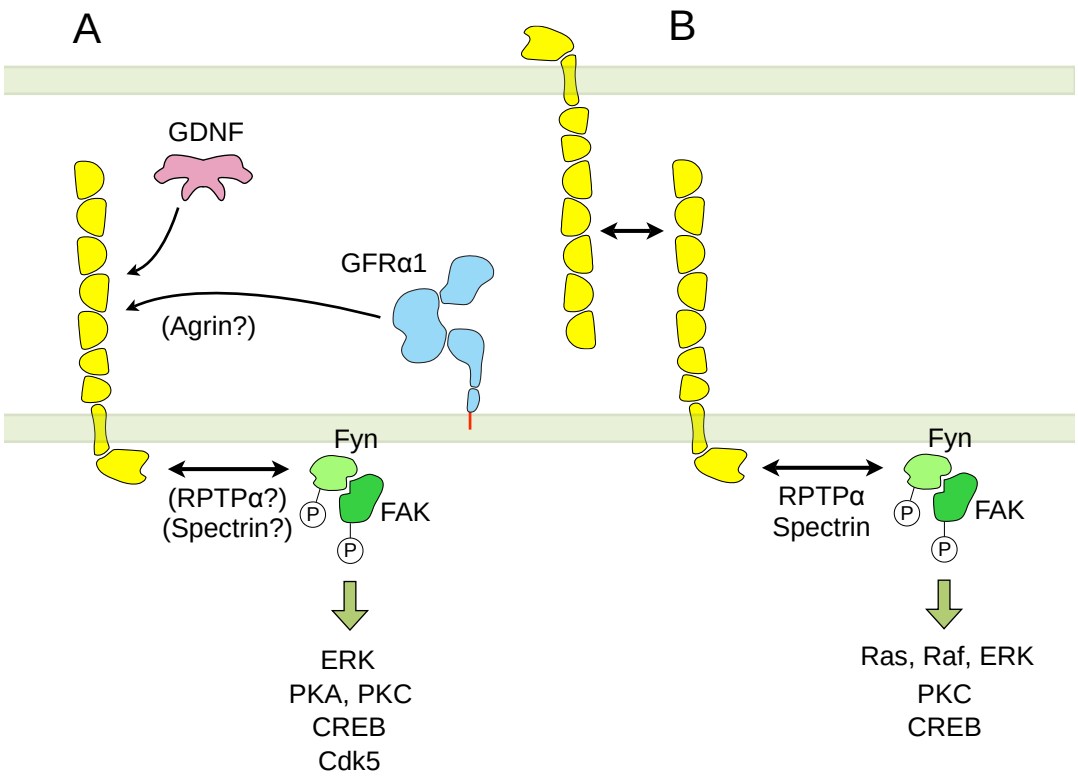

Figure 5. NCAM signaling. (A): GDNF-NCAM signaling. (B): NCAM-NCAM signaling in lipid rafts, showing striking similarities with the GDNF-NCAM signaling in (A).

associated protein Spectrin (Bodrikov 2005) and, interestingly, the spectrin-associated protein GAP-43 has been postulated to regulate the effects of the two different transmembrane isoforms. In this model, NCAM-dependent neurite outgrowth is mediated by NCAM-180 in the presence of GAP-43, while in the absence of GAP-43 NCAM-140 is the outgrowth-inducing isoform (Korshunova 2007). Several other downstream effectors have been shown to play a role in NCAM signaling, including CaMKII, cyclic GMP and the transcription factor NF-κB (Ditlevsen 2008). Additional extracellular molecules involved in NCAM signaling include the growth factors PDGF and BDNF (Walmod 2004), and the prion protein PrsPc, which has been shown to interact with NCAM and to induce neurite outgrowth by stimulation of NCAM-mediated Fyn activation in lipid rafts (Schmitt-Ulms 2001, Santuccione 2005).

Structural features

GDNF structure

GDNF is a small extracellular protein that is initially synthesized as a 211-residue pre-propeptide. It is then converted to its mature form by proteolytic cleavage of the 56-residue propeptide and the dimerization of two GDNF protomers, forming the active dimer (Airaksinen 2002). The GDNF protomers are held together by an arrange-

ment of three disulfide bridges known as the cystine knot, a structural motif shared among the TGF-β superfamily of ligands. The GDNF molecule has some intrinsic flexibility, as is evident from the different conformations of the protein obtained by X-ray crystallography (Fig. 6A-B) (Eigenbrot 1997). More specifically, the ”wings” of the molecule in the two structures adapt different angles in respect to the vertical axis, which appears to be due to a certain degree of flexibility in the loop regions adjacent to the α-helix of each protomer. In accordance with this, the Artemin structure displays an even more strongly bent dimer (Fig. 6C) (Silvian 2006, Wang 2006). Mutagenesis studies of the GDNF molecule clearly show that the tips of the dimer ”wings” is where GDNF binds to GFRα1, with both loops in each wing contributing to the interaction (Eketjäll 1999). This arrangement was also confirmed by the structure of Artemin in complex with GFRα3 (Fig. 6D) (Wang 2006). The more central regions of the ligand could thus be suspected to host the interaction site for a second receptor, as is the case for BMP-2, another ligand of the TGF-β superfamily (Allendorph 2006).

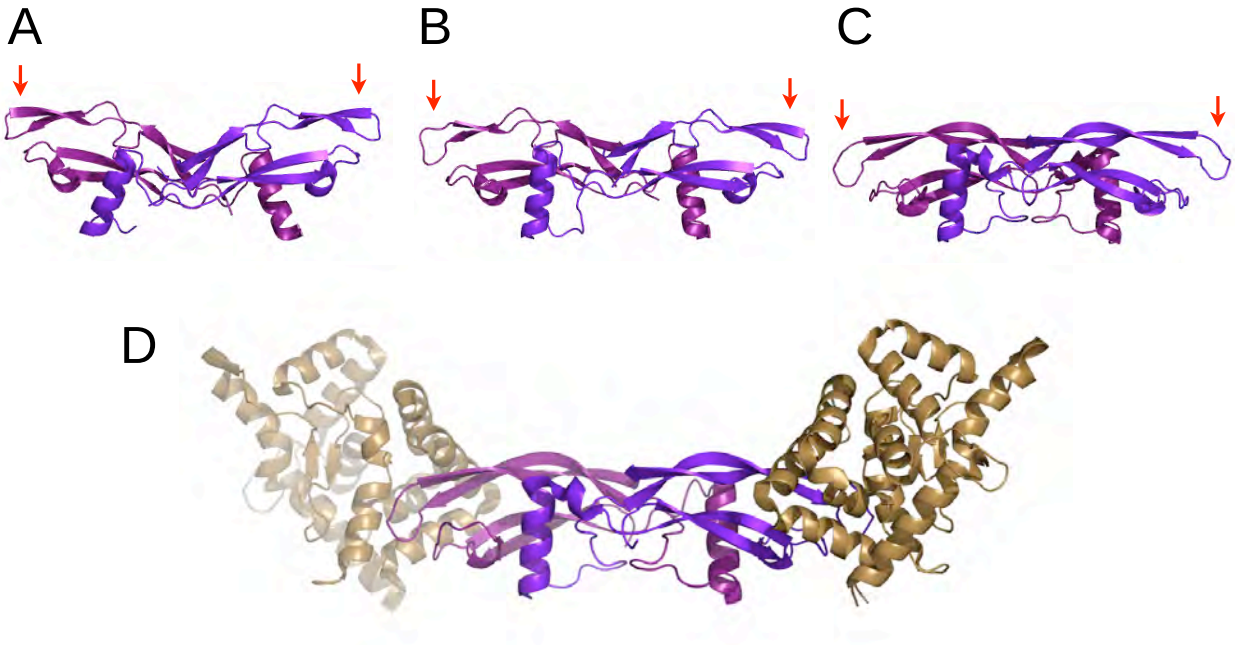

Figure 6. Structures of GDNF family ligands display some intrinsic flexibility. (A-B): Two different crystal forms of GDNF. Note how the ”wings” (red arrows) are bent differently with respect to the vertical axis in the two forms. (C): The structure of Artemin displays an even more bent molecule than the GDNF in (B). (D): The structure of Artemin in complex with the GFRα3 ligand-binding domain.

GFRα structure

Although the structure of the GFRα3 ligand-binding domain is known from X-ray crystallography studies (Wang 2006), to date no complete structure of a GFRα ECD has been determined. The importance of various structural features in GFRα receptors for ligand binding has however been extensively studied in structure-function studies, using deletion mutants and GFRα receptor chimeras (Scott 2001). The GFRα molecule consists of an N-terminal domain of unknown structure, a larger central domain, and a C-terminal domain, the end of which harbors the GPI-anchoring site (Fig.

8A). GDNF was found to bind to the central domain of the GFRα1 molecule (Scott 2001), although the presence of the N-terminal GFRα1 domain has been suggested to stabilize this interaction (Virtanen 2005). Although some earlier studies had suggested that the ligand-binding core domain of GFRα1 consists of two separate domains (Airaksinen 1999), with one study even reporting a crystal structure of the second half of the central domain alone (Leppänen 2004), the structure of Artemin in complex with GFRα3 clearly shows that the central part of the GFRα molecule comprises one single folding unit (Fig. 6D) (Wang 2006). Interestingly, while the chicken Persephin receptor GFRα4 is similar to the other GFRα molecules, mammalian GFRα4 lacks the N-terminal domain of the molecule (Lindahl 2001). GFRα1 has been predicted to be glycosylated in four positions, with one N-linked glycosylation site in each of the N-terminal, central, and C-terminal domains, and one O-linked glycosylation site, residing in the C-terminal domain (Fig. 8A).

Ret structure

The structure of the extracellular domain of the receptor tyrosine kinase Ret is so far known only from structure-function studies and molecular modeling. Homology comparison suggests the presence of four cadherin-like domains (Fig. 7A), as determined by comparing Ret orthologs to a consensus amino acid pattern derived from various cadherin molecules (Anders 2001). In a structure-function study, the first cadherin-like domain was shown to contain the ligand-binding site (Kjaer 2003b). As Ret is easily misfolded upon mutation or deletion, ending up trapped in the endoplasmic reticulum (Kjaer 2003a), this was accomplished by assessing the GDNF binding capabilities of chimeric proteins of human Ret and its *Xenopus* paralog, the latter being unable to bind mammalian GDNF (Kjaer 2003b). The Ret molecule has 12 predicted N-linked glycosylation sites. 11 are distributed over the four cadherin-like domains, with a single site residing in the cysteine-rich domain. Mutations in two of these glycosylation sites have been linked to Hirschsprung's disease (Kjaer 2003a). Molecular modeling of the Ret ECD further revealed a calcium binding site in the interface between the second and third cadherin-like domains (Fig. 7A), concordant with the requirement of calcium for Ret activation (Nozaki 1998, Anders 2001). The molecule also contains a membrane-proximal cysteine-rich domain responsible for dimerization (Asai 1995), and mutations in this region are commonly found in multiple endocrine neoplasias and medullary thyroid carcinomas (Mulligan 1993, Donis-Keller 1993). Recently, residues in the transmembrane domain have also been shown to be important for the dimerization of Ret and, interestingly, two of these residues are mutated in a sporadic case of thyroid medullary carcinoma (Kjaer 2006). The structure of the intracellular tyrosine kinase domain has recently been determined by x-ray crystallography (Knowles 2006), and shown to contain some unexpected features. Receptor tyrosine kinases are usually activated by dimerization followed by autophosphorylation, resulting not only in a lot of phosphorylated residues for intracellular adaptors to bind, but also in a strong increase of the kinase activity itself (Schlessinger 2003). Phosphorylation of the Ret TK domain, however, induces only a moderate increase in kinase activity. Interestingly, one crystal structure displays a dimer conformation with the active kinase

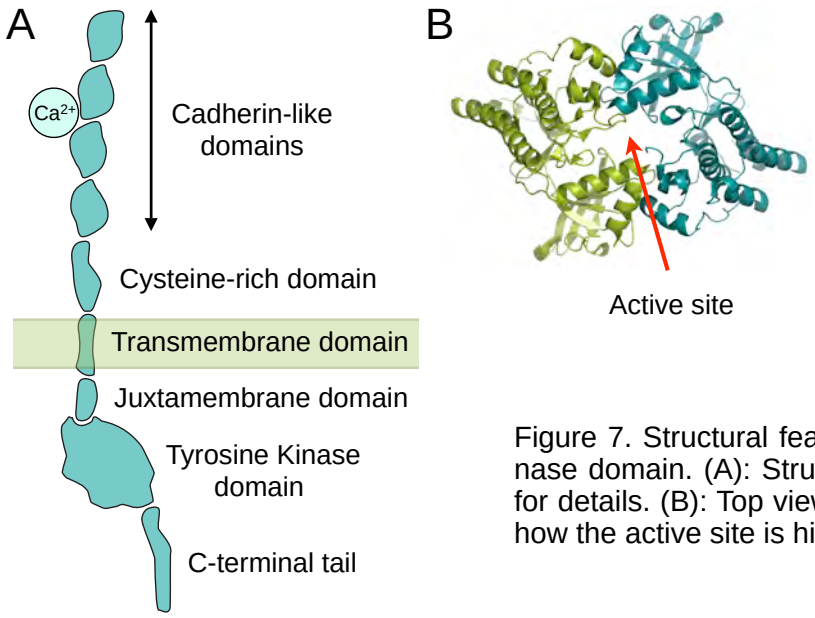

Figure 7. Structural features of Ret and structure of its kinase domain. (A): Structural features of Ret. See the text for details. (B): Top view of the kinase domain dimer. Note how the active site is hidden inside the dimer structure.

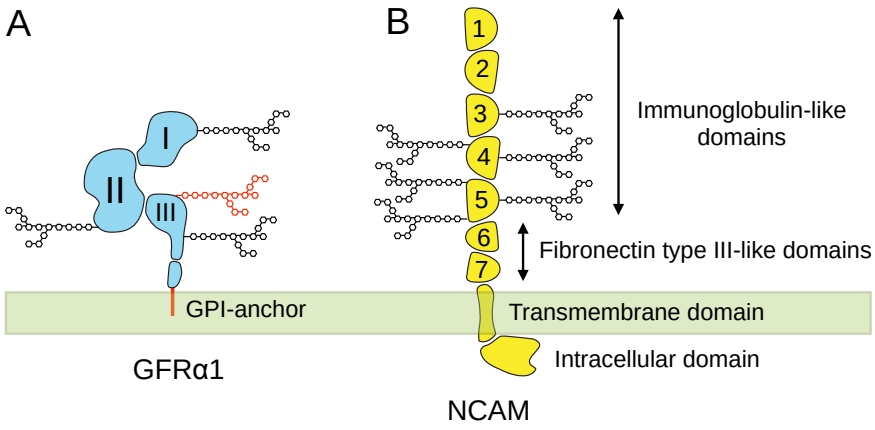

Figure 8. Structural features of GFRα1 and NCAM-140. The domain numbering used in the papers of this thesis are shown, together with determined and predicted glycosylation sites. The putative O-linked glycosylation site in GFRα1 is shown in red.

site hidden inside (Fig. 7B), suggesting that receptor activation could cause a conformational change in a preformed Ret dimer, unmasking the active site. Although many Ret mutations involved in multiple endocrine neoplasias are believed to cause dimer formation (Asai 1995), an alternative possibility is that these mutations change the conformation of a preformed dimer, rendering Ret constitutively active.

NCAM structure

The three major isoforms of NCAM share the same extracellular domain, but differ in their modes of membrane anchoring. NCAM-120 is GPI-anchored, just like GFRα, and is thus incapable of transmitting signals into the cell on its own, while NCAM-140 has a transmembrane α-helix and a short intracellular domain. NCAM-180 is similar to the NCAM-140 ICD but with the addition of a large insertion in the intracellular domain (Maness 2007, Reyes 1993). All three isoforms have identical extracellular regions, comprised of seven domains (Fig. 8B). The five N-terminal domains share the same immunoglubulin-like C2-type fold while the two membrane-proximal domains sha-

re the Fibronectin type III-like fold. NCAM is glycosylated on six sites in its third, fourth and fifth Ig-like domains (Fig. 8B) (Albach 2003), the fifth domain harboring the polysialylation site (Nelson 1995). In addition to this, a muscle-specific splice variant of NCAM has been shown to contain an O-glycosylation site (Suzuki 2003). The crystal structure of domains 1-2 together includes homophilic contacts in the interdomain area, suggesting a role for this interaction in adhesion (Fig. 9A) (Jensen 1999). However, the structure of domains 1-3 displays two other modes of dimerization, both involving domain 3 in the interaction interface (Fig. 9B) (Soroka 2003). In the proposed model, the domain 1-2 interaction is involved in cis-dimerization, implying a potential zipper-like arrangement of NCAM molecules (Fig. 9C). Direct force measurements also show that there may indeed be more than one way for NCAM to make ho-

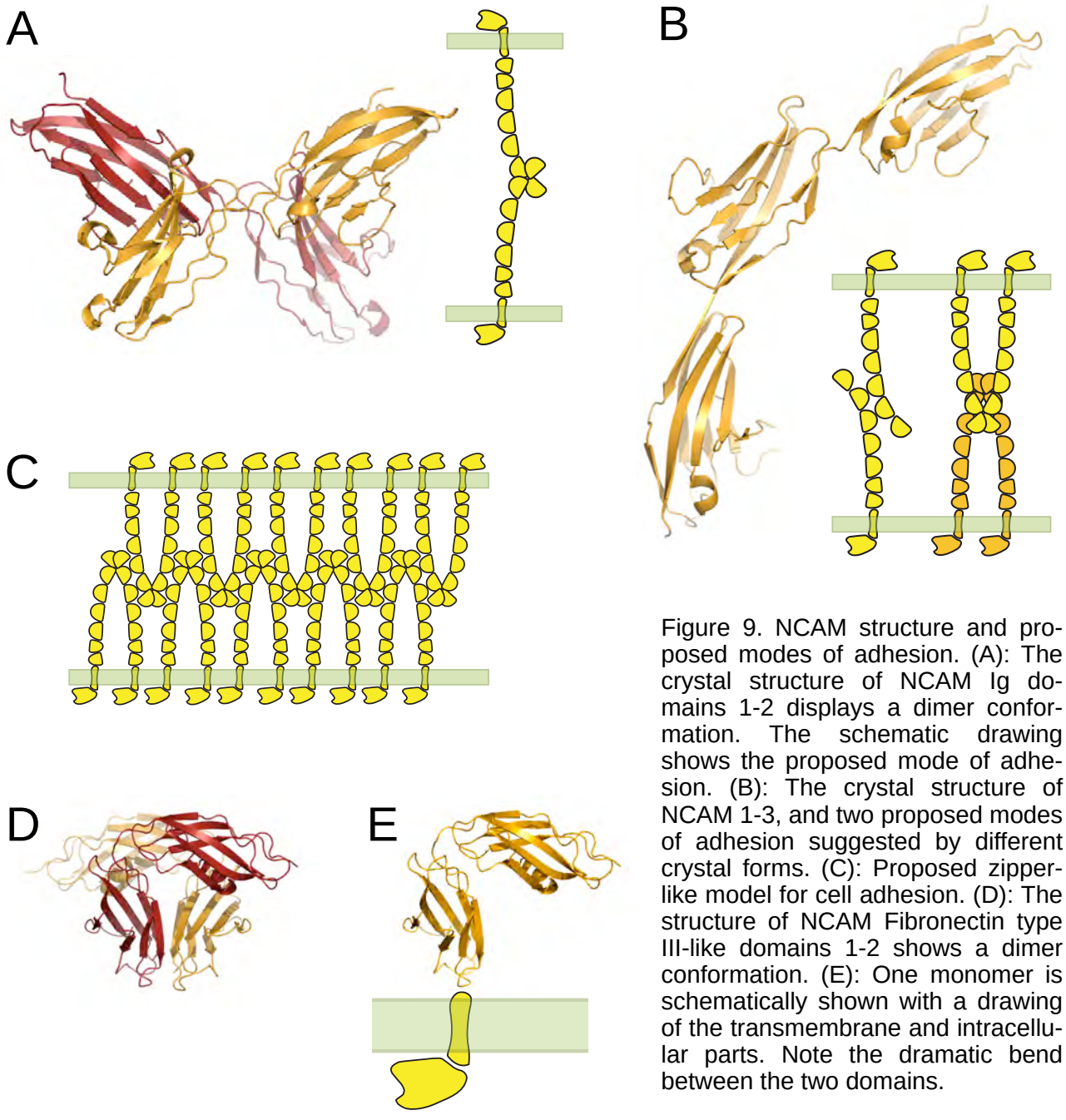

Figure 9. NCAM structure and proposed modes of adhesion. (A): The crystal structure of NCAM Ig domains 1-2 displays a dimer conformation. The schematic drawing shows the proposed mode of adhesion. (B): The crystal structure of NCAM 1-3, and two proposed modes of adhesion suggested by different crystal forms. (C): Proposed zipper-like model for cell adhesion. (D): The structure of NCAM Fibronectin type III-like domains 1-2 shows a dimer conformation. (E): One monomer is schematically shown with a drawing of the transmembrane and intracellular parts. Note the dramatic bend between the two domains.

mophilic interactions, suggesting that domains 1-2 are responsible for one and domain 3 for the other (Johnson 2004, Wieland 2005). Other reports suggest that all five Ig domains are involved in the interaction with domain 3 being especially important, or that the integrity of intramolecular domain-domain interactions is important and that domain 3 is not involved in the actual interaction (Ranheim 1996, Atkins 2004). Domain 2 of NCAM contains the binding site for heparin (Kulahin 2005), and the fibronectin-like domains have been shown to harbor the binding site for FGF receptor 2 (Kiselyov 2003, Christensen 2006). The first fibronectin domain also contains the binding site for polysialyl transferase, the enzyme responsible for polysialylation of NCAM. A recent study reports the structure of the two fibronectin domains together, showing a dramatic bend between these two domains, and suggesting an additional site for cis-dimerization (Fig. 9D-E) (Carafoli 2008). Substantial bends in the NCAM molecule are also evident from electron microscopy studies of purified NCAM molecules (Hall 1987, Becker 1989). Despite the large amount of structural data available for the extracellular domain, the structures of the intracellular domains of NCAM-140 and -180 are as yet unknown.

Evolution

GDNF family ligands in evolution

In the vertebrates, the four paralogs in the GDNF family of ligands are present in all classes from bony fishes to mammals. Chicken, however, lacks the GFRα4 ligand Persephin, and clawed frog lacks the GFRα2 ligand Neurturin (Fig. 10), although all four GFRα receptors are clearly present in both genomes (Hätinen 2006). A plausible explanation for this is that the two rounds of gene duplication that occured early in vertebrate evolution (Dehal 2005) introduced a certain amount of redundancy among the ligands, allowing for the specific loss of one ligand in frog and chicken. As a result of the additional round of gene duplication in teleost fishes (Hoegg 2004), zebrafish has two copies of three of the GDNF family ligands. No GDNF-like molecules have been found in other phyla than the chordates, and no chordate GFL outside the vertebrate lineage has been described to date (Airaksinen 2006). The function of GDNF in ENS development is conserved among vertebrates (Shepherd 2001), but its role for renal development in non-mammals is less clear.

GFRα receptors and GFRα-related molecules

Although the ligands appear to be missing in other lineages than the vertebrates, putative orthologs of the GFRα receptors are present in insects, sea urchins, *C. elegans* and *Amphioxus*, while the absence of GFRα-like genes in *Ciona intestinalis* suggests a urochordate-specific loss (Fig. 10) (Hätinen 2006). Zebrafish has duplicate copies of GFRα1 and GFRα2 due to the teleost fish genome duplication. GFRα1 is required for ENS development in zebrafish, suggesting a highly conserved function of the GDNF-

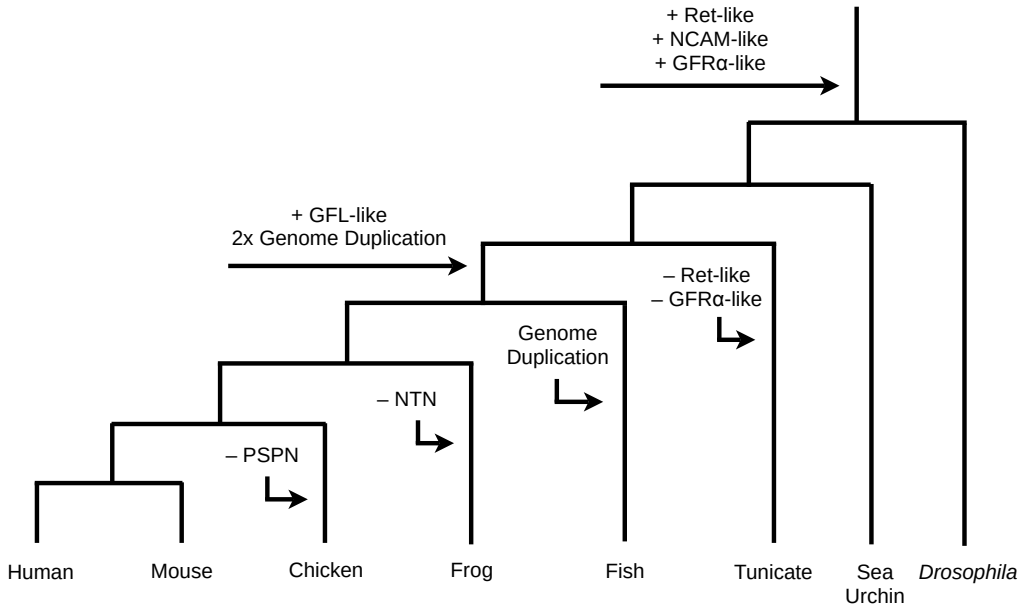

Figure 10. The evolutionary origins of GDNF family ligands and their receptors, and the lineage-specific loss of ligands and receptors in evolution. See text for details.

GFRα1-Ret complex in this developmental process among the vertebrates (Shepherd 2004). Interestingly, additional GFRα-like sequences have been found in the vertebrate genome. One such gene, GRAL, was described to be expressed in the CNS of the adult mouse and to promote survival of cultured PC12 cells and Hippocampal neurons, although GDNF family ligands were not able to activate Ret via GRAL (Li 2005, Airaksinen 2006). Recently Gas1, a more distantly related GFRα-like protein, has been found to modulate Ret signaling. Gas1 appears to bind Ret in the absence of ligand, and a very recent report suggests that it inhibits GDNF-mediated phosphorylation of Ret tyrosine 1062 (Cabrera 2006, López-Ramírez 2008).

Ret in evolution

Ret is present in all examined vertebrates from fish to human, and in addition to this a Ret ortholog has been found in *Drosophila* (Hahn 2001). No Ret-like molecule is present in *Ciona*, in accordance with the loss of GFRα in urochordates (Airaksinen 2006). In zebrafish development Ret is expressed in the excretory system, CNS and PNS, including the enteric nervous system. The latter is concordant with the observed importance of GDNF and GFRα for ENS development. Despite the teleost fish genome duplication, to date only one Ret gene has been found in zebrafish. Interestingly, *Drosophila* Ret is expressed in neurons of the foregut, the excretory system and parts of the central and peripheral nervous systems, implying functional similarities to mammalian Ret in development (Sugaya 1994, Hahn 2001). Ret is structurally related to the cadherin family (Anders 2001), but it is not known whether invertebrate Ret paralogs can play a role in cell adhesion.

NCAM and NCAM-2

NCAM belongs to the immunoglobulin superfamily of cell adhesion molecules. This family includes a large number of molecules, characterized by their extracellular do-

mains comprised of tandem arrays of immunoglobulin-like and fibronectin type III-like folds. Many of these molecules are involved in development and maintenance of the nervous system, playing diverse roles in processes such as precursor migration, axon guidance, synapse formation and maintenance of neural connections in the adult (Fusaoka 2006, Rougon 2003). Two paralogous NCAM molecules have been found in all vertebrates, with additional paralogs in bony fishes. NCAM-2 isoforms corresponding to NCAM-120 and -140 are expressed in mouse, but the gene lacks an exon corresponding to the NCAM-180-specific insertion. Although knowledge about NCAM-2 function is very sparse, NCAM-2 is abundantly expressed in the olfactory bulb suggesting a role in development of the olfactory system (Kulahin 2008, Alenius 2003). NCAM orthologs are also present in *C. elegans*, several insects and sea urchin, with the major difference being that invertebrates appear to have only one NCAM paralog. In addition to this, the *Drosophila* NCAM ortholog Fasciclin II has been demonstrated to play a role in axonal growth and synaptic plasticity. Interestingly, Fasciclin II has also been suggested to signal via the FGF receptor (Kristiansen 2008), suggesting that this mode of NCAM signaling is evolutionarily conserved.

Aims of this thesis

The principal aims of this thesis were:

1. To describe the interaction determinants of the GDNF-NCAM-GFRα complex.

2. To assess the role of these determinants for various aspects of complex formation and receptor function.

3. To study the evolutionary conservation of GDNF receptor function, by examining a *Drosophila* Ret othologue for its functional properties.

4. To examine potential GDNF receptor candidates in GABAergic neurons from the medial ganglionic eminence.

Comments on materials and methods

The materials and methods used in this thesis are in general well described in the four papers. In some cases, however, these descriptions leave out some aspects of the methodology, or the rationale for using a particular method.The following few comments have therefore been added for clarification.

Deletion constructs

In order to make deletion mutants of NCAM and GFRα1, boundaries between the different folding domains had to be chosen. The GFRα1 domain boundaries were determined by inspecting the sequence of GFRα1 for sequences corresponding to the N-and C-terminal ends of the crystal structure of the central, ligand-binding domain GFRα3 (Wang 2006). For NCAM, a series of constructs was originally generated, some of which had intrinsic folding problems. This was assessed by treatment with the endoclycosidase EndoH, to see whether the proteins were stuck in the endoplasmic reticulum or not. When the exon structure of the chicken NCAM gene (Owens 1987) was compared to the rat NCAM sequence, and the boundaries between exons were used for the definition of domain boundaries in the rat polypeptide, the folding of individual constructs was markedly improved. Figure 11 shows schematic illustrations of the NCAM and GFRα1 deletion constructs used in papers I and II of this thesis.

Selective immunoprecipitation of surface molecules

The observed folding problem highlighted the issue of quantification of ligand binding. A normalization to the total expression level could be misinformative, lowering the apparent binding capacity of constructs for which some of the protein is arrested in the ER. We therefore developed a one-step protocol for the immunoprecipitation of surface molecules. Intact cell monolayers transfected with N-terminally HA-tagged constructs were incubated with 10ug/ml anti-HA antibodies in binding buffer (PBS, 0.1% BSA, 0.1% Glucose, 1mM $MgCl_2$, 1mM $CaCl_2$) for 1-2 hours at 4°C, and washed exten-

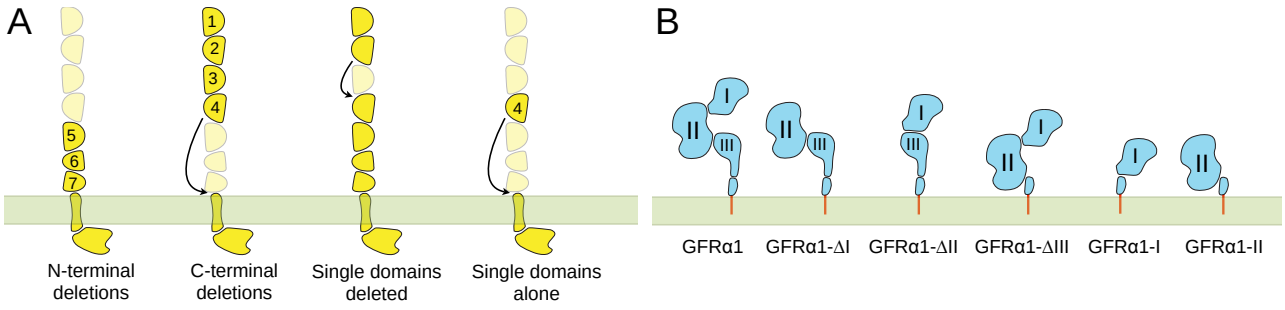

Figure 11. The NCAM and GFRα1 constructs used for mapping of the interaction sites in papers I and II. (A): Rationale for the NCAM deletions. (B): The GFRα1 constructs used in paper II.

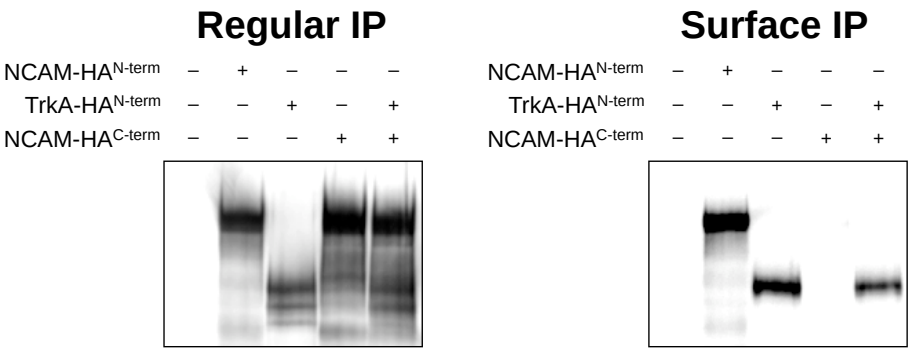

Figure 12. Proof-of-concept of the surface immunoprecipitation protocol. The left panel shows a standard IP, while the right panel shows a surface IP. Note that the C-terminally tagged NCAM is not pulled down in the surface IP.

sively with PBS to remove excess antibody. The cells were then lysed in lysis buffer containing 2% Octyl-β-glucoside, followed by pull-down of the antibody complex with protein G sepharose. The specificity of this protocol for only immunoprecipitating surface molecules was assessed in a proof-of-concept experiment (Fig. 12), where N-terminally tagged TrkA was efficiently immunoprecipitated, while co-transfected C-terminally tagged NCAM was not. In the standard-type IP where antibody was added to the lysates, however, both proteins were immunoprecipitated.

Radiolabeling of GDNF and Chemical crosslinking

GDNF was radioiodinated using a redox reaction catalyzed by lactoperoxidase. Carrier-free GDNF was mixed with hydrogen peroxide, ^{125}I and lactoperoxidase, and incubated at room temperature for 30 minutes. The reaction was stopped by the addition of Sodium Iodide and Glycine, and the labeled ligand was purified from the free ^{125}I using Sephadex G25 gel filtration media. The choice of EDC as crosslinking reagent was based on the fact that it is very short, a so called zero-length crosslinker, and

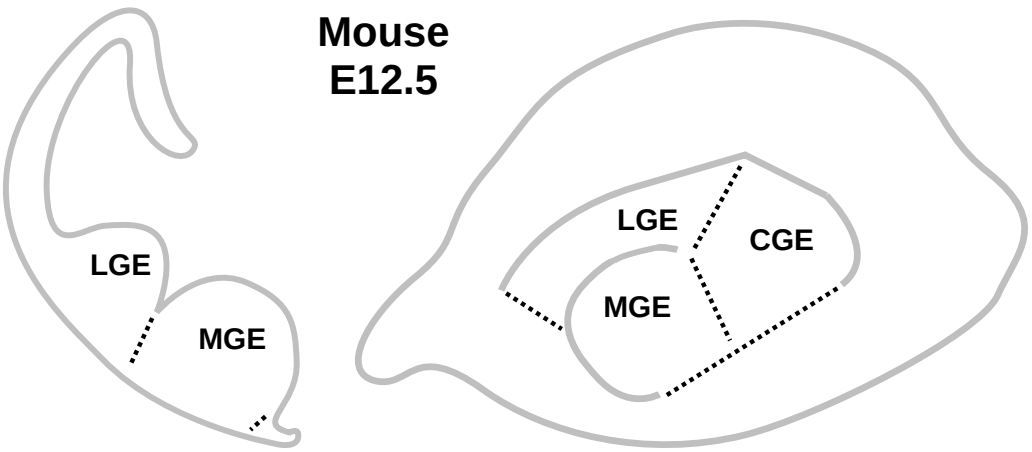

Figure 13. Schematic drawing illustrating the approximate borders used for dissection of the MGE.

should thus primarily pick up direct interactions. It is also non-reducible, enabling detection of the crosslinked complex with monomeric receptors in western blots after reducing SDS-PAGE.

Dissection and neurite measurements

The medial ganglionic eminence was dissected from E12.5 mouse brains in PBS+0.6% glucose, and dissociated by treatment with trypsin in PBS+0.6% glucose for 20 minutes. The reaction was stopped with horse serum, DNase was added, and the cells were dissociated by pipetting up and down. The cells were then washed three times with PBS+0.6% glucose before counting and plating. For clarity, Figure 13 shows approximate borders used for dissection of the MGE. Pictures were taken in an Axiovert 200 inverted microscope (Zeiss) using OpenLab software (Improvision Ltd.) and collected in a systematic fashion to avoid selection bias. Neurites were measured manually in ImageJ (NIH). A freeform line was drawn along the longest process of every cell, even when this process was very short, and the length of this line was subsequently measured using the ImageJ software.

Results and discussion

Paper I

In the first paper of this thesis, our aim was to describe the interaction interface between GDNF and its receptor NCAM. As the different domains of the NCAM ECD have been shown to harbor different functional properties of NCAM, we first set out to determine what domains were required for the ligand-receptor interaction. We created a large set of mutant NCAM molecules, where the extracellular region either had one or several domains deleted, or consisted of a single domain on its own. NCAM binds to GDNF in the absence of GFRα1 (although the latter strongly potentiates binding), which allowed for studying the NCAM-GDNF interaction in our deletion constructs without having to take into account how the deletions would affect GFRα1 binding. COS-7 cells transfected with the various mutant NCAM constructs were incubated with radioiodinated GDNF and subjected to chemical crosslinking with EDC/S-NHS. Our results show that the immunoglobulin-like domain 3 of NCAM is required for efficient GDNF interaction, and that it is also the only one of the seven domains that is capable of forming a strong interaction with the ligand on its own.

In order to get a more detailed view of the interaction, we decided to fine-tune the binding site. Since the crystal structures of GDNF and domain 3 of NCAM have been determined previously, we took advantage of molecular dynamics software to create a model of the interaction. Interestingly, all the resulting conformations, representing the calculated energy minima for the interaction, placed the NCAM domain 3 in the same position onto the GDNF molecule, with positively charged amino acid side chains in GDNF in close proximity of negatively charged residues in NCAM (Fig. 14). The unanimousity of these results already suggested that this model is a good representation of the real interaction interface, but in order to test our hypothesis we mutated the four amino acids in the NCAM molecule that were predicted to be the most important for the interaction in our model. The resulting 4x mutant full-length NCAM molecule was expressed well, but did not bind GDNF, a result that was phenocopied by a construct based on domain 3 on its own harboring the four mutations. Our results show that these four mutated amino acids are necessary for the interaction, and support the validity of our model.

In order to see whether this impaired ability to bind GDNF would also affect NCAM-mediated cell adhesion, we compared our 4x mutant NCAM construct with the wild-type molecule in a Jurkat cell adhesion assay. Our results show that the 4x mutant NCAM and wild-type NCAM promote cellular aggregation equally well, but that an NCAM molecule lacking domain 3 altogether has decreased adhesion-promoting properties. The results suggest that NCAM's function as a GDNF binding molecule involves structural features in its third domain that are different from those required for cellular adhesion, and that these two functions can be genetically separated.

Finally, we modeled the interaction between GDNF and GFRα1, using the Artemin-GFRα3 structure as a template. The GDNF-GFRα1 interaction was then added to the

GDNF-NCAM domain 3 complex. Moreover, domains 1-2 of NCAM were added with guidance from the structure of domains 1-3. Our model of the NCAM-GDNF interface allowed for the formation of such a tripartite 2:2:2 complex (Fig. 15B-D), but we had no biochemical proof for its existence. Having noticed several high molecular weight complexes in crosslinkings with all three molecules present, we ran crosslinkings of GFRα1/NCAM co-transfected cells on low percentage SDS-PAGE gels. Several bands were found to contain all three molecules, in various stoichiometrical combinations including the 2:2:2 complex suggested by our model. Although this supports the existence of a tripartite complex such as the one depicted in Figure 15B-D, it should be added that the actual complex may look quite different, due to the lack of x-ray data on substantial parts of the receptors.

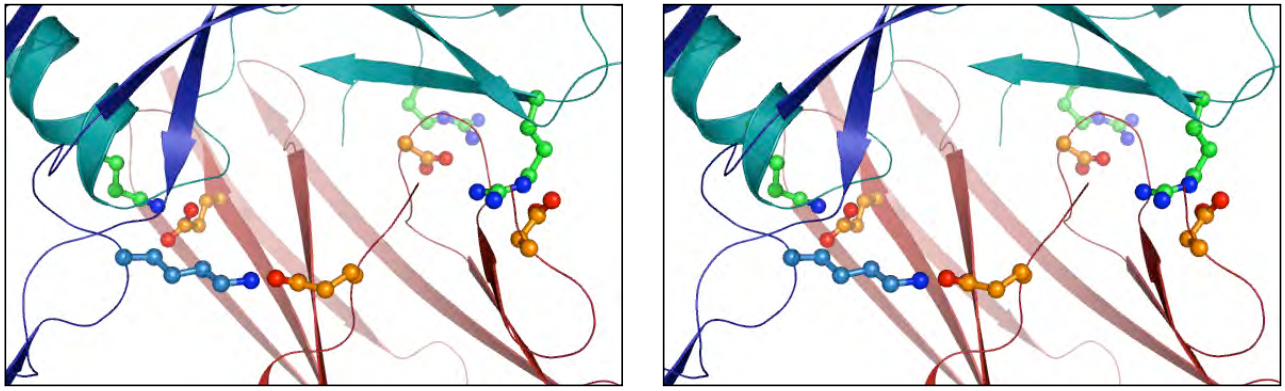

Figure 14. Cross-eyed wiew of the interaction interface proposed in paper I. If the reader is able to cross his/her eyes to let the pictures overlap, a three-dimensional view of the interface appears. Note that in this picture NCAM is coloured red, and the GDNF protomers are blue and green.

Paper II

For the second paper, we set out to map the determinants for the NCAM-GFRα1 interaction, and to find out how these determinants affect GDNF signaling. Both NCAM and GFRα1 are known to be glycosylated at several sites, and such post-translational modifications could potentially affect the interaction, either negatively or positively. In order to assess the role of N-linked glycosylations for the interaction, we co-immunoprecipitated GFRα1 in the presence or absence of the N-glycosidase PNGaseF, using full-length NCAM as a bait. Although the N-glycosylations were efficiently cleaved off by the PNGaseF treatment, the GFRα1 molecule stayed bound to the NCAM molecule despite thorough washing of the immunoprecipitates. Interestingly, when N-glycosylations were cleaved off before the immunoprecipitation step, more GFRα1 remained bound to NCAM, suggesting that N-linked glycosylations may actually have a negative effect on NCAM-GFRα1 binding. Since GFRα1 has a potential site for O-linked glycosylations, we also made a GFRα1 construct lacking the entire membrane-proximal domain, including the O-glycosylation site. This construct (GFRα1-Δ3) had a binding capacity comparable to the wild-type molecule, showing that the membrane-

proximal domain, including the O-glycosylations, is dispensable for the interaction with NCAM.

Taking advantage of the large set of NCAM deletion constructs generated in paper I, we used co-immunoprecipitation to study the interactions between full-length GFRα1 and various NCAM deletion constructs. We discovered that the deletion of NCAM domain 4 severely impaired binding, and that a smaller negative effect was also seen upon the deletion of domain 2. In order to see if these domains were able to interact on their own, we performed co-immunoprecipitation experiments with single domains as the sole NCAM extracellular sequence. The results clearly show that domain 4 is the only NCAM domain that strongly binds GFRα1 on its own, suggesting that this domain harbors the major interaction site. Next, we made a series of GFRα1 deletion constructs in order to map the other side of the interaction interface. Deletion of the N-terminal domain in GFRα1 impaired its interaction with NCAM in our co-immunoprecipitation assay, and this domain also bound to NCAM very effectively on its own. This suggests that the N-terminal domain is the major NCAM-binding region in the GFRα molecule. Figure 15A is a schematic illustration of the front view of the tripartite complex proposed in paper II. Figure 15E-F shows schematic drawings of how the complete complex may possibly look. Figure 15E shows the complex with the NCAM domains 1-2 oriented as in the model proposed in paper I. Figure 15F includes the dramatic bend obsereved in the structure of the fibronectin-like domains (Carafoli 2008) and in addition to this takes into account the observed, much weaker interaction between GFRα1 and NCAM domain 2, at the expense of the NCAM domain 1-2 homodimerization orientation.

In addition to mapping the interaction interface, it was also interesting to see how these interaction determinants affect GFRα1-dependent NCAM-GDNF binding potentiation. In order to do this, we compared GFRα1 molecules including or excluding the N-terminal domain for their ability to potentiate NCAM-GDNF crosslinking. If GFRα1 needs to bind to NCAM in order to potentiate GDNF binding, the potentiating capability of a GFRα1 molecule lacking the N-terminal domain should be severely impaired. If however GFRα1 potentiates GDNF-NCAM binding by concentrating the ligand in the plasma membrane and presenting it to NCAM, only the ligand-binding domain of GFRα1 should be required for potentiation. Cells co-transfected with NCAM and the respective GFRα1 constructs were incubated with radiolabeled GDNF and subjected to chemical crosslinking. Intriguingly, the autoradiographs clearly showed that the N-terminal domain of GFRα1, despite being the major NCAM-binding determinant in the molecule, was not necessary for potentiation of GDNF binding to NCAM while the N-terminal domain alone displayed no potentiating properties. Our results suggest that the potentiation effect is achieved by GFRα1 concentrating GDNF close to the membrane, presenting it to the NCAM molecule, rather than allosterically changing the GDNF binding properties of NCAM.

Finally, as GFRα1 has previously been shown to modulate NCAM-mediated cell adhesion (Paratcha 2003), we determined the role of the N-terminal domain of GFRα1 in this process. The effects of the different GFRα1 constructs on NCAM-mediated cell

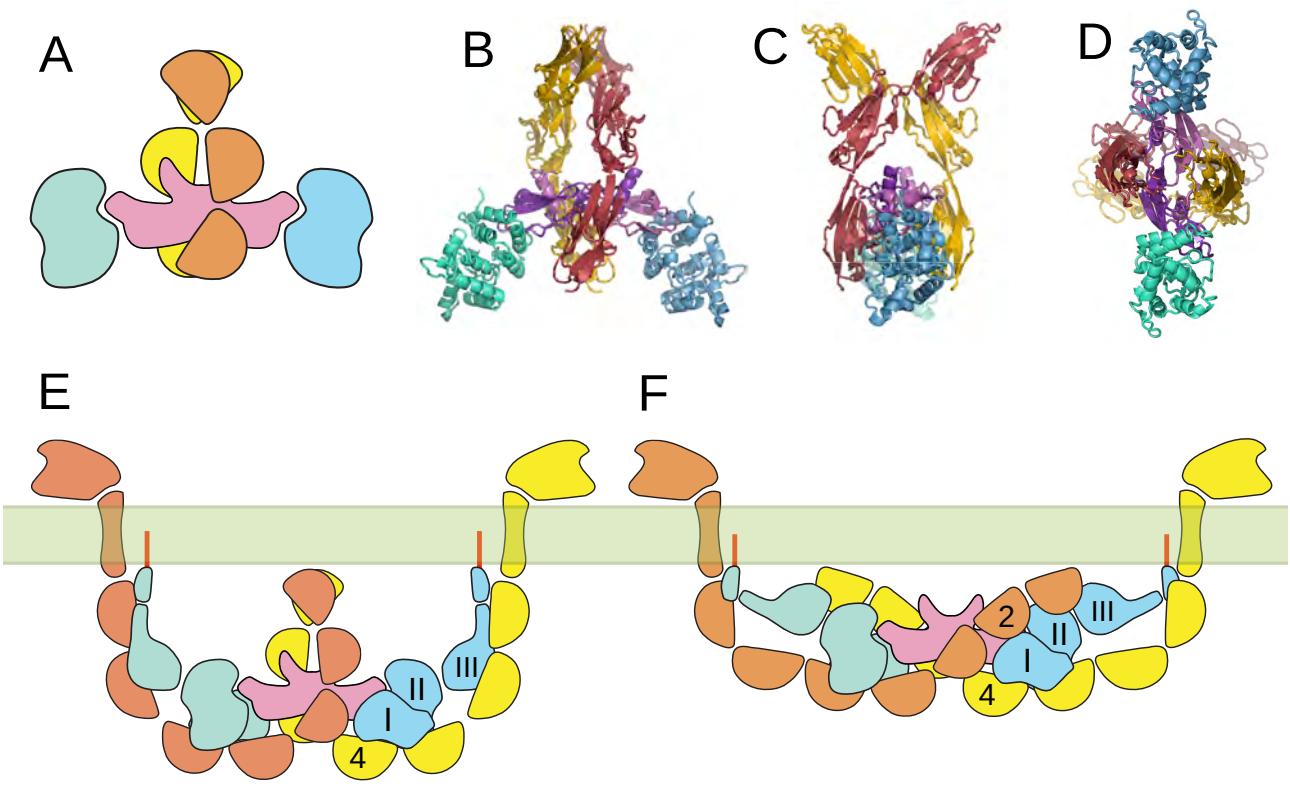

Figure 15. The GDNF-NCAM-GFRα1 complex. (A): Schematic drawing of the model of the tripartite complex proposed in paper I. (B-D): Front, side and bottom views of the model. (E): Schematic drawing of how the entire complex may possibly look, including the observed interaction between NCAM domain 4 and the N-terminal domain of GFRα1. (F): Similar to E, but including the observed (much weaker) NCAM domain 2 interaction and the dramatic bend described in the crystal structure of the two fibronectin domains.

adhesion were assessed using a Jurkat cell aggregation assay. The N-terminal domain potently decreased NCAM-mediated cell adhesion on its own, while the deletion of this domain markedly decreased its inhibitory effect. Our results indicate that the N-terminal domain of GFRα1 is not only the major NCAM interacting site, but that it is also required for efficient modulation of NCAM-mediated cell adhesion. This feature of GFRα1 is independent of its function as a potentiator of GDNF-NCAM binding.

Paper III

The aim of paper III was to find out whether the functional properties of the GDNF receptor Ret are evolutionarily conserved from insects to mammals. While the intracellular tyrosine kinase domain is 65% identical between *Drosophila* and mammalian Ret (Anders 2001), the extracellular domain is not so well conserved, implying that the ligand binding function of Ret may have appeared later during evolution, and that the primordial Ret-like molecule of the last common ancestor of insects and vertebrates had another mechanism for activation of the tyrosine kinase. Interestingly though, the four cadherin-like folding domains in the ECD are conserved in *Drosophila* Ret, which indicated a potential cadherin-like role as an adhesion molecule.

In order to assess the GDNF-binding properties of *Drosophila* Ret, we used two different approaches. First, the extracellular domains of human Ret (hRet) and *Drosophila* Ret (dRet) were immobilized, and tested for their ability to bind to a preformed complex of GFRα1 and GDNF in a sandwich ELISA assay. Although human Ret bound strongly to the GDNF-GFRα1 complex in our assay, dRet displayed no binding above background levels. We also performed a ligand crosslinking assay, with COS-7 cells cotransfected with rat GFRα1 and either *Drosophila* or human Ret. While radioiodinated GDNF was efficiently crosslinked to hRet, no such band was detectable in the autoradiographs for the dRet transfected samples. Taken together, our data show that *Drosophila* Ret is unable to bind to the mammalian GDNF-GFRα1 complex, and that it is thus most likely not functional as a GDNF receptor.

In order to determine whether *Drosophila* Ret can mediate cell adhesion, we performed a cell adhesion assay. While Jurkat cells transfected with the positive control NCAM gave rise to large aggregates, *Drosophila* Ret displayed no aggregation above control levels, suggesting that dRet does not have a function as a cell adhesion molecule in the fly.

Finally, we set out to determine whether the more homologous kinase domain in *Drosophila* Ret would show some degree of functional conservation. We found that dRet overexpression resulted in weak background autophosphorylation, indicating a functional tyrosine kinase. In order to test for ligand-dependent activities, we made a chimeric construct consisting of the extracellular, transmembrane and juxtamembrane regions of hRet fused to the kinase domain of dRet. *T*he chimeric molecule was then tested for its ability to induce PC12 cell differentiation. The chimeric protein was autophosporylated upon overexpression, and this effect was abolished in a kinase dead mutant version, clearly showing that the kinase domain of dRet is functional. In order to see if the dRet kinase domain in the chimeric molecule could be activated upon ligand stimulation, PC12 cells were stimulated for three days with GDNF and soluble rat GFRα*1*. Stimulation of PC12 cells transfected with the chimeric construct gave a differentiation response similar to hRet-transfected cultures, an effect that was not seen in cells expressing a kinase dead mutant version of the chimeric protein. The same cells expressing wild-type *Drosophila* Ret only displayed background levels of differentiation. Altogether, our results suggest that although the extracellular domains of the insect and mammalian orthologs seem to have divergent properties, the function of the intracellular domain of Ret appears to be well conserved from insects to mammals.

Paper IV

The last paper of this thesis is a study on GDNF signaling in interneuron precursors from the medial ganglionic eminence (MGE). GDNF and GFRα1 have previously been shown to promote tangential migration and differentiation of GABAergic interneurons from the MGE (Pozas 2005), but the receptor responsible for transmitting the signal through the plasma membrane is unknown. We have examined two interesting candidate molecules for their role in GDNF signaling in MGE neurons: The Neuregulin-1

receptor ErbB4 and the HGF receptor Met. In order to see whether GDNF could activate these receptors, we transiently stimulated mouse E12.5 MGE cultures with GDNF and subjected the samples to SDS-PAGE followed by Western blotting with antibodes specific for either phospho-ErbB4 or phospho-Met. Our data showed that although ErbB4 was expressed in our cells, and was strongly phosphorylated upon stimulation with its endogenous ligand Neuregulin-1, the GDNF stimulated cultures displayed no increase in ErbB4 phosphorylation above the level of untreated control cultures. HGF stimulation of our cultures resulted in a weak but clear Met phosporylation, confirming the expression of Met. Treatment with GDNF, however, gave no increased phosphorylation of Met above background levels. As the phosporylation of the downstream effector ERK1/2 was much stronger in the HGF-stimulated samples, we made a titration of HGF concentrations in order to exclude the possibility that GDNF induced Met phosporylation, but that the level of induction was too weak to be detected in our immunoblots. Using an HGF concentration resulting in phospho-ERK levels comparable to the GDNF-stimulated sample, HGF still induced Met phosphorylation while GDNF did not, clearly showing that GDNF does not exert its effects through Met phosphorylation.

In order to see if any of these receptors were involved in the morphological differentiation of MGE interneurons, we cultured our cells in the presence or absence of specific ErbB4 or Met inhibitors. Cells stimulated with GDNF for 2 days showed a two-fold increase in neurite outgrowth (determined by assessing the number of cells harboring a neurite of a length above a chosen cutoff), and this effect was not affected by the presence of the ErbB4 inhibitor AG1478. This strongly suggests that ErbB4 activation is not a step in the signaling pathway for GDNF-induced neurite outgrowth. To our surprise, we found that the Met inhibitor SU11274 displayed a clear outgrowth-promoting effect of its own. Adding GDNF to the cultures further increased the number of neurite-bearing cells, showing that Met activation is not necessary for the outgrowth-promoting activity of GDNF. Moreover, the effects were additive and not synergistic, suggesting that the effects of GDNF and SU11274 are caused by the activation of separate signaling pathways. In order to exclude the possibility that the unexpected effect of the Met inhibitor was due to off-target effects of SU11274 (which is sometimes common among kinase inhibitors), we tested another Met inhibitor: PHA665752. This Met inhibitor had a strong (three-fold) positive effect on neurite outgrowth even at concentrations as low as 200 nM, which suggests that the observed effect could indeed be due to Met inhibition.

Finally, we examined the effects of the endogenous ErbB4 and Met ligands on our cultures. Although Neuregulin-1 stimulated phosphorylation of ErbB4 in our cells, it only displayed a weak outgrowth-promoting effect which we found not to be statistically significant. HGF stimulation had no effect on the number of neurite-bearing cells. Although the effects of Met inhibition would suggest that HGF could potentially have a negative effect on neurite outgrowth, no such effect was seen. However, it should be noted that the baseline in our cultures was quite low, and that a negative effect of added ligand could therefore be difficult to detect. It is also possible that the observed

effects are due to the inhibition of an HGF-independent Met signaling pathway, activated by other, as yet unknown factors.

Taken together, our data in paper IV show that neither ErbB4 nor Met are involved in GDNF signaling in mouse E12.5 MGE cultures, and that neither HGF nor Neuregulin-1 display any significant effect on the morphological differentiation of the same cells. We also suggest that, surprisingly, inhibition of Met may have a positive effect on neurite outgrowth in cortical GABAergic interneurons.

Concluding remarks and future perspectives

In the first two papers of this thesis, we describe the binding determinants of the NCAM-GDNF-GFRα1 complex. We also describe the functional implications of these molecular features for GDNF signaling and NCAM-mediated cell adhesion, and find that these two processes depend on separate structural determinants of the molecules.

GDNF was found to bind to the third Ig-like domain of NCAM, and modeling of the interaction site revealed four charged contacts, the mutation of which abolished the interaction. Interestingly though, NCAM-mediated cell adhesion was unaffected by the same mutations, suggesting that these two functions of NCAM depend on different molecular determinants. A number of complexes of high molecular weight were found in crosslinking assays, and some of these were shown to contain all three molecules, proving the existence of a tripartite complex.

The fourth Ig-domain of NCAM was shown to be both necessary and sufficient for GFRα1 binding, with a possible small contribution from the second Ig-domain, and the N-terminal end of GFRα1 was shown to be the major NCAM-binding determinant. Enzymatic removal of N-glycosylations increased NCAM-GFRα1 binding, suggesting that this could be a mechanism to regulate complex formation, with possible effects on cell adhesion and GDNF-NCAM signaling. Intriguingly, in the proposed model of the complex the NCAM molecule and the GFRα1 molecule have their C-terminal ends in opposite directions. Although surprising, this is quite plausible, considering the fact that the structure of the N- and C-terminal parts of GFRα1 presently are unknown and may show to contain regions of high flexibility. Without this structural information, it is thus hard to tell how the ligand-binding domain is oriented with respect to the membrane. In addition to this, several studies suggest that the NCAM molecule adapts a heavily kinked conformation (Hall 1987, Becker 1989), further supported by the sharp bend in the interdomain region of the recently determined structure of the two fibronectin domains (Carafoli 2008). This implies that the N-terminal part of NCAM could potentially fold back towards the membrane in order to form the complex with GDNF and GFRα1, as in the schematic drawings in Figure 15E-F.

GFRα1 was shown to strongly potentiate GDNF-NCAM binding, and also to form a tripartite complex, even when its major NCAM-binding determinant (the N-terminal domain) was deleted. This strongly suggests that GFRα1 does not need to bind directly to NCAM in order to potentiate ligand binding and GDNF-NCAM signaling. The same deletion construct, however, showed a markedly impaired capability to modulate NCAM-mediated cell adhesion, while the N-terminal domain alone displayed wild-type effects. We therefore postulate that the ligand-binding part of GFRα1 is responsible for modulation of NCAM signaling while the N-terminal domain is responsible for modulating NCAM-mediated cell adhesion.

The last two papers shed some light on other, as yet unknown, modes of GDNF signaling. A *Drosophila* Ret-like molecule (dRet) was found to have at least part of its function (the tyrosine kinase activity), conserved from fruit fly to human. dRet did however

not bind mammalian GDNF. Since *Xenopus* Ret has also been shown not to bind mammalian GDNF (Kjaer 2003b), and since the extracellular domain of Ret is not very well conserved (Anders 2001), this is however not entirely surprising. *Drosophila* Ret did not mediate cell adhesion in a Jurkat cell aggregation assay, despite its proposed cadherin-like ECD structure. Interestingly, however, *Drosophila* Ret displayed weak kinase activity upon overexpression. Since mammalian GDNF did not bind to dRet in our assays a chimeric construct was generated, comprised of the extracellular, transmembrane and juxtamembrane domains of human Ret and the kinase domain of dRet. Upon stimulation with mammalian GDNF in the presence of soluble GFRα1, the chimeric molecule induced differentiation of PC12 cells similarly to wild-type human Ret. This shows that the kinase domain of Ret is evolutionarily conserved, not only in terms of sequence but also in terms of functionality. We do however not know what extracellular factors are responsible for its activation in *Drosophila*, and considering the considerable evolutionary divergence of the extracellular domain these factors may even be unrelated to the GDNF family of ligands.

Furthermore, ErbB4 and Met, two very interesting candidates for transferring the GDNF signal through the plasma membrane during interneuron development, were found not to be involved in this mechanism. Neurite outgrowth experiments with Met inhibitors, however, yielded some quite unexpected results, with two different Met kinase inhibitors stimulating neurite outgrowth on their own. Since Ret is known as a potent motogen for neurons, one could speculate that the opposing effects of GDNF on neurite outgrowth may reflect a mechanism for the definition of different interneuronal subsets. Since kinase inhibitors are known to often display off-target effects, however, other types of Met inhibition experiments would be useful, in order to see if the observed effects on neurite outgrowth can be confirmed.

Obviously, a lot more work needs to be done on GDNF and its receptors in order to fully understand how the structural features of the molecules affect their various functions. First, structural data has to be obtained for the missing pieces of the receptors and, most importantly, for the entire receptor complexes. While the classical methods of X-ray crystallography and solution NMR may well be the most appropriate methods for the former, a complete understanding of the receptor complex would ideally involve information on real-life conformation and complex dynamics. Since this information will most like be more important than high resolution for understanding the function of the receptor complex as a whole, new methods such as cryo-electron tomography of single molecules and molecules in living cells may be the best way to go.

Another interesting project would be to study the functional importance of the different structural features in an *in vivo* context. The generation of a knock-in mouse where the endogenous receptor is exchanged for a mutant construct lacking the N-terminal of NCAM would allow us to specifically study the physiological role of the NCAM-binding, cell adhesion modulatory function of GFRα1. Furthermore, a knock-in mouse with the 4x mutant NCAM construct inserted would enable us to assess the importance of NCAM in its role as a GDNF receptor, without adversely affecting its function as a cell adhesion molecule.

Although the ICD of *Drosophila* Ret is fully functional, we do not know how it is activated. One obvious possibility is that extracellular domain of dRet binds to another, as yet unidentified, ligand. Another possibility would be that dRet does not have a ligand, and that its function is to assist in the signaling pathway of another, dRet interacting receptor. In order to test the first possibility, a number of extracellular factors could be tested for their ability to activate phosphorylation of the wild-type dRet.

The search goes on for the elusive signal transducing GDNF-receptor in GABAergic interneurons from the MGE. Phage display with GFRα1 as bait or immunoprecipitation of GFRα1 from MGE neurons followed by mass spectrometry are two possible ways to screen for GFRα1-interacting proteins. There are however still some promising candidates for a receptor that should be examined for their role in this mechanism, in parallell with a more systematic screening approach.

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