CELLULAR REGULATION OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR

Maria Lume

Institute of Biotechnology
Helsinki Institute of Life Sciences
&
Faculty of Biological and Environmental Sciences
Department of Biosciences
Division of Physiology and Neuroscience
&
Doctoral Programme Brain & Mind
Doctoral School of Health Sciences
University of Helsinki

ACADEMIC DISSERTATION

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ABSTRACT

Neurotrophic factors are small secretory proteins with important functions both in the nervous system and in peripheral tissues. Glial cell line-derived neurotrophic factor (GDNF) is best known for its ability to support the survival of midbrain dopaminergic neurons and enteric neurons. Also, GDNF is essential for the development of kidney and testis. It has been shown that both the absence and excessive amounts of GDNF protein negatively regulate kidney morphogenesis, highlighting the importance of proper spatiotemporal regulation of GDNF. Despite the wealth of knowledge regarding GDNF functions both in and outside the nervous system, relatively little is known about the trafficking mechanisms of GDNF.

GDNF is synthesized as a precursor protein, proGDNF. In this thesis, we characterized the cellular localization and secretion of two GDNF splice variants, pre-(α)pro-GDNF and pre-(β)pro-GDNF, that differ in their pro-regions. Both precursor forms were shown to be secreted from cell lines. However, while (α)pro-GDNF co-localized mainly with the Golgi markers, the (β)pro-GDNF was found primarily in the secretogranin-II positive vesicles of the regulated secretory pathway. In accordance, the two splice isoforms responded differently to KCl-induced depolarization that is known to trigger the secretion of neurotrophin family members in neuronal cells. Only (β)pro-GDNF and corresponding mature GDNF were secreted activity-dependently, whereas (α)pro-GDNF and its corresponding mature GDNF were secreted via the constitutive secretory pathway. In addition, we determined which enzymes are responsible for the proteolytic cleavage of proGDNF into mature GDNF.

To elucidate, whether secreted proGDNF has any biological activity, the recombinant cleavage-resistant proGDNF mutant protein was expressed in mammalian CHO cells and next purified from the media. Our results demonstrate that proGDNF is biologically active. Furthermore, similarly to mature GDNF, proGDNF can signal via the GDNF receptor α1/RET receptor tyrosine kinase complex and activate downstream MAPK and AKT pathways. Interestingly, proGDNF is not able to activate RET via the GFRα2 receptor.

Finally, we identified a novel sorting receptor for GDNF and its receptors. Our results show that SorLA, a member of the vacuolar protein sorting 10-p domain receptor family, can internalize GDNF and GFRα1. While GDNF is subsequently degraded in lysosomes, GFRα1 is recycled back to the cell membrane. In the presence of SorLA and GFRα1, also RET is internalized and directed to early endosomes. By regulating the availability of GDNF and its co-receptors, SorLA can inhibit GDNF-induced neurotrophic activity in SY5Y cells. Moreover, SorLA seems to regulate intracellular localization of GFRα1 in hippocampal neurons.

In summary, results of this thesis characterize the cellular regulation of GDNF regarding its secretion, processing, internalization and subsequent degradation. Furthermore, this is the first time that biological functions of the GDNF precursor protein proGDNF are described. Our findings indicate that the trafficking of GDNF is very different from that of other neurotrophic factors, and in contrast to apoptotic proneurotrophins, proGDNF is a trophic protein with increased specificity to GDNF receptor complex GFRα1-RET.
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REFERENCES

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LIST OF ORIGINAL PUBLICATIONS

This thesis work is based on the following original articles and an unpublished manuscript, which are referred in the text by their roman numerals I-III.


*Equal contribution

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ABBREVIATIONS

aa  amino acid
AD  Alzheimer’s disease
ARTN artemin
BDNF brain-derived neurotrophic factor
CNS central nervous system
CRD cysteine-rich domain
EGFR epidermal growth factor receptor
ELISA enzyme-linked immunosorbent assay
ER  endoplasmic reticulum
ERK extracellular signal-regulated kinase
FMTC familial medullary thyroid carcinoma
GDNF glial cell line-derived neurotrophic factor
GFL GDNF family ligand
GFRα GDNF receptor alpha
GPI glycosylphosphatidylinositol
HC  hippocampus
HS  heparan sulfate
HSCR Hirschsprung’s disease
kDa kilodalton
Kd dissociation constant
KO knock-out
MAPK mitogen-activated protein kinase
MEN2 multiple endocrine neoplasia type 2
MMP matrix metalloproteinase
NCAM neuronal cell adhesion molecule
NGF nerve growth factor
NRTN neurturin
NTF neurotrophic factor
NTR neurotrophin receptor
PC proprotein convertase
PD Parkinson’s disease
PI3K phosphatidylinositol-3-kinase
PKC protein kinase C
PLCγ phospholipase C gamma
PNS peripheral nervous system
PSPN persephin
RET rearranged during transfection
RTK receptor tyrosine kinase
SorCS sortilin related receptor CNS expressed
SorLA sorting-protein related receptor with type-A repeats
TGF-β transforming growth factor beta
TGN trans-Golgi network
UTR untranslated region
Trk tropomyosin-related kinase
VPS vacuolar protein sorting
wt wild-type
INTRODUCTION

The nervous system has been of great interest to mankind for thousands of years, but modern neuroscience as a separate field of study was established only during the last century. Despite its “young” age, significant development of methods and tools have exponentially increased the amount of knowledge of how the nervous system works. Nevertheless, deciphering the precise regulation of molecular mechanisms within neurons and neuronal circuits continues to challenge scientists worldwide.

The following literature review aims to give an overview of what is currently known about the regulation of neurotrophic factors, concentrating on the best-studied neurotrophin family members, the glial cell line-derived neurotrophic factor (GDNF) and its receptors.

1. Discovery and classification of neurotrophic factors

Neurotrophic factors (NTFs) are small secretory proteins with a broad range of functions in the development and maintenance of the nervous system. NTFs act by binding to their cognate receptors on the cell surface and can support for example the survival, migration, and differentiation of neurons, as well as modulate neuronal connectivity.

The first NTF was discovered and purified by Rita Levi-Montalcini, Viktor Hamburger, and Stanley Cohen back in the 1950-s. These scientists elegantly demonstrated that when certain mouse tumors were implanted in developing chick embryos, they released a soluble diffusible factor that induced extensive neurite outgrowth from cultured sensory and sympathetic ganglia (reviewed in Levi-Montalcini, 1964). This factor was soon found to be highly expressed in snake venom and male mouse salivary gland, and this discovery enabled the biochemical purification and functional characterization of the protein (Cohen and Levi-Montalcini, 1956; Cohen, 1960). The potent molecule was called nerve growth factor (NGF), which is the founding member of the neurotrophin protein family and the best studied neurotrophic factor so far.

Based on their experimental data, Levi-Montalcini and Hamburger proposed a hypothesis, currently known as the classical “Neurotrophic Factor Hypothesis,” which in short states that during neuronal development neurons are born in excess, and target-derived neurotrophic factors are necessary for the proper formation of neuronal connections. NTFs are released by the targets in very low concentrations, and neurons of the same type compete for the supply. Successful neurons survive whereas others undergo programmed cell death (Hamburger and Levi-Montalcini, 1949). This hypothesis, supported today by massive amounts of experimental data, holds true for NGF both in the peripheral and the central nervous system (Bothwell,
However, in the case of other neurotrophic factors, for example, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), there is evidence suggesting that neurotrophic factors may function differently from what was postulated in the original hypothesis. Thus, neurotrophic factors should be considered as a heterogeneous group of trophic molecules based on the function and mode of action (Conner et al., 1998).

Based on the structure of the ligands, NTFs are broadly classified in the following manner:

1) Neurotrophins
2) GDNF family ligands
3) Neurokines
4) MANF and CDNF protein family

Neurotrophins and GDNF family ligands will be described in more detail in sections 2 and 3 of the introduction, respectively. Neurokines or neuropoietic cytokines are small molecules that act as monomers, signaling via common cytokine receptor components. Members of the neurokine protein family include ciliary neurotrophic factor (CNTF), interleukin 6 (IL-6), cardiotrophin 1 and 2, and leukemia inhibitory factor (LIF). In the nervous system, CNTF and other neurokine family members promote the survival of motor neurons (Sendtner, 2014). In addition, these factors play a role in metabolism and lipid homeostasis and are therefore candidates for treating obesity and obesity-related metabolic diseases (Pasquin et al., 2016).

Mesencephalic astrocyte-derived neurotrophic factor (MANF) and its homologue CDNF (cerebral dopamine neurotrophic factor) are members of the most recently discovered neurotrophic factor family (reviewed in Lindholm and Saarma, 2010). Although the receptor for MANF and CDNF has not yet been discovered, evidence indicates that these proteins may have a unique mode of action compared to other NTFs. Crystal structures of MANF and CDNF revealed that both proteins have an amino-terminal saposin-like domain for possible interactions with lipids or membranes, and a carboxy-terminal domain that may protect cells against endoplasmic reticulum (ER) stress (Lindahl et al., 2017). So far, both proteins have been shown to support the survival of midbrain dopaminergic neurons, and CDNF will be used in Phase I/II clinical trial for the treatment of Parkinson’s disease starting in September 2017 (Herantis Pharma Press Release 23.3.2017, http://herantis.com/release/herantis-pharmas-clinical-study-with-cdnf-in-parkinsons-disease-authorized-in-sweden/?lang=fi). Furthermore, MANF is an important regulator of endocrine islet beta cells and a potential therapeutic candidate for the treatment of diabetes mellitus (Lindahl et al., 2014).
2. Neurotrophins and their receptors

While working with NGF, Rita Levi-Montalcini observed that although NGF was critical for the developmental survival of neural crest-derived peripheral sensory neurons, it did not support the survival of the cranial sensory ganglia, hinting the possible existence of another protein with trophic function (Levi-Montalcini, 1964). At the beginning of 1980-s, brain-derived neurotrophic factor (BDNF) was purified from pig brain (Barde et al., 1982), and nucleotide sequence analysis revealed that BDNF was structurally similar to NGF (Leibrock et al., 1989). At present, there are four members in the mammalian neurotrophin protein family: NGF, BDNF, neurotrophin 3 (NT-3) (Hohn et al., 1990; Jones and Reichardt, 1990; Maisonpierre et al., 1990b; Rosenthal et al., 1990), and neurotrophin 4 (NT-4, also known as NT-4/5) (Berkemeier et al., 1991; Hallböök et al., 1991; Ip et al., 1992).

Similarly to most growth factors, neurotrophins are first synthesized as precursor proteins (proneurotrophins) that undergo proteolytic processing to generate dimeric mature protein forms (Seidah et al., 1996). Mature neurotrophins signal via the members of tropomyosin-related kinase (Trk) receptor family as well as p75 neurotrophin receptor (p75NTR). While p75NTR is a common low-affinity receptor for all neurotrophins, their binding to the Trk receptors is more ligand-specific: NGF binds preferentially to TrkA, BDNF, and NT-4 to TrkB, and NT-3 to TrkC (Klein et al., 1991a, 1991b, 1992; Lamballe et al., 1991) (Figure 1). Ligand binding initiates phosphorylation of Trk receptors and regulates cell growth and survival by activation of downstream signaling pathways (reviewed by Reichardt, 2006).

![Figure 1. Mature neurotrophins, proneurotrophins, and their preferred receptors.](image)

While mature neurotrophins (i.e., mNGF) bind either a specific high-affinity Trk receptor or common low-affinity p75NTR, proneurotrophins signal via a receptor complex comprising of p75NTR and either Sortilin or SorCS2. Figure adapted from Gibon and Barker, 2017.
For a long time, proneurotrophins were considered inactive precursor molecules and the proposed role of the pro-domain was to ensure proper folding and secretion of the mature NTF (Suter et al., 1991). In 2001, however, Lee and colleagues demonstrated for the first time that proneurotrophins are secreted, biologically active proteins that bind with high affinity to p75NTR and induce apoptosis in cultured neurons (Lee et al., 2001). Since then, many groups have investigated the function of those proteins and currently it is known that proneurotrophins bind a receptor complex comprising of p75NTR and either sortilin or SorCS2 (members of the vps10p domain protein family that will be discussed in section 1.5 of this thesis) (Figure 1), and modulate complex signaling regulating synaptic activity, pruning and network reorganization (Nykjaer et al., 2004; Teng et al., 2010; Costa et al., 2017; Gibon and Barker, 2017).

2.1. Expression and structure of neurotrophins

Neurotrophins have a wide variety of functions in the peripheral and central nervous systems as well as in non-neuronal tissues. These include regulating neuronal development, differentiation, survival, and plasticity of certain neuronal populations (Bothwell, 2014). Furthermore, neurotrophins modulate retinal, cochlear and heart development, and participate in muscle development and function. BDNF, for example, has been shown to regulate the regeneration of myogenic progenitor cells in vivo (Clow and Jasmin, 2010).

The expression of neurotrophin encoding genes, *bdnf* in particular, is tightly regulated, allowing precise temporal and spatial expression of the protein. BDNF is the most abundantly expressed neurotroph in the central nervous system (CNS) and has been detected in the hippocampus, cerebral cortex, amygdala, and hypothalamus (Hofer et al., 1990; Ernfors et al., 1992; Conner et al., 1997). BDNF expression levels increase substantially after birth (Katoh-Semba et al., 1997). Human *bdnf* gene comprises nine exons, eight of which are noncoding 5′ exons, each controlled by a distinct promoter that is induced by different stimuli (Figure 2A). For instance, promoter IV can be induced by neural activity, calcium influx, and activation of either NMDA receptor or cAMP-responsive element-binding protein (CREB). All promoters are linked by alternative splicing to exon IX encoding the protein and the 3′ untranslated region (UTR) (West et al., 2014). Since there are two polyadenylation sites in the *bdnf* 3′UTR, each transcript can exist in two forms, one with a short and the other with a long 3′UTR, generating a total of 34 possible transcripts in humans (Pruunsild et al., 2007) (Figure 2A).

Several groups have reported that in neurons BDNF mRNA is distributed both in somatic structures and in dendritic compartments, where it can undergo local translation (reviewed in Edelmann et al., 2014). Recent work by Tongiorgi and colleagues suggests that spatial segregation of BDNF mRNA variants depends rather on sequences located in the 5′UTR region of BDNF mRNA than in the 3′UTR as proposed before. More specifically, neuronal activity drives relocation of transcripts.
encoding exons 2, 4 or 6: exon 4 containing transcripts localize to proximal dendrites and exon 2 or 6 containing transcripts to the distal dendrites (Tongiorgi and Baj, 2008; Baj et al., 2011). All in all, though various stimuli regulate the transcription of bdnf and the transcripts differ in stability and localization, in the end, all of them give rise to the same BDNF protein (West et al., 2014) (see Figure 2A).

To add up to the complexity, BDNF is also regulated at the translational level. There are multiple conserved sequences in the 3’UTR of bdnf mRNA, and several RNA binding proteins such as tristetraprolin and microRNAs have been identified that bind to these regions and regulate the expression of BDNF protein either positively or negatively (Kumar et al., 2014; Varendi et al., 2014).

NGF is expressed in both neuronal and non-neuronal cells of the peripheral nervous system (PNS) and CNS (Sofroniew et al., 2001). Similarly to BDNF, NGF expression can be regulated by neuronal activity as well as stimuli related to inflammation. Four different splicing patterns have been identified in mouse ngf gene consisting of five exons and a single polyadenylation site. The gene structure and synthesis of NT-3 and NT-4 is quite similar but their expression sites are very different: while NT-3 is highly expressed in the developing CNS, the levels of NT-4 are highest in testis, skeletal muscle, and spinal cord but relatively low in the CNS. Both genes comprise three exons and three (NT-3) or four (NT-4) polyadenylation sites, giving rise to multiple mRNA transcripts. Unlike BDNF and NGF, the expression of NT-3 and NT-4 is not induced by neuronal activity (West et al., 2014).

Figure 2. The structure of BDNF gene, NGF homodimer, and proNGF homodimer. A) The schematic structure of bdnf with 5’UTR, protein-encoding region (exon IX) and 3’UTR with two alternative polyadenylation (pA) sites. Exons inducing bdnf mRNA translocation to dendrites are marked with an asterisk. All transcripts give rise to identical BDNF protein. B) The crystal structure of mature NGF, with one monomer colored blue and the other purple. C) Predicted structure of proNGF dimer, the pro-region is depicted in red and furin cleavage site is shown. The figures are modified from Maynard et al., 2016 (A); Butte et al., 1998 (B); Paoletti et al., 2011 (C).
High-resolution crystal structures have been determined for all neurotrophins, and analysis of the structural data revealed a novel type of protein fold, referred to as a cysteine-knot structure (McDonald et al., 1991; Robinson et al., 1995; Butte et al., 1998). Highly conserved cysteines form three disulfide bridges that stabilize two pairs of antiparallel β-strands in each neurotrophin subunit (Figure 2B). In addition to neurotrophins, a similar folding pattern has been observed in other growth factors, for example in GDNF family ligands, and platelet-derived growth factor (PDGF) family members (McDonald and Chao, 1995).

Attempts to model the structure of the neurotrophin pro-domain indicated that the pro-region is largely disordered as it contains features typical of an intrinsically unfolded region (Anastasia et al., 2013). Crystallization of pro-NGF has proved difficult due to its dynamic nature (Feng et al., 2010). Nevertheless, a recent study provides evidence that pro-domain of NGF assumes globular conformation in solution (Paoletti et al., 2011) (Figure 2C).

2.2. Trafficking and processing of neurotrophins

Like neuropeptides, neurotrophins are synthesized as preproproteins in the endoplasmic reticulum (ER). The pre-region is cleaved off co-translationally, and next, the proneurotrophins ranging from 210 to 270 amino acids in length dimerize via the mature region and can undergo post-translational modifications (i.e., glycosylation, amidation) while being transported through the Golgi complex and packed into the secretory vesicles (Bradshaw et al., 1993). Glycosylation of the NGF pro-domain and trimming of the oligosaccharide chains have been shown to be important for the precursor to exit ER and subsequent processing and secretion of the protein (Seidah et al., 1996b).

Two major secretory pathways exist in the cell, and these are the constitutive pathway, present in all cell types, and the regulated pathway that is employed by excitable cells like neurons. In the constitutive pathway, vesicles release their cargo by default when reaching the plasma membrane, whereas large dense core vesicles used by the regulated secretory pathway need elevated Ca^{2+} for exocytosis. The secretion mechanisms of neurotrophins have been studied extensively. In neurons, NGF and NT-4 are predominantly secreted via the constitutive pathway but can also be found in the secretory granules of the regulated pathway in both axons and dendrites of the CNS neurons (reviewed in Leßmann and Brigadski, 2009). In cultured hippocampal neurons, removal of the pro-region is essential for the regulated secretion of mature NGF (Lim et al., 2007).

Neuronal BDNF and NT-3 are secreted mainly in an activity-dependent manner with similar efficiency (Mowla et al., 1999; Brigadski et al., 2005). The precise molecular mechanism determining which proteins in the trans-Golgi network (TGN) are directed to either of the two pathways described or secreted via other, unconventional secretory pathways, remains largely elusive. It is suggested that sequence information within the prodomain is necessary for protein sorting (Ma et
al., 2008), and this is partly true for BDNF. Vps10p domain receptor family member sortilin has been reported to guide BDNF to the activity-dependent secretory pathway by interacting with BDNF prodomain (Chen et al., 2005). A single-nucleotide polymorphism (SNP) in bdnf gene leading to a substitution of valine to methionine at codon 66 (Val66Met) can alter the binding affinity to sortilin and compromise the trafficking of proBDNF (Chen et al., 2008) (Figure 3). Importantly, more than 25% of the human population is either homozygous or heterozygous for this mutation that is strongly associated with deficits in episodic memory, reduced hippocampal volume and a higher risk of depression (Egan et al., 2003).

In addition to sortilin, carboxypeptidase E (CPE) has been shown to bind BDNF and direct the protein to the regulated pathway (Figure 3). Unlike sortilin, CPE interacts with the mature domain of BDNF (Lou et al., 2005). This interaction may be necessary for the activity-dependent secretion of mature BDNF that has undergone proteolytic processing in the trans-Golgi network.

Cleavage of the pro-domains is highly regulated and can occur in multiple places along the secretory pathways or in the extracellular space, depending on the cellular context and the expression of proteases. Proneurotrophins contain conserved dibasic amino acid sequences in their prodomain and a consensus motif (K/R)-(X)-(K/R) - (R), where X is any amino acid, is recognized by the proprotein convertase (PC) family members (Figure 3). There are nine members in the mammalian PC serine proteinase family that cleave various precursor proteins.

![Figure 3. Potential sites of post-translational modifications in BDNF precursor protein. BDNF pre-, pro- and mature domains are drawn in scale. Different members of the proprotein convertase (PC) family can cleave BDNF in addition to subtilisin/kexin, and furin. BDNF can also be cleaved by matrix metalloproteinases (MMP) and plasmin. In addition, a putative N-glycosylation site, carboxypeptidase E sorting signal, and the position of the Val66Met single nucleotide polymorphism (SNP) are shown. Figure adapted from Lessmann and Brigadski, 2009.](image)

All PC-s are initially synthesized as inactive zymogens that are activated by cleavage of the prosegment. PCs differ in expression, and subcellular localization:
ubiquitously expressed membrane-bound furin and PC7 are activated in the TGN, soluble PC1/3 and PC2 are expressed mainly in neurons and activated specifically in dense core secretory granules. PC4, PCSK9, PACE4, and PC5/6A are secreted via the constitutive pathway, and the latter two interact with heparan sulfate proteoglycans via their cysteine-rich domains (reviewed in Seidah et al., 2008). PCs can hydrolyze proneurotrophins either in the Golgi apparatus, in the TGN or in the lumen of the secretory vesicles, to release the mature protein and the prodomain.

If proneurotrophins are secreted, they can be cleaved extracellularly by serine protease plasmin or by selective matrix metalloproteinases (MMP3, MMP7, and MMP9) (Lee et al., 2001; Mizoguchi et al., 2011) (Figure 3). Cultured hippocampal neurons, for instance, secrete a substantial proportion of proBDNF compared to mature BDNF and cleavage of proBDNF by plasmin is essential for the expression of late-phase long term potentiation and hence hippocampal plasticity and formation of memory (Pang et al., 2004; Barnes and Thomas, 2008).

After secretion, neurotrophins signal by binding to their preferred transmembrane Trk receptor tyrosine kinase and elicit signaling cascades promoting survival and differentiation, BDNF-TrkB signaling also modulates synaptic plasticity (Costa et al., 2017). When high concentrations of mature neurotrophins engage with the p75NTR receptor, a stress kinase c-Jun is activated, leading to activation of the apoptotic pathway (Teng et al., 2010). Once the ligand is bound to either of the receptors, the activated complex is quickly internalized. Interestingly, Yang and colleagues showed that proBDNF could be cleaved after being internalized in complex with p75NTR and yield mature BDNF that can either activate endocytosed TrkB or be recycled back to the cell surface (Yang et al., 2009).

A characteristic feature of the neurotrophins is their dynein-dependent retrograde transport in signaling endosomes. In this way, neurotrophins exert many of their functions including regulation of neuronal survival and specification, modulation of both axonal and dendritic growth, and regulation of the degree of connectivity between neurons by promoting postsynaptic density formation (Zweifel et al., 2005; Bronfman et al., 2014). Neurotrophins are degraded in lysosomes, and unexpectedly, trafficking of BDNF to the lysosome requires the cytoplasmic tail of sortilin - the same sorting receptor that regulates BDNF secretion (Evans et al., 2011).

2.3. Biological functions of proneurotrophins

Historically, pro-domain containing precursor proteins have been considered biologically inactive. As already mentioned, the pro-region was suggested to contain information needed for proper folding, intracellular trafficking and protection from proteolytic degradation of the protein and these assumptions were shown to be true for proneurotrophins (Suter et al., 1991; Seidah et al., 1996b; Paoletti et al., 2011). Moreover, the pro-domain was thought to be involved in the inactivation of the mature domain, but studies investigating the role of proneurotrophins proved
otherwise. The biological activity of proneurotrophins was first demonstrated in 2001 and in subsequent years, several groups intensively studied the functions of proneurotrophins and the role of the pro-region of neurotrophins as well as other growth factors (Lee et al., 2001; Hillger et al., 2005; Hempstead, 2006; Bradley et al., 2010; Costa et al., 2017).

To date, proNGF and proBDNF have been characterized thoroughly, proNT-3 to a lesser extent and nothing has been reported on a role for proNT-4. Two main findings concerning proneurotrophins except proNT-4 are the following: they can be secreted, and they all bind with high affinity to p75NTR via their mature domain (Lee et al., 2001; Hasan et al., 2003; Bruno and Cuello, 2006; Yang et al., 2009; Yano et al., 2009). In addition, despite low sequence homology of the pro-domains, proNGF, proBDNF and proNT-3 but not proNT-4 can bind to sortilin via their pro-region (Lee et al., 2001; Hasan et al., 2003; Bruno and Cuello, 2006; Yang et al., 2009; Yano et al., 2009). Signaling via a receptor complex comprising of p75NTR and sortilin, proneurotrophins can induce apoptosis in different neuronal populations as well as in oligodendrocytes (Costa et al., 2017). Recently another member of the vps10p sorting receptor family, SorCS2, was shown to mediate the actions of proneurotrophins when in complex with p75NTR. ProNGF and proBDNF can initiate growth cone retraction, while proNT-3 modulates the proliferation of cerebellar neurons via this complex (Deinhardt et al., 2011; Anastasia et al., 2013; Zanin et al., 2016).

The levels of proneurotrophins change during development. While proNGF is expressed at low level in non-injured or young animals, and the levels are upregulated in aging rodents, the amount of proBDNF decreases during aging and in adult mouse brain, where the mature form dominates (Fahnestock et al., 2001; Al-Shawi et al., 2008; Yang et al., 2009). ProNGF levels are increased upon injury and in patients suffering from Alzheimer's disease (Fahnestock et al., 2001; Harrington et al., 2004). In rat cortical neurons, proNGF secretion occurs in an activity-dependent manner (Bruno and Cuello, 2006). Its function on the cell surface depends on the availability of signal-mediating receptors (Ioannou and Fahnestock, 2017). It is important to mention that in addition to interacting with p75NTR and sortilin, proNGF can also bind to TrkA, although with much lower affinity than NGF (Fahnestock et al., 2004). In this way, proNGF can exhibit neurotrophic activity similarly to the mature form (Clewes et al., 2008; Masoudi et al., 2009). Recent work analyzing the effects of proNGF and NGF in PC12 cell line suggests that proNGF can be trophic in the presence of TrkA, p75NTR, and sortilin but when TrkA levels are reduced, proNGF activity shifts from survival signaling to induction of cell death (Ioannou and Fahnestock, 2017).

ProBDNF can be released from cultured hippocampal neurons in response to depolarization similarly to mature BDNF (Yang et al., 2009). The electrical signal triggering their secretion differs in that mature BDNF secretion is regulated by high-frequency stimulation while proBDNF responds to low-frequency stimulation (used
to induce long-term depression (LTD)) (Nagappan et al., 2009). Since proBDNF can be cleaved by proconvertases in the secretary vesicles, also the isolated pro-peptide can be secreted from neurons (Lee et al., 2001; Dieni et al., 2012). The effects of secreted proBDNF are opposite to those of mature BDNF. While BDNF elicits survival and differentiation signals and is needed for neurogenesis and long-term potentiation (LTP), proBDNF binds to a complex of p75NTR and sortilin or sorCS2, to promote cell death, growth cone retraction and LTD (reviewed in Costa et al., 2017; Kojima and Mizui, 2017). Nevertheless, similarly to proNGF’s ability to signal via TrkA, proBDNF can bind to and activate TrkB receptor with a slightly lower affinity compared to mature BDNF, suggesting that proBDNF could also participate in trophic signaling (Fayard et al., 2005). Generation of pro-region specific antibodies revealed that in the brain, the expression levels of BDNF N-terminal propeptide are greater than that of proBDNF (Dieni et al., 2012). Further studies investigating the biological activity of this fragment showed that BDNF propeptide acts as a monomer and can elicit LTD similarly to proBDNF protein (Mizui et al., 2015; Zanin et al., 2017). Moreover, when analyzing the structure of recombinant Val66 and Met66 propeptide variants, a shift in conformation from β-strand to helical conformation was observed in the Met66 propeptide. This conformational change was suggested to account for the enhanced ability of the Met-type BDNF propeptide to bind to SorCS2 and promote growth cone retraction in a p75NTR-dependent manner (Anastasia et al., 2013).

ProNT-3 is secreted activity-dependently and induces cell death in superior cervical ganglion neurons by signaling via p75NTR and sortilin (Yano et al., 2009). Also, proNT-3 can bind to a complex of p75NTR and SorCS2 expressed in cerebellar granule cells to modulate Ca²⁺ homeostasis and mitochondrial potential as well as to regulate cell cycle exit of these cells (Safina et al., 2015; Zanin et al., 2016).

In conclusion, proneurotrophins are biologically active ligands with important cellular functions. Although proneurotrophins are best known for promoting cell death, they can also induce survival signals in Trk-dependent manner and should not be considered solely apoptotic molecules.

2.4. General characterization of neurotrophin receptors

As mentioned at the beginning of section 2, neurotrophin receptors include p75NTR of the tumor necrosis factor (TNF) superfamily and members of the Trk receptor family.

p75NTR was initially identified as a low-affinity receptor for NGF, but in the following years, it was shown to bind all neurotrophins with the similar affinity via the cysteine-rich domains (CRDs) in its extracellular domain (Rodriguez-Tébar et al., 1990; Rodríguez-Tébar et al., 1992; Baldwin and Shooter, 1995). Today, the pan-neurotrophin receptor p75NTR is also known as a high-affinity receptor for proneurotrophins (Lee et al., 2001; Yano et al., 2009). p75NTR is widely expressed in both central and peripheral neurons and glia of the developing nervous system.
For example, in the CNS it is mainly found in the striatum, some brainstem nuclei and the cholinergic neurons of the basal forebrain, the latter continue to express high levels of p75NTR through adulthood (Pioro and Cuello, 1990). Importantly, p75NTR expression increases in neurons, macrophages, microglia, astrocytes, and Schwann cells in response to injury, seizures and neurodegenerative diseases. In non-neuronal tissue, p75NTR is detected in heart and muscle (Meeker and Williams, 2015).

Structurally, p75NTR comprises the extracellular domain with four CRDs, transmembrane domain and the intracellular domain (ICD) (He and Garcia, 2004) (Figure 4). Two domains have been identified in the p75NTR ICD: a Chopper domain in the juxtamembrane region that is able to induce cell death when bound to the membrane (Coulson et al., 2000; Underwood et al., 2008) and a C-terminal region that resembles the death domain present in TNF receptor (TNFR) and the Fas antigen, used for mediating apoptotic signals. p75NTR undergoes a two-step regulated intracellular proteolysis whereby it is first cleaved by the \( \alpha \)-secretase TACE/ADAM17 and subsequently by presenilin-dependent \( \gamma \)-secretase, releasing the ICD of p75NTR to the cytosol for signaling (Skeldal et al., 2011). The ICD of p75NTR receptor does not contain catalytic activity but is able to recruit a number of cytosolic signaling adaptor proteins and promote downstream signaling (Kraemer et al., 2014).

p75NTR has been attributed numerous functions that modulate survival, differentiation or death of the cell depending on whether p75NTR is expressed independently or in association with different co-receptors on the plasma membrane and which ligand it binds to (Meeker and Williams, 2015) (see Figure 4). Strikingly, for a long time, there was no consensus whether p75NTR signals as a monomer or a dimer (He and Garcia, 2004; Feng et al., 2010). Results of a very recent publication demonstrate that on the cell surface p75NTR can co-exist both as a monomer or a trimer (Anastasia et al., 2015).

Binding of neurotrophins results in p75NTR mediated activation of the nuclear factor-\( \kappa \)B (NF-\( \kappa \)B) and c-Jun N-terminal kinase (JNK) pathways, inducing cell death. When co-expressed with the Trk receptors, p75NTR can enhance the binding affinity between neurotrophins and the Trk receptors, supporting survival and growth signaling via an unknown mechanism since the direct interaction between p75NTR and Trk receptors has not been demonstrated (Hempstead et al., 1991; Esposito et al., 2001). When in complex with the vps10p domain receptors sortilin or sorCS2, p75NTR mediates proneurotrophin signaling. The interaction of p75NTR and sortilin occurs via the extracellular domains of the receptors (Skeldal et al., 2012). ProNGF induces apoptosis of the sympathetic as well as basal forebrain neurons when it binds to the complex of p75NTR and sortilin (Lee et al., 2001; Nykjaer et al., 2004). ProBDNF signaling via p75NTR/SorCS2 can induce long-term depression (LTD) in hippocampal neurons, while proNT3 reduces proliferation of cerebellar cells through the same receptor complex (Gibon and Barker, 2017).
Finally, p75NTR interactions with the Nogo receptor and Lingo-1 regulate cell growth. Myelin-derived ligands Nogo, MAG, and MOG bind to the receptor complex, activating RhoA and leading to growth cone collapse, neurite retraction and decreased spine density (Meeker and Williams, 2015) (Figure 4).

**Figure 4. Neurotrophin receptor p75NTR and its co-receptors.** Figure adapted from Meeker and Williams, 2015.

There are three members in the Trk family of tyrosine kinase receptors: TrkA, TrkB, and TrkC. The expression patterns of Trk receptors do not overlap significantly: TrkB is primarily expressed by both neuronal and glial cells of the CNS, while TrkA and TrkC can be found mainly in neurons of the PNS and less in the CNS. Each Trk receptor selectively binds to different neurotrophin family members with nanomolar affinity. TrkA is the preferred receptor of NGF but can also be activated by NT-3 and NT-4, TrkB binds BDNF and NT-4, and TrkC is the receptor for NT-3 (Klein et al., 1991a, 1991b, 1992; Lamballe et al., 1991).

Trk receptor family belongs to the receptor tyrosine kinase (RTK) superfamily. They are type-1 transmembrane receptors with a large, heavily glycosylated extracellular domain followed by a single-pass transmembrane domain and an intracellular tyrosine kinase domain. The extracellular domain comprises one CRD, three N-terminal leucine-rich repeats (LRR), another CRD and two immunoglobulin-C2 (Ig) domains. Binding of the ligands occurs via the second Ig domain, triggering receptor dimerization and consequent trans-activation of the kinase domain, followed by activation of signaling pathways. In addition to direct activation by neurotrophins, Trk receptors can be intracellularly transactivated *in vivo* by epidermal growth factor (EGF), glucocorticoids and zinc (reviewed in Deinhardt and Chao, 2014).

Major pathways activated by the phosphorylation of the tyrosine residues in the intracellular kinase domain of Trk receptors include i) the mitogen-activated protein kinase - extracellular signal-regulated kinase (MAPK-ERK) pathway mediating neuronal survival and differentiation, ii) the phosphatidylinositol 3-
Trk signaling may be compromised by truncated isoforms of TrkB and TrkC lacking the tyrosine kinase domain. These splice variants can form heterodimers with full-length monomers and have dominant negative effects, sequester neurotrophins, and signal independently (Eide et al., 1996; Fenner, 2012). Upon ligand engagement, Trk receptors are quickly internalized and can either undergo recycling or degradation, or form signaling endosomes that are retrogradely transported and mediate signaling from axons to cell soma and dendrites (Grimes et al., 1996; Ginty and Segal, 2002; Barford et al., 2017).

2.5. Trafficking of neurotrophin receptors

Trk receptors are synthesized at the ER, and their expression can be triggered by neuronal activity similarly to their ligands. TrkB mRNA has been shown to translocate to the dendrites for local translation in response to BDNF and neuronal activity (Tongiorgi et al., 1997; Tongiorgi and Baj, 2008). Trk receptors are glycosylated post-translationally and transported to the cell membrane by microtubule-dependent kinesins (Deinhardt and Chao, 2014). More specifically, the interaction of TrkB cytoplasmic region and kinesin-1 is mediated by a complex comprising of collapsin response mediator protein-2 (CRMP-2), a small GTPase Rab27 and its effector Slp1 (Arimura et al., 2009). In sensory neurons, anterograde transport of the Trk receptors is facilitated by sortilin (Vaegter et al., 2011).

Both plasma membrane and intracellular membranes contain asymmetrically distributed clusters of sphingolipids and cholesterol are called lipid rafts. They are suggested to be important for cell adhesion, axon guidance, and synaptic transmission by forming a signaling hub for transmembrane receptors with adaptor and signaling proteins (Simons and Ikonen, 1997; Ikonen and Simons, 1998; Tsui-Pierchala et al., 2002b). Despite abundant literature characterizing the lipid rafts in in vitro settings, there has been a long debate whether they exist in vivo. In a recent publication, lipid rafts were detected for the first time in vivo when the structure of the biological membranes was analyzed by small-angle neutron scattering (SANS) (Nickels et al., 2017).

NTF receptors can localize to lipid rafts before ligand binding (i.e., GDNF receptor α family members) or move to these microdomains upon ligand engagement. TrkA and p75NTR are concentrated in caveolae-containing lipid rafts at the plasma membrane. Moreover, caveolin-1 and caveolin-2 differentially regulate Trk signaling and subsequent cell differentiation (Spencer et al., 2017). TrkB, in turn, translocates to lipid rafts of the intracellular compartments in
response to BDNF in a tyrosine kinase Fyn-dependent manner (Pereira and Chao, 2007).

Internalization of the Trk receptors can occur in two different ways: one is clathrin and dynamin dependent, whereas the other is an actin-dependent macropinocytotic process. Both types of internalization can take place in axons but also in the cell body. After internalization of the activated ligand-receptor complex, Trk receptors continue signaling from early endosomes, and small Rab GTPases regulate the dynamics of intracellular trafficking (Grimes et al., 1996; Bronfman et al., 2014). Some endosomes are recycled, others are sorted to late endosomes and lysosomes. TrkA receptor contains a post-endocytic recycling signal in its juxtamembrane domain, and hence it is recycled back to the plasma membrane more efficiently than TrkB or TrkC. Furthermore, in developing sympathetic neurons, somatic TrkA can be endocytosed in the absence of NGF and resides in endosomes in cell soma.

NGF signaling at distal axons triggers the anterograde transport of endocytosed TrkA and exocytosis of the receptor into axon growth cones (Ascano et al., 2009). This process whereby an endocytosed receptor is anterogradely transported from somatodendritic compartments to axon terminals is called transcytosis (Horton and Ehlers, 2003). When the Trk receptors are expressed in distal axons, target-derived ligand engagement leads to the internalization of the signaling complex and retrograde transport of the signaling endosome (Ye et al., 2003; Howe and Mobley, 2005).

Rab5 and Rab7 have been implicated to be important for guiding the signaling endosome retrograde transport. For example, the signaling endosome containing BDNF-TrkB receptor undergoes a conversion from Rab5-positive early endosome to Rab7-positive late endosome, and the retrograde transport depends on an adaptor protein snapin linking TrkB to dynein and microtubules (Deinhardt et al., 2006; Bronfman et al., 2014; Barford et al., 2017). It is not well understood, what happens to the signaling endosome when it has reached cell soma. Suo and colleagues showed recently that TrkA containing signaling endosomes were active at the cell soma for up to 25 hours, with persistent signaling inducing transcriptional changes by controlling nuclear transactivation of genes such as CREB (Suo et al., 2014) (Figure 5). Instead of subsequent degradation, the signaling endosome was exocytosed on the soma membrane and later re-internalized (Suo et al., 2014). By this mechanism, some signaling endosomes are thought to switch compartment identity from Rab7-positive late endosomes to Rab11-positive recycling endosomes, but further studies are needed to confirm this hypothesis (Barford et al., 2017).

The degradation of Trk receptors occurs mainly in lysosomes. As mentioned before, due to a particular recycling signal, TrkA is preferentially sorted to the recycling pathway and thus escapes lysosomal degradation, while TrkB is sorted primarily to the degradative pathway (Chen et al., 2005a).
Figure 5. Model for the retrograde transport of Trk receptors and neurotrophins. Target-derived neurotrophins bind to Trk receptors expressed in distal axons. Activated TrkA can induce axon extension via Erk1/2 and PI3K signaling pathways. Alternatively, the signaling complex of NGF-TrkA is internalized, and, subsequently, retrogradely transported to convey trophic signals to the cell body. Figure adapted from Ginty and Segal, 2002.

The rate of degradation is decreased when TrkB interacts with a recently identified regulator Slitrk5 that targets the receptor to recycling endosomes (Song et al., 2015). Trk receptor turnover and degradation can also be regulated via ubiquitination and deubiquitination, but the exact mechanisms and outcomes behind these processes remain unresolved (Sánchez-Sánchez and Arévalo, 2017).

Despite the wealth of knowledge regarding p75NTR functions, detailed characterization of its cellular trafficking remains to be studied. Before reaching the cell surface, p75NTR is glycosylated posttranslationally as it possesses both N-glycosylation and O-glycosylation sites, and its activity can be regulated by neurotrophins (Skeldal et al., 2011).

Similarly to Trk receptors, p75NTR concentrates to lipid rafts in response to neurotrophins, implicating the importance of this membrane microdomain in p75NTR signaling. When exposed to NGF or BDNF, p75NTR is internalized in a clathrin-dependent manner in PC12 cell line but with slower kinetics compared to TrkA. Clathrin-dependent endocytic pathway targets p75NTR to retrograde transport (Deinhardt et al., 2007). In motor neurons, p75NTR internalization mechanism is site-specific: in soma p75NTR is endocytosed in the absence of the ligand in a clathrin-independent manner while in axons the two pathways co-exist. After internalization, p75NTR undergoes proteolytic processing, giving rise to C-terminal fragments that are critical for signaling. P75NTR continues to signal in recycling endosomes but is also detected from multivesicular bodies targeted for exosomal release (Escudero et al., 2014). In motor neurons, p75NTR is recycled both in the somatodendritic compartment and axons to a similar extent (Deinhardt et al., 2007).
The significance of p75NTR retrograde transport is not well understood. A few recent studies indicate that retrograde transport of p75NTR in complex with either BDNF or proNT3 can lead to apoptosis in sympathetic neurons (Hibbert et al., 2006; Yano et al., 2009).

3. GDNF family ligands

GDNF family ligands (GFLs) include GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN). They are distant members of the TGF-β superfamily due to a conserved pattern of seven cysteine (Cys) residues in their primary sequence (Airaksinen and Saarma, 2002) (Figure 6). All GFLs are synthesized as precursor proteins, containing a signal sequence and a pro-region similarly to neurotrophins. Interestingly, GDNF is the only member of the GFLs that is known to be glycosylated (Lin et al., 1993; Piccinini et al., 2013) (see Figure 6). GFLs are biologically active as homodimers and signal preferentially via a receptor complex comprising one of four cognate co-receptors known as GDNF family receptor-α (GFRα), and transmembrane Rearranged during transfection (RET) receptor tyrosine kinase.

Figure 6. Schematic representation of GDNF family ligands (GFLs). All four GFL members encode a signal sequence marked with light green, followed by a pro-region (yellow) of variable length. The mature domain of GFLs is highly conserved, containing seven cysteine residues depicted as black lines. N marks putative N-linked glycosylation sites found in GDNF.

In addition, GFLs bind to heparin and their signaling can be mediated by alternative receptors like neuronal cell adhesion molecule (NCAM) and syndecan-3 (Paratcha et al., 2003; Bespalov et al., 2011). GFLs are involved in the development, differentiation and maintenance of multiple neuronal populations including dopaminergic, sensory, motor, sympathetic, parasympathetic and enteric neurons (Airaksinen and Saarma, 2002). Outside the nervous system, members of the GFLs are important for example in kidney development, regulation of spermatogenesis and lung pathophysiology (Moore et al., 1996; Davies et al., 1999; Meng et al., 2001; Mauffray et al., 2015).
3.1. Glial cell line-derived neurotrophic factor (GDNF)

GDNF is a founding member of the GDNF family ligands that was initially discovered as a survival factor for dissociated rat embryonic midbrain dopaminergic neurons (Lin et al., 1993). Over the years, numerous other functions have been attributed to this NTF and those will be discussed in section 3.1.3, but to name a few, GDNF is critical for the development of kidney and the enteric nervous system. Moreover, it modulates survival, migration, and differentiation of several neuronal populations such as sensory, sympathetic and motor neurons (reviewed in Airaksinen and Saarma, 2002). In line with that, GDNF-deficient mice completely lack the enteric nervous system, and kidneys, and they die shortly after birth. Notably, whereas the knockout animals have deficits in dorsal root ganglion, sympathetic and nodose neurons, their midbrain dopamine neurons do not differ from wild-type (wt) littermates (Moore et al., 1996; Pichel et al., 1996). These results suggest that GDNF is not a critical survival factor for embryonic development of dopaminergic neurons.

In the brain, GDNF expression has been detected in several regions including parvalbumin positive interneurons of the striatum, hypothalamus, hippocampus, cerebellum and olfactory bulb (Trupp et al., 1997; Golden et al., 1998, 1999; Hidalgo-Figueroa et al., 2012). A recent study, where a transgenic mouse model with GDNF overexpression from the native locus was used, confirmed the reported expression pattern (Kumar et al., 2015). Outside the CNS, GDNF is expressed more abundantly and is found in sensory neurons, sciatic nerve, developing kidney, ovary, gastrointestinal tract, testis, heart, lung, and liver for example (Suter-Crazzolara and Unsicker, 1994; Trupp et al., 1995; Suvanto et al., 1996; Kumar et al., 2015).

GDNF is initially synthesized as a 211 amino acids long precursor protein which is cleaved, yielding a mature form of 134 amino acids (Trupp et al., 1995) (Figure 6). Mature homodimeric GDNF binds preferentially to the GFRα1 receptor and activates the transmembrane RET receptor (Jing et al., 1996; Treanor et al., 1996) or NCAM (Paratcha et al., 2003). In vitro studies suggest that GDNF can also bind to GFRα2 co-receptor and signal via RET (Balah et al., 1997; Jing et al., 1997; Sanicola et al., 1997; Cik, 2000). Compared to other GFLs, GDNF contains a long N-terminal region rich in basic amino acids that form a consensus heparin-binding site (Alfano et al., 2007). In addition to heparin, GDNF can bind to a heparan sulfate proteoglycan syndecan-3 (Bespalov et al., 2011). Binding to polysaccharides decreases the diffusion of GDNF and may be necessary to concentrate the ligand at certain locations of the extracellular space.

3.1.1. GDNF expression and structure

According to the traditional view, the genomic structure of human gdnf includes large 5′UTR, three exons and over 2kb long 3′UTR (Grimm et al., 1998). However, this gene locus was recently re-analyzed, and the results revealed the existence of 6
exons that can be alternatively spliced to generate 5 GDNF isoforms, which all yield an identical mature GDNF protein (Airavaara et al., 2011).

The gene is driven by at least one inducible promoter containing a TATA-box, located upstream of exon 1 (Tanaka et al., 2000). The promoter activity can be induced by various agents such as phorbol ester, fibroblast growth factor 2 (FGF2) and cyclic adenosine monophosphate (cAMP) (Grimm et al., 1998). Furthermore, GDNF expression can be upregulated by neurotransmitters like dopamine and serotonin, antidepressants, and pro-inflammatory molecules (reviewed in Saavedra et al., 2008). In addition to promoter induction, GDNF levels can be regulated by several miRNAs that bind to specific regions in the evolutionarily conserved 3′UTR and repress GDNF translation (Kumar et al., 2015).

Five human GDNF mRNA transcripts have been identified, but only two of them are well characterized (Airavaara et al., 2011). In addition to the wild-type transcript described in the seminal paper by Lin et al, alternative splicing gives rise to a shorter GDNF variant, which contains an in-frame 78bp deletion (Lin et al., 1993; Suter-Crazzolara and Unsicker, 1994; Trupp et al., 1995; Grimm et al., 1998). This deletion results in the loss of 26 amino acids and a single amino acid change in the pro-region of the shorter transcript. The two splice isoforms are called pre-(α)pro-GDNF and pre-(β)pro-GDNF or GDNFFL (full-length) and GDNFΔ78, respectively (Wang et al., 2008) (Figure 7). These splice variants are expressed slightly differently in tissues outside the CNS, with the shorter isoform being more abundant in kidney and the longer isoform in lung (Suter-Crazzolara and Unsicker, 1994).

As mentioned before, GDNF is a distant member of the TGF-β superfamily and structurally very similar to TGF-β2 and bone morphogenetic protein-7 (BMP-7) (Eigenbrot and Gerber, 1997). GDNF monomer has a central α-helix called the heel region surrounded by less-ordered stretches from where mainly β-sheet-containing fingers emerge.

![Figure 7. Pre-(α)pro-GDNF and pre-(β)pro-GDNF differ in the length of the pro-region.](image)

Three intramolecular disulfide bridges support the monomeric structure. Negatively charged amino acids at the tip of finger two were determined important for binding to GFRα1/RET complex, but insufficient to activate GFRα2/RET (Balah et al., 2000). Two GDNF monomers dimerize in a head-to-tail fashion, and the dimer is stabilized by an interchain disulfide bond (Eigenbrot and Gerber, 1997).
Notably, this GDNF structure lacked the N-terminal region that is very mobile by nature, and was cleaved off in crystals.

### 3.1.2. Cellular regulation and processing of GDNF

The most common isoform of GDNF is synthesized as a 211 amino acids long inactive preproprotein. The pre-region that is cleaved off immediately after synthesis contains a signal sequence that targets the protein to the secretory pathway. In the ER and Golgi compartments, GDNF is folded and glycosylated. Although human GDNF contains two putative N-glycosylation sites at N\(^{49}\) and N\(^{85}\), only N\(^{49}\) seems to be in use (Piccinini et al., 2013). Glycosylation increases the stability of GDNF but at the same time regulates its processing as glycosylation-deficient GDNF mutant (N\(^{49}\)A) does not undergo proteolytic cleavage in contrast to the wild-type glycosylated protein (Piccinini et al., 2013). Proteolytic processing mechanisms that convert precursor proGDNF to the mature form are not well understood. GDNF has a putative furin cleavage site (KRLKR) that can be recognized by members of the proprotein convertase (PC) family (Lin et al., 1993), but the specific enzymes responsible for the propeptide cleavage have not been determined. As different PCs are distributed in several organelles as well as at the cell surface, the cleavage of proGDNF can possibly take place anywhere between the TGN and the extracellular space (Seidah et al., 2008). In addition to the best-characterized furin cleavage site, additional processing sites have been predicted in GDNF primary sequence that could give rise to several neuropeptides (Immonen et al., 2008; Bradley et al., 2010).

Interestingly, two of the predicted neuropeptides are specific for (α)pro-GDNF (Immonen et al., 2008). Although the predicted neuropeptides have not been detected in vivo, the activity of synthetic neuropeptides derived from GDNF sequence has been studied. Characterization of the synthetic 11-mer called Brain excitatory peptide 2 (BEP-2) in rats and Dopamine neuron stimulating peptide 11 (DNSP-11) in humans indicated that it could induce synaptic excitation in rat hippocampus and support the survival of dopamine neurons (Immonen et al., 2008; Bradley et al., 2010). Curiously, the putative PEP-3 and PEP-4 neuropeptides are located at the very N-terminal region of mature GDNF (Immonen et al., 2008). Recent N-terminal amino acid sequence analysis revealed the existence of two N-terminal sequences. Surprisingly, the novel cleavage site is located only six amino acids downstream of the conventional furin cleavage site and partially overlaps with the predicted PEP-3 (Piccinini et al., 2013). Whether the fragment of PEP-3 is released and functional in vivo remains to be studied.

In contrast to neurotrophins, relatively little is known about the role of GDNF pro-region. It has been suggested to play a role in GDNF secretion efficacy as a construct encoding only the mature GDNF was retained mostly within the cell (Grimm et al., 1998). In addition, the two splice isoforms may be secreted via different mechanisms, as the (β)pro-GDNF was detected mainly in the TGN...
compartment and was secreted less efficiently than (α)pro-GDNF (Wang et al., 2008). Further studies by the same group showed that (α)pro-GDNF, but not (β)pro-GDNF, interacted with the sorting protein SorLA via the pro-domain, and was secreted in an activity-dependent manner as a result of this interaction (Geng et al., 2011). Nevertheless, Piccinini and colleagues demonstrated that presence of the pro-region was not an absolute requirement for proper folding and secretion of GDNF. They showed that human GDNF lacking the pro-domain could be detected from CHO media as a consequence of transient overexpression, though in smaller quantities compared to wild-type GDNF (Piccinini et al., 2013). To my knowledge, there is just one publication where proGDNF was found to be the predominant form of GDNF in aging rodent brains (Sun et al., 2014). While proGDNF was shown to be secreted from primary astrocytes in response to lipopolysaccharide (LPS) stimulation, the authors did not characterize the potential functions of the precursor protein (Sun et al., 2014). In addition, the in vivo specificity of the proGDNF antibody should have been characterized more thoroughly. Therefore, further studies are needed to clarify the possible biological activity of proGDNF.

When mature GDNF is released from the cell, it binds preferentially to the cognate GFRα1 receptor to elicit neurotrophic signaling via RET RTK. Subsequently, RET is activated, and the ternary signaling complex is internalized. Notably, GDNF can be internalized by GFRα1 also in the absence of RET (Vieira et al., 2003). Endocytosed GDNF is either degraded or similarly to neurotrophins, retrogradely transported to convey the survival signal over long distances. In motoneurons, for example, a significant portion of internalized GDNF escapes degradation and is transported not only to the cell soma but also to the dendrites, where it accumulates in the multivesicular bodies at postsynaptic sites of afferent synapses and can undergo transsynaptic transcytosis (Rind et al., 2005). Dorsal root ganglion neurons can also retrogradely transport both GDNF as well as its family member NRTN in vivo (Leitner et al., 1999). In sympathetic neurons of the superior cervical ganglion, in contrast, GFLs are not retrogradely transported due to fast degradation of activated RET in these neurons (Leitner et al., 1999; Tsui and Pierchala, 2010).

3.1.3. GDNF functions in and outside the nervous system

The pioneering study characterizing the function of GDNF demonstrated the ability of this neurotrophic factor to support the survival of cultured embryonic midbrain dopaminergic neurons (Lin et al., 1993). Since midbrain dopaminergic neurons of GDNF knock-out animals are indistinguishable from wild-type littermates, GDNF does not seem to be critical in the development of these neurons (Moore et al., 1996; Pichel et al., 1996; Kopra et al., 2015). However, as conventional GDNF-deficient mice do not survive to adulthood, it was long unclear whether GDNF has any effect on postnatal and mature dopaminergic neurons in vivo. To date, results of four studies, where either RET or GDNF is ablated specifically from dopaminergic neurons, have been published. Only one of the reports found GDNF to be critically
important for the survival of the catecholaminergic neurons (Pascual et al., 2008). Results of other studies indicate that GDNF signaling is not required for the developmental survival of these neurons but may be required for the maintenance of adult dopamine neurons (Jain et al., 2006b; Kramer et al., 2007; Kopra et al., 2015). In addition, in a very recent report, GDNF was proposed to be an essential modulator of striatal dopamine homeostasis (Kopra et al., 2017). The authors demonstrated that GDNF could regulate the localization and protein levels of the dopamine transporter (DAT) and thus affect the rate of dopamine reuptake. Furthermore, mice with no GDNF in their brain displayed decreased hyperactivity in response to amphetamine (Kopra et al., 2017).

In the ventral tegmental area (VTA) of the mesolimbic system, GDNF is proposed to be an important protector of dopaminergic neurons against excessive alcohol consumption and addiction caused by the drugs of abuse (Messer et al., 2000; He et al., 2005; Barak et al., 2015; Koskela et al., 2017). Taken together, these results highlight the importance of GDNF in controlling the homeostasis of dopamine neurons and elucidate the therapeutic potential of this neurotrophic factor in neuropsychiatric and neurodegenerative disorders that are often a consequence of abnormal functionality of the dopaminergic system.

In addition to being a dopaminotrophic factor, GDNF regulates synapse formation in hippocampal neurons together with GFRα1 and NCAM (Ledda et al., 2007). Furthermore, GDNF is required for the differentiation of ventral precursor cells to inhibitory gamma-aminobutyric acid (GABA) expressing neurons and for the migration of these interneurons from the medial ganglionic eminence to the cortex (Pozas and Ibáñez, 2005; Canty et al., 2009; Perrinjaquet et al., 2011). Results by the same research group demonstrate the importance of GDNF signaling also in the development and function of the olfactory bulb (Marks et al., 2012). Finally, according to a recent publication, GDNF expressed by Purkinje cells functions as a survival factor for cerebellar molecular layer interneurons (Sergaki et al., 2017).

While NGF is the major survival factor for the developing superior cervical ganglion (SCG) neurons in the PNS, these cells also express GFL receptors. ARTN is the most potent GFL in supporting the survival of the SCG during development, whereas GDNF together with NRTN support only a small subset of these neurons (Trupp et al., 1995; Kotzbauer et al., 1996; Baloh et al., 1998b). Parasympathetic neurons, in turn, require GDNF during early embryogenesis for proper migration and proliferation (Enomoto et al., 2000; Rossi et al., 2000). GDNF is expressed by astrocytes and Schwann cells and has been established as an important regulator of both developing and adult motor neurons in vivo (Henderson et al., 1994; Yan et al., 1995). Hence, GDNF is a potential therapeutic candidate for the treatment of neuromuscular diseases such as amyotrophic lateral disease (ALS), characterized by loss of motor neurons and subsequent progressive muscle atrophy leading to paralysis and death in a few years after diagnosis (Zinman and Cudkowicz, 2011).
The survival of embryonic sensory neurons depends largely on NGF, but postnatally the expression of TrkA is downregulated and, instead, RET is expressed in IB4-positive non-peptidergic sensory neurons (Bennett et al., 1996). GDNF can act as a trophic factor for sensory neurons, inducing neurite outgrowth and ensuring proper target innervation (Trupp et al., 1995; Fundin et al., 1999; Paveliev et al., 2007).

As evident from knock-out mice deficient in either GDNF, GFRα1 or RET, GDNF signaling is essential for proper survival, migration, and differentiation of enteric neuronal precursors (reviewed in Airaksinen and Saarma, 2002). Equally important is the role of this signaling complex in kidney development. GDNF knock-out mice lack kidneys, and this is the main reason for the premature death of the pups (Moore et al., 1996; Pichel et al., 1996). Recent work by Kumar et al. showed that, perhaps unexpectedly, overexpression of GDNF from its native locus had a negative effect on kidney size and maturation (Kumar et al., 2015). This finding demonstrates the importance of proper spatiotemporal regulation of GDNF protein since only the correct concentration of the neurotrophic factor ensures the development of functional kidneys.

In addition to aforementioned functions, GDNF also participates in the regulation of spermatogenesis, and several reports have associated GDNF with diabetes (Meng et al., 2000; Mwangi et al., 2011; Abadpour et al., 2017). Diabetes is a metabolic disease described by high blood glucose levels as a consequence of insufficient insulin secretion by pancreatic beta cells. In type 1 diabetes mellitus, the beta cells in islets of Langerhans are attacked by body’s immune system, and one of the therapies involves transplantation of donor islets (Mwangi et al., 2011). GDNF expression was upregulated in the vicinity of beta cells following islet injury, indicating that it could partake in modulating islet survival and repair (Teitelman et al., 1998). Moreover, GDNF was shown to increase the function and viability of isolated human islets in vitro and alleviate thapsigargin-induced ER stress in transplanted islets (Abadpour et al., 2017). In conclusion, pretreatment of isolated human islets with GDNF is essential for the survival and functionality of the islets post transplantation.

3.1.4. **GDNF and Parkinson’s Disease**

Parkinson’s Disease (PD) is a neurodegenerative disorder affecting over 10 million people worldwide. PD incidence increases with age and is slightly more common in males than in females (Miller and Cronin-Golomb, 2010). Less than 10% of PD cases are young-onset, found in people of 40 years or less. PD is best characterized by motor problems caused by the loss of dopaminergic neurons in the midbrain region called substantia nigra pars compacta (SNpc). The motor symptoms include resting tremor, rigidity, postural instability, and difficulties in walking. Also, non-motor symptoms such as depression, sleep disorders, cognitive impairment, lack of motivation, pain, and constipation have been reported (Rana et al., 2015; Knudsen
et al., 2017; Wu et al., 2017). Recent data demonstrate that at the time of motor symptoms onset, about 30-40% of neurons in SNpc have lost their functional connections and degenerated (Kurowska et al., 2016). However, the disease is progressive, and although currently available medication suppresses movement symptoms, there is no treatment available that would stop or slow down the degeneration of dopaminergic neurons.

As animal experiments have shown, GDNF can protect and restore the dopaminergic function both in vitro and in vivo. Since GDNF receptors GFRα1 and RET are expressed by dopamine neurons both in rodents and in human, GDNF is a potential drug candidate for the treatment of the PD (reviewed by Tenenbaum and Humbert-Claude, 2017). There have been several clinical trials where GDNF protein infusion was administered to the parkinsonian patients (Gill et al., 2003; Nutt et al., 2003; Slevin et al., 2005; Lang et al., 2006), but unfortunately, the promising results obtained in preclinical testing were not reproduced. Several factors could explain the modest outcome of the clinical trials, and they are thoroughly reviewed by Tenenbaum and Humbert-Claude (2017). Shortly, since GDNF protein does not pass the blood-brain-barrier and has to be administered intracranially via a mechanical pump or using viral vectors, optimal delivery parameters are crucial. Since GDNF is a heparin-binding protein, which does not diffuse well in brain tissue, developing a biologically active GDNF variant with reduced heparin affinity could be useful for improved therapeutic effect. At least, using this approach, a prominent functional difference was shown in animal models using NRTN variants (Runeberg-Roos et al., 2016). Also, the stability of GDNF should be taken into account and therefore mammalian glycosylated GDNF could be a better candidate for the clinical trials compared to bacterially-produced GDNF used so far (Piccinini et al., 2013).

At the time of writing this thesis, results from the third GDNF Phase II clinical trial conducted in Bristol (UK) with 41 patients are being analyzed. Although the primary efficacy endpoint was not met (http://medgenesis.com/news.htm#top-line), it will be interesting to see, whether GDNF that was administered for the first time via an innovative Renishaw delivery system, had any beneficial effect this time.

3.2. Neurturin

The second member of the GFL family, NRTN was first discovered as a survival factor for the cultured sympathetic neurons (Kotzbauer et al., 1996). Similarly to GDNF, NRTN is synthesized as a preproprotein, having a 19-amino-acid signal sequence and a 76-amino-acid pro-region (Figure 6). The role of the pro-region is not clear, but it seems to be involved in the trafficking and activity of NRTN protein (Fjord-Larsen et al., 2005). The pro-domain is followed by a conserved furin cleavage site and a 100-amino-acid mature region that is 42% homologous to the mature domain of GDNF (Kotzbauer et al., 1996). Mature NRTN homodimer signals preferentially via its cognate receptor GFRα2 and RET receptor tyrosine kinase, but can also activate RET by binding to GFRα1 (Baloh et al., 1997; Jing et al., 1997; Klein
et al., 1997; Suvanto et al., 1997). In addition, a number of other receptors exist: NRTN can bind to GFRα2 and NCAM, direct interaction with syndecan-3 has been demonstrated, and NRTN’s affinity to heparin is higher than that of GDNF (Paratcha et al., 2003; Alfano et al., 2007; Bespalov et al., 2011). NRTN KO mice are viable and fertile, but show deficits in enteric and parasympathetic innervation (Heuckeroth et al., 1999; Enomoto et al., 2000; Rossi et al., 2000).

NRTN mRNA is widely expressed in a variety of neuronal and non-neuronal tissues both in embryos and adults, indicating a plethora of possible functions for this protein all over the organism (Kotzbauer et al., 1996; Widenfalk et al., 1997; Golden et al., 1998). Like GDNF, NRTN can modulate the development of the kidney, promoting epithelial branching by inducing branch initiation in vitro (Davies et al., 1999). In addition, NRTN is expressed in embryonic liver, where it has been shown to direct liver bud migration (Tatsumi et al., 2007) and suggested to participate in erythropoiesis (Golden et al., 1999). Overexpression of NRTN in basal keratinocytes modulates mechanical responsiveness to peripheral stimuli in primary sensory neurons (Jankowski et al., 2017). GFRα1, GFRα2 and RET receptor are all expressed in lung, but their function in that tissue has remained elusive. Recently, Mauffray and colleagues discovered that NRTN is involved in the pathophysiology of asthma by influencing inflammatory responses and increasing markers of airway remodeling in several mouse asthma models (Mauffray et al., 2015). Finally, a novel function was demonstrated for NRTN in preventing the development of diabetes in a rat model. When NRTN was administered together with a glucagon-like peptide 1 receptor agonist liraglutide, metabolic parameters and glucose homeostasis were restored in hyperglycemic animals (Trevaskis et al., 2017).

Within the nervous system, NRTN is important for a number of neuronal populations in addition to sympathetic neurons. Ablation of nrtn gene demonstrated a critical role for NRTN in the development and maintenance of the enteric neurons of the gut (Heuckeroth et al., 1999). In addition, developing parasympathetic and sensory neurons of the dorsal root, trigeminal and nodose ganglia require NRTN for survival (Kotzbauer et al., 1996; Heuckeroth et al., 1999). Besides being a survival factor for the neurons, NRTN together with GDNF plays a critical role in the selective maintenance of herpes simplex viruses (HSV) 1 and 2 latency, inhibiting viral DNA replication in primary adult sensory neurons of peripheral ganglia (Yanez et al., 2017).

In the CNS, NRTN together with GDNF and IFNγ was recently shown to promote the differentiation of cortical neural precursor cells in vivo (Yuzwa et al., 2016). Furthermore, NRTN can protect embryonic motor neurons and basal forebrain cholinergic neurons in vitro (Klein et al., 1997; Golden et al., 2003) and dopaminergic neurons in the ventral midbrain both in vitro and in vivo in a 6-OHDA model of PD (Horger et al., 1998; Akerud et al., 1999). Based on the neuroprotective effects on nigrostriatal dopamine neurons, NRTN has been tested
in clinical trials as a candidate for the treatment of PD. Unlike GDNF, which has been administered to the patients by recombinant protein infusion, NRTN trials have been carried out using mainly a recombinant adeno-associated virus serotype-2 (rAAV2) mediated gene transfer (CERE-120)(Marks et al., 2010; Bartus et al., 2015). So far, the results of NRTN clinical trials have been inconclusive. On the positive side, the surgical procedure is well tolerated, and there are no clinically significant adverse events related to AAV2-neurturin. Motor status of patients was stable or even modestly improved (relative to baseline) over the course of the open-label, long-term follow-up (Warren Olanow et al., 2015; Marks et al., 2016). Although NRTN gene therapy did not improve the condition of all tested PD patients significantly, post-hoc analysis suggested a significant therapeutic effect on early stage PD patients (Bartus and Johnson, 2017). Post-mortem brain analysis of the PD patients that had received NRTN-gene therapy over 4 years before autopsy revealed a mild but persistent NRTN expression level. However, the number of NRTN-positive nigral neurons was extremely small (Bartus et al., 2015). It is tempting to speculate that if NRTN variants with reduced heparin affinity developed by Runeberg-Roos and colleagues were used in future clinical trials with less advanced PD patients, NRTN would prove to be a potent therapeutic agent for the treatment of the PD (Runeberg-Roos et al., 2016).

3.3. Artemin

ARTN was discovered as a result of a database homology search. Protein alignment analysis revealed that ARTN has a relatively long pre-pro-region, multiple putative furin cleavage sites for removal of the pro-part and the mature region of this factor is \(~45\%\) identical to those of NRTN and PSPN (Baloh et al., 1998b). Like other GFLs, ARTN acts as a dimer and activates RET receptor tyrosine kinase by binding to the GFRα3 co-receptor. Baloh and co-workers initially proposed that ARTN could also signal via GFRα1 and RET \textit{in vitro}, but further studies concluded that this interaction was not likely to be functional \textit{in vivo} (Baloh et al., 1998b; Carmillo et al., 2005). Similarly to GDNF and NRTN, ARTN can also signal in a RET-independent manner via GFRα3/NCAM, and bind with high affinity to heparin and syndecan-3 (Alfano et al., 2007; Bespalov et al., 2011; Schmutzler et al., 2011).

ARTN is expressed at a relatively low level both in the brain and in peripheral tissues (Baloh et al., 1997; Masure et al., 1999; Quartu et al., 2005, 2007). Functionally, ARTN was first identified as a survival factor for sensory and sympathetic peripheral neurons \textit{in vitro} (Baloh et al., 1998b). Ablating GFRα3 or ret from mice proved that ARTN-GFRα3 signaling is indeed crucial for the migration of sympathetic precursors as well as for the survival of mature sympathetic neurons \textit{in vivo} (Nishino et al., 1999; Enomoto et al., 2001). Since GFRα3 is expressed mostly in nociceptive sensory neurons of the dorsal root ganglia, there is abundant literature linking ARTN to neuropathies (Orozco et al., 2001; Merighi, 2016). Furthermore, ARTN seems to play a role in atopic dermatitis,
caused by air pollutants and warm environment (Murota et al., 2012; Hidaka et al., 2016), and is in clinical trials for the treatment of neuropathic pain (Rolan et al., 2015).

3.4. Persephin

The fourth member of the GFL family was identified by homology cloning. PSPN is expressed as a 156 aa long pre-pro-form, which is cleaved, yielding a 96 aa long mature protein that is 40% identical to the mature regions of GDNF and NRTN proteins (Milbrandt et al., 1998). PSPN signals mainly through a receptor complex comprising of GFRα4 and RET receptor tyrosine kinase but it can also bind to GFRα1 (Milbrandt et al., 1998; Lindahl et al., 2001; Sidorova et al., 2010). PSPN is the only member of the GFLs that does not bind to heparan sulfate proteoglycan syndecan-3 (Bespalov et al., 2011).

PSPN is expressed at low levels in the central nervous system of rodents and primates (Jaszai et al., 1998; Tomac et al., 2002; Quartu et al., 2005, 2007). Similarly to other GFLs, PSPN promotes the survival of ventral midbrain dopaminergic neurons in culture and prevents their degeneration after 6-hydroxydopamine treatment in vivo. Furthermore, PSPN can attenuate ischemic neuronal cell death both in vitro and in vivo (Tomac et al., 2002) and it also supports the survival of motor neurons in culture and in vivo after sciatic nerve axotomy (Milbrandt et al., 1998). Mice lacking PSPN show normal development and behavior but are hypersensitive to cerebral ischemia (Tomac et al., 2002).

In contrast to GDNF or NRTN, PSPN has no survival-promoting effect on peripheral neurons, including SCG and sensory neurons in dorsal root ganglion (Milbrandt et al., 1998). However, it can promote neurite outgrowth of the SCG neurons (Sidorova et al., 2010). PSPN might also contribute to the survival of precursor cells during enteric nervous system development since in some patients with Hirschsprung’s disease (HSCR), a point mutation (R91C) in the mature region of PSPN was found to be associated with the HSCR phenotype (Ruiz-Ferrer et al., 2011).

Outside the nervous system, PSPN like GDNF, promotes ureteric bud branching (Milbrandt et al., 1998) and regulates calcitonin synthesis and release by the C-cells in the thyroid (Lindfors et al., 2006). In addition, PSPN was recently found to be overexpressed in oral squamous cell carcinomas and to be strongly associated with tumoral progression by promoting cell-cycle progression in the G1 phase through the RET receptor and the RTK signaling pathway, and by decreasing the expression of cyclin-dependent kinase inhibitors (Baba et al., 2015).
4. GDNF family receptors

GDNF ligands signal through a tetrameric receptor complex comprising of two GFRα molecules and two RET receptors (Durbec et al., 1996; Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996). Each of the ligands binds preferentially to a different GFRα receptor: GDNF to GFRα1, NRTN to GFRα2, ARTN to GFRα3 and PSPN to GFRα4 (reviewed in Airaksinen and Saarma, 2002) (Figure 8). Furthermore, in vitro studies have shown that GDNF can also bind to GFRα2 and subsequently activate RET, and neurturin in turn is able to signal via GFRα1 (Baloh et al., 1997; Jing et al., 1997; Sanicola et al., 1997; Trupp et al., 1998; Cik et al., 2000). Also ARTN and PSPN have been shown to bind to GFRα1 in the presence of RET (Baloh et al., 1998b; Sidorova et al., 2010). Since all GFRα receptors are linked to the plasma membrane via a glycosylphosphatidylinositol (GPI)-anchor and do not contain an intracellular domain, additional receptor is needed to convey the signal into the cell. RET is the signaling receptor shared by all the GFLs (Jing et al., 1996; Trupp et al., 1996). Interestingly, RET is the only known receptor tyrosine kinase that does not bind its ligands directly and requires a co-receptor for activation. Importantly, the structure of the extracellular domain of RET in complex with GFRα1 and GDNF was recently solved by combining cryo-electron microscopy and low-angle X-ray scattering (SAXS) data (Goodman et al., 2014).

In some cells of the nervous tissue i.e. neocortex and hippocampus, GFRα receptors are expressed abundantly, but RET protein is not present (Golden et al., 1999). This observation suggested the existence of RET-independent signaling, and indeed, by now, several alternative receptors have been discovered. First, neuronal cell adhesion molecule (NCAM) was identified as a co-receptor for GFRα1-3, then a heparan sulfate proteoglycan syndecan-3 was shown to bind GDNF, NRTN and ARTN (Paratcha et al., 2003; Bespalov et al., 2011; Schmutzler et al., 2011). Interestingly, the latter interaction between syndecan-3 and the GFLs does not require the presence of either of the traditional receptors GFRα or RET (Bespakov et al., 2011). In addition, there is some evidence that GDNF could also signal through N-cadherin and integrins and thus protect the dopamine neurons but these interactions have not been characterized in detail and will not be discussed in this thesis (Chao et al., 2003; Cao et al., 2008; Zuo et al., 2013).

4.1. GFRα receptors

There are four members in the GFRα receptor family: GFRα1 (Jing et al., 1996; Treanor et al., 1996), GFRα2 (Baloh et al., 1997; Buj-Bello et al., 1997; Jing et al., 1997; Klein et al., 1997; Suvanto et al., 1997), GFRα3 (Jing et al., 1997; Baloh et al., 1998a; Masure et al., 1998; Naveilhan et al., 1998; Worby et al., 1998) and GFRα4 (Enokido et al., 1998; Thompson et al., 1998; Lindahl et al., 2000; Masure et al., 2000). As mentioned already in section 1.4, each GFRα receptor functions primarily
as a dimeric co-receptor for RET receptor tyrosine kinase when ligated to a preferred high-affinity binding GDNF family ligand (Figure 8).

The length of mature GFRα receptors is about 400 amino acids, and they share 30%–45% sequence identity. GFRαs have three cysteine-rich domains (CRD1, CRD2, and CRD3) and a C-terminal extension, except for GFRα4 that lacks CRD1 (Airaksinen and Saarma, 2002). Leppänen and colleagues solved the crystal structure of GFRα1 CRD3, and proposed that CRD2 and CRD3 have a similar structure with five α-helices, and five disulfide bridges and the domains are located close to each other, whereas CRD1 is linked to CRD2 by a flexible hinge region (Leppänen et al., 2004). Mutational analysis indicates that for ligand interaction, domains CRD2 and CRD3 are required, while CRD1 has been proposed to stabilize the forming complex (Scott and Ibanez, 2001; Virtanen et al., 2005). In the case of GFRα1, residues F213, R224, R225 and I229, located in CRD2, are important for GDNF binding and further RET activation (Leppänen et al., 2004). Furthermore, length of the hinge region seems to be equally important for GFRα1 functioning, as the splice variant lacking five amino acids of that area (GFRα1b) binds GDNF with higher affinity and promotes stronger RET phosphorylation compared to GFRα1a, where exon 5 encoding these amino acids is included (Charlet-Berguerand et al., 2004). In addition to their cognate ligands and RET, GFRα receptors 1, 2 and 3 bind also to NCAM, and this interaction is likely to be mediated by the N-terminal CRD1 domain (Sjostrand and Ibanez, 2008).

Analysis of the crystal structure of GFRα1 comprising CRD2 and CRD3 in complex with GDNF confirmed the results of earlier mutational studies. The fingertips of GDNF and CRD2 of the GFRα1 are essential for the high-affinity ligand-receptor binding (Parkash et al., 2008). Comparison of the GDNF-GFRα1 crystal structure with that of ARTN-GFRα3 demonstrated that the ligand-receptor binding site is highly conserved. However, there is a difference in the bend angle of GDNF and ARTN, and this feature affects the formation of the ligand-receptor complex and further activation of RET (Parkash et al., 2008; Parkash and Goldman, 2009).

Not much is known about the trafficking of the GFRα receptors. All of them contain putative N-glycosylation sites and undergo glycosylation in the Golgi complex. Sugar moieties have been suggested to play a role in protein trafficking (Hart and Copeland, 2010) but currently, no evidence has been provided for GFRαs. TGF-β is crucial for recruiting GFRα1 to the plasma membrane in primary neurons, but trafficking of GFRα2 does not depend on TGF-β, indicating differential regulatory mechanisms for each of the GDNF receptors (Peterziel et al., 2002).

GFRα receptors are linked to the cell membrane by a GPI-anchor and thus lack the cytoplasmic domain. GPI-anchored proteins along with doubly acylated proteins (e.g. cytoplasmic Src-family kinases), cholesterol-linked and palmitoylated proteins are enriched in lipid rafts. GFRα receptors are localized to lipid rafts, and upon GDNF binding, GFRα1 has been shown to recruit RET to lipid rafts where the signaling occurs (Tansey et al., 2000). When RET localization is disrupted using
either transmembrane or soluble GFRα1, GDNF-stimulated intracellular signaling events, as well as neuronal differentiation and survival, are markedly attenuated (Tansey et al., 2000; Pierchala et al., 2006). Interestingly, in the presence of GDNF, GFRα1 can activate Src family kinases in lipid rafts also independently of RET (Poteryaev et al., 1999). GFRα receptors can be cleaved by the phosphoinositide-specific phospholipase C (PI-PLC) (Yu et al., 1998), and soluble forms of GFRα have been shown to activate RET receptor tyrosine kinase in trans (Trupp et al., 1997). Indeed, GFRα1 can be cleaved by neurons and Schwann cells and together with GDNF, soluble GFRα1 can recruit RET to the lipid rafts (Paratcha et al., 2001). However, since transgenic mice expressing GFRα1 in ret locus did not show deficits in enteric or motor neurons, neither in kidney or Schwann cells where trans-signaling has been implicated in vitro, the physiological relevance of this signaling in vivo could be restricted to CNS (Enomoto et al., 2004).

The tissue expression pattern of the GFRα receptors is very similar to their corresponding ligands. During mouse embryonic development GFRα1 and GFRα2 are highly expressed in the mammalian brain but are also detected in the urogenital system, the digestive system, the respiratory system, developing skin, bone, muscle, and endocrine glands (Golden et al., 1999). GFRα3 is mostly found in the nociceptive sensory neurons and superior cervical ganglion neurons of the peripheral nervous system, while GFRα4 is expressed in other organs like thyroid, pituitary and adrenal glands (Nishino et al., 1999; Lindahl et al., 2000; Orozco et al., 2001). The expression level of GFRα receptors decreases in adult mouse compared to the embryo (Golden et al., 1999).

Soon after the discovery of the four receptor family members, conventional knock-out mice were generated for each receptor, and in short, their phenotype was rather similar to that of their cognate ligands (Airaksinen and Saarma, 2002). GFRα1 knock-out mice die at birth due to uremia similarly to GDNF knock-out animals; they lack kidneys and enteric neurons below stomach (Enomoto et al., 1998; Tomac et al., 1999). GFRα2-deficient animals are viable but have retarded growth as well as severely reduced parasympathetic innervation of the lacrimal and salivary glands and the myenteric plexus of the intestine (Rossi et al., 1999). Also, GFRα2 KO mice show a deficit in parasympathetic innervation of pancreatic islets and impaired vagal tone but respond normally to exogenous glucose (Rossi et al., 2005). GFRα3-ARTN signaling is critical for the migration of sympathetic neurons. Thus, not surprisingly, GFRα3 KO mice display abnormal innervation of the entire sympathetic nervous system (Nishino et al., 1999; Tanaka et al., 2011). Mice lacking GFRα4, created by Lindfors and colleagues, are viable and fertile without any gross defects. Further analysis of these mice revealed, however, that they do have reduced thyroid calcitonin levels and therefore increased bone formation (Lindfors et al., 2006).

There are two GFRα related receptors: GDNF family receptor α-like (GFRAL) and GAS-1. GFRAL does not bind GDNF family members but very recently, Hsu and
colleagues (Hsu et al., 2017), and three other research teams (Emmerson et al., 2017; Mullican et al., 2017; Yang et al., 2017) have identified GFRAL as the receptor for growth differentiation factor 15 (GDF15), and demonstrate that GDF15-GFRAL-RET signaling controls food intake and animal body weight.

Figure 8. GDNF family ligands and their receptors. GFLs bind to GPI-linked GFRα-receptors and signal via RET receptor tyrosine kinase. Complex formation is calcium-dependent. Preferred receptors for each GFL are indicated with bold arrows, secondary receptors are marked with dotted arrows. GFRα receptors (except GFRα4) can mediate GFL signaling also in complex with NCAM. Adapted from Kramer and Liss (2015).

4.2. RET

RET is a RTK superfamily member with essential functions in kidney morphogenesis, mediating spermatogonial stem cell maintenance, body weight control, and development of the nervous system (reviewed in Mulligan, 2014). The gene encoding RET (REarranged during Transfection) receptor tyrosine kinase was initially characterized as a gene activated by DNA rearrangement (Takahashi et al., 1985). Further studies revealed that the ret gene consists of 20 exons that give rise to several splice isoforms (Tahira et al., 1990; Carter et al., 2001; De Graaff et al., 2001). For example, alternative splicing of intron 19 generates three splice isoforms: RET9, RET43, and RET51 that are identical until Tyr1062 but have a unique C-terminal amino acid sequence. RET9 and RET51 are evolutionarily highly conserved, most abundantly expressed isoforms of RET protein and therefore best-studied, RET43 has been detected in low levels only in primates (Tahira et al., 1990; Myers et al., 1995; Carter et al., 2001; De Graaff et al., 2001). Recently, it was shown
that exon skipping in the 5' region of the RET gene produces two novel splice isoforms \( \text{RET}^{\Delta E3} \) and \( \text{RET}^{\Delta E345} \), which are found both in human and in lower organisms (Gabreski et al., 2016).

Mutations in the \( \text{ret} \) gene are linked with several pathologies. Gain of function mutations lead to endocrine cancers, including multiple endocrine neoplasia type 2A and 2B (MEN2A and MEN2B), and familial medullary thyroid carcinoma. Germline loss of function mutations, in turn, are strongly associated with Hirschsprung's disease (HSCR) and can also be found in patients with congenital abnormalities of the kidney and urinary tract (CAKUT) (Mulligan, 2014). HSCR is a gut syndrome characterized by the absence of enteric neurons in the distal part of the colon and small intestine (Amiel et al., 2008).

RET is expressed at highest levels during the embryogenesis in neural crest-derived cells and tissues (Pachnis et al., 1993; Tsuzuki et al., 1995). These include dopaminergic, noradrenergic and motor neurons, sympathetic, parasympathetic and sensory neurons and the enteric nervous system (Arighi et al., 2005). In adult brain, RET mRNA expression is mainly restricted to the midbrain, cerebellum, pons, and thalamus (Kramer and Liss, 2015). Outside the nervous system, RET is for example expressed by a population of ureteric bud tip cells (Shakya et al., 2005), as well as in lung, testis, thyroid and adrenal gland (Tsuzuki et al., 1995).

Similarly to GDNF and GFR \( \alpha_1 \) knock-out animals, ablation of the \( \text{ret} \) gene in mice leads to death shortly after birth due to renal agenesis. Moreover, those mice lack enteric neurons throughout the digestive tract, and their sympathetic precursor cells fail to migrate properly (Schuchardt et al., 1994; Enomoto et al., 2001). To address, whether the KO phenotype is splice isoform-dependent, mice monoisoformic for either RET9 or RET51 were generated. Both mouse-lines are viable with normal kidney, indicating redundant roles for RET isoforms in kidney development (Jain et al., 2006a). Since GDNF is a prominent survival factor of the midbrain dopamine neurons in numerous \textit{in vitro} assays, and its signal transduction is mediated mainly by RET receptor tyrosine kinase, the role of RET in the dopaminergic neurons of adult animals was studied. For that, conditional RET-KO mouse line was created where RET was specifically ablated from dopaminergic neurons (Jain et al., 2006b; Kramer et al., 2007). The first study did not observe differences in the survival of dopaminergic neurons in 1-y-old RET conditional knock-out mice compared to the age-matched controls (Jain et al., 2006b). However, gradual loss of dopamine neurons in SNpc and reduction of dopaminergic nerve terminals in the striatum of transgenic mice was reported by the other group. The change was observed at nine months and peaked at two years of age, indicating that RET signaling is required for maintenance of target innervation of midbrain DA neurons in aged animals (Kramer et al., 2007).
4.2.1. RET structure and signaling

Typically, RTKs are type-1 transmembrane receptors with a large extracellular part for interacting with ligands, a single transmembrane helix and a cytoplasmic kinase domain for the signal transduction (Figure 9). The extracellular domain (ECD) of RTKs commonly contains leucine-rich repeats, immunoglobulin or fibronectin-like domains (Lemmon and Schlessinger, 2010). RET, however, differs from other RTKs as its ECD comprises of 4 cadherin-like domains (CLD) with a calcium binding site between CLD2 and CLD3 and a membrane-proximal cysteine-rich domain (Anders et al., 2001). Cadherins are transmembrane proteins that mediate Ca^{2+}-dependent homophilic cell adhesion, and RET is classified as a distant member of the cadherin superfamily (Hulpiau and van Roy, 2009). Unlike traditional cadherins with linear organization of cadherin domains, RET CLD1-2 forms a clamshell arrangement (Kjaer et al., 2010). Functionally, CLD motifs are required for stabilizing RET dimers. The presence of calcium ions has been reported crucial for proper folding and cell surface expression of RET and necessary for GDNF signaling (van Weering et al., 1998; Anders et al., 2001). Taking that into account, it is surprising that the novel splice isoforms where either exon 3 (RETΔE3) or exons 3, 4, 5 are skipped (RETΔE345), leading to large deletions in the ECD structure, can still bind to all GFRα receptors to similar extent as the wild-type RET (Gabreski et al., 2016).

The cysteine-rich region is required for protein conformation and ligand binding (Amoresano et al., 2005), while the transmembrane (TM) domain of RET is essential for dimer association and hence for intracellular signaling (Kjaer et al., 2006). The intracellular part of RET comprises of a 50-residue-long juxtamembrane domain, a highly conserved tyrosine kinase domain and a C-terminal tail of 100 residues. Within the cytoplasmic domain of RET, there are multiple tyrosines (Tyr) and one serine (Ser) residue that can become phosphorylated upon activation of the RET dimer and facilitate either direct interaction with signaling molecules like Src and phospholipase Cγ (PLCγ) or serve as docking sites for numerous adaptor proteins which activate downstream signaling pathways, promoting cell growth, proliferation, survival or differentiation (reviewed in Ibáñez, 2013; Mulligan, 2014) (Figure 9). The structure of the human RET tyrosine kinase domain has been solved (Knowles et al., 2006).

Tyr^{687} and Ser^{696} are located in the juxtamembrane region, and both residues are involved in cyclic adenosine monophosphate (c-AMP)-mediated modulation of RET activity (Fukuda et al., 2002). By creating a knock-in mutant mouse, Ser^{696} was shown to be involved in the migration of the enteric neural crest cells in mouse developing gut (Asai et al., 2006). Tyr^{687} also has an established role in the integration of RET and protein kinase A (PKA) signals (Ibáñez, 2013). Phosphotyrosines Tyr^{752} and Tyr^{928} can both activate Signal Transducer and Activator of Transcription (STAT3) and downstream Janus kinase (JAK)-STAT pathway, resulting in enhancement of proliferation and differentiation. Tyr^{905} is located in the kinase activation loop and is activated upon ligand binding. Structural
analysis of the RET kinase domain confirmed that this phosphotyrosine is necessary for full kinase activation of the protein (Knowles et al., 2006). Tyr981 binds the proto-oncogene tyrosine protein kinase Src, thereby promoting GDNF-induced survival (Encinas et al., 2004). Phosphorylated Tyr1015 serves as a high-affinity docking site for PLCγ and its mutation leads to a decrease in RET signaling (Borrello et al., 1996). Kidney defects were found in mice when Tyr1015 of either Ret9 or Ret51 was mutated to phenylalanine (Jain et al., 2006a).

Figure 9. Intracellular signaling pathways mediated by RET. Adapted from Mulligan, 2014.

Tyr1062 is the major signaling hub of RET protein and mice with a point mutation in Tyr1062 have a severe loss-of-function phenotype (Ibanez, 2013) (see Figure 9). Autophosphorylation of Tyr1062, present in all splice isoforms of RET, is required for activation of rat sarcoma (RAS)/MAPK and PI3K/AKT pathways. The MAPK signaling pathway mediates neurite outgrowth but also contributes to the neuronal survival. The PI3K pathway, in turn, is essential for neuronal survival but can also stimulate neurite growth. Signaling pathway activated by the Dok proteins regulates neuronal differentiation (Ibanez, 2013).

When Tyr1062 was mutated in mice expressing either RET9 or RET51 selectively, defects in kidney branching morphogenesis were observed. However, the phenotype was much milder in RET51 mice since presumably the signaling was partly compensated by Tyr1096 (Jain et al., 2006a). Tyr1096, present only in RET51 isoform, is phosphorylated upon ligand binding, and binds growth factor receptor-bound protein Grb2, contributing to RAS/MAPK and
PI3K/AKT pathways. Mutation of this residue has been shown to increase the activity of an oncogenic MEN2B form of RET (Liu et al., 1996).

The splice isoform specific amino acid sequence in the C-terminus of the RET protein is proposed to be responsible for certain distinct properties of RET9 and RET51. Indeed, differences in gene expression induced by RET9 and RET51 have been described (Hickey et al., 2009). Furthermore, there is evidence of differential binding of RET9 and RET51 to adaptor proteins Shc (Src homology 2 domain-containing protein), SHANK3 (SH3 and multiple ankyrin repeat domains protein 3), Enigma, Grb2, and c-Cbl (E3 ubiquitin-protein ligase) that support this idea (Mulligan, 2014) and will be further discussed in the next section.

4.2.2. Cellular regulation of RET

The first translated N-terminal amino acids (1-28) of RET encode a signal sequence, which targets the nascent protein to the endoplasmic reticulum (ER). RET monomers have 12 putative N-linked glycosylation sites in the extracellular domain, and they undergo rapid glycosylation in ER to produce an immature 150 kDa glycoprotein (Takahashi et al., 1993a). Proper processing of the precursor protein in ER is dependent on calcium as the lack of it negatively affects maturation and trafficking of RET to the plasma membrane (van Weering et al., 1998). Next, the immature form of RET is further processed within the trans-Golgi network (TGN) and secretory pathway, and this results in a mature fully glycosylated plasma membrane-localized protein with a mass of approximately 170 kDa (Cosma et al., 1998). Ret conversion from immature to mature form most likely takes place in the acidic trans-Golgi apparatus since the terminal glycosylation of the receptor is pH sensitive (Hirata et al., 2010). RET is believed to be synthesized as an inactive monomer that can get into contact with ligands and the co-receptors only on the cell surface. This is probably correct for the wild-type RET, but a constitutively active RET MEN2B mutant can be phosphorylated and interact with Grb2 and Shc already in the ER, further activating AKT, ERK, and STAT3 pathways, at least in cellular assays (Runeberg-Roos et al., 2007).

The rate of maturation differs between RET isoforms. Though RET9 is expressed at higher levels relative to RET51, the immature form of RET9 accumulates in a perinuclear region that co-localizes with areas of TGN (Richardson et al., 2012). Interestingly, RET9 binds to and co-localizes with Enigma, an adaptor protein acting between the kinases and cytoskeleton, with stronger affinity than RET51 (Borrello et al., 2002). This finding may partly explain why the majority of RET9 protein is retained inside the cell while RET51 matures faster and is localized mostly on the plasma membrane (Richardson et al., 2012). RET surface levels can also be modulated by protein kinase C (PKC) and high K+ depolarization via phosphorylation of the Thr675 residue in the juxtamembrane region of RET (Li et al., 2012).
Differently from neurotrophin receptors, plasma membrane-localized RET is found outside the lipid rafts, where it interacts with Shc upon activation (Paratcha et al., 2001). RET can be recruited to the rafts by the GDNF-GFRα1 complex, thus enabling the association of RET and Src (Tansey et al., 2000; Pierchala et al., 2006). Activation of RET results in recognition of the receptor by the endocytic machinery of the cell, leading to internalization primarily through clathrin-coated pits (Richardson et al., 2006; Crupi et al., 2015). RET localization to endosomes is necessary for complete MAPK activation and provides an important link between receptor down-regulation and activation of the proliferative signaling cascade (Richardson et al., 2006). Interestingly, RET9 and RET51 have been shown to assemble unique signaling complexes in cells as well as primary neurons (Tsui-Pierchala et al., 2002a). Although RET51 is internalized to endosomes more rapidly than RET9 (Crupi et al., 2015), and it has been shown to associate more strongly with the ubiquitin ligase Cbl than RET9 (Scott et al., 2005), this does not lead to the faster degradation of the longer splice variant. On the contrary, a portion of RET51 escapes degradation and is recycled back to the plasma membrane through a Rab11-positive recycling pathway, while RET9 is delivered to lysosomes (Richardson et al., 2012). By this virtue, RET51 is present and active within the plasma membrane and cytoplasm longer than RET9 and mediates prolonged ERK1/2 activation compared to the shorter isoform (Richardson et al., 2012). In summary, alternative splicing of RET protein generates isoforms with notably different localization and trafficking properties, which affect their signaling capacities and thus may contribute to differences in their biological functions.

4.3. NCAM

Neuronal cell adhesion molecule (NCAM) is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily. The ectodomain consists of five N-terminal immunoglobulin-like domains (Ig1-5), followed by two membrane-proximal fibronectin domains. NCAM is posttranslationally modified and carries an unusual carbohydrate – polysialic acid (PSA) - at its Ig5 domain. PSA is a highly negatively-charged sugar polymer and may comprise above 90 sialic acid residues in postnatal mouse brains, increasing the hydrodynamic radius of their carriers dramatically. To date, only eight proteins expressed in vertebrates have been identified to undergo polysialylation, neuropilin-2, synaptic cell adhesion molecule, and C-C Chemokine receptor type 7 being some examples in addition to NCAM (reviewed in Galuska et al., 2017). NCAM is expressed on neuronal cell surface, and it is shown to play an important role in neural development, promoting cell adhesion and migration, axon pathfinding and synaptic plasticity (Fields and Itoh, 1996; Maness and Schachner, 2007). While the role of NCAM in the developing brain is rather well studied, a recent review proposed that NCAM could also regulate the development of other organs like kidney, liver, heart, and testis (Galuska et al., 2017).
Alternative mRNA splicing gives rise to three NCAM isoforms: NCAM-180, NCAM-140 and NCAM-120, the numbering referring to the molecular weight of the isoforms in kDa. NCAM-180 and NCAM-140 are transmembrane proteins, whereas the shortest isoform is a GPI-linked receptor similarly to GFRα receptors. Unlike RET, NCAM-140 can bind GDNF directly with a $K_d$ of about 5nM (Paratcha et al., 2003; Nielsen et al., 2009) but for intracellular signaling the presence of GFRα1 is required. The interaction site has been identified, and it involves the heel region of GDNF and the third Ig domain of NCAM (Sjöstrand et al., 2007; Nielsen et al., 2009). NCAM140 also interacts physically and functionally with GFRα via its Ig4 domain (Paratcha et al., 2003; Sjöstrand et al., 2007).

According to current understanding, the two receptors seem to interact in cis only (Paratcha et al., 2003; Ledda et al., 2007). Interestingly, GFRα1 binding decreases NCAM’s ability to trigger homophilic cell-cell interactions and thus mediate cell adhesion, a phenomenon that does not depend on GDNF (Ibáñez, 2010). On the other hand, in the presence of GFRα co-receptor, the binding affinity of NCAM and GDNF increases to $K_d = 1$nM. As a result of this binding, Src-like tyrosine kinase Fyn and downstream focal adhesion kinase (FAK) are activated, leading to GDNF-stimulated Schwann cell migration and axonal growth of cortical and hippocampal neurons (Paratcha et al., 2003). NCAM-140 expression is upregulated at presynaptic sites in hippocampal neurons during the early postnatal period, and in addition to regulating neurite outgrowth, NCAM can modulate also synapse formation together with GDNF and GFRα1 (Ledda et al., 2007). In addition to NCAM-140, also NCAM-180 is involved in mediating GDNF signaling (Nielsen et al., 2009). So far, there is little evidence of other GFLs signaling through NCAM. Schmutzler and coworkers found that NRTN and ARTN are able to signal through the complex comprising of their cognate receptor and NCAM independently of RET, mediating sensitization of sensory neurons (Schmutzler et al., 2011). In another study, overexpressed GFRα4 was shown to interact with NCAM, suggesting that also PSPN may use this alternative signaling pathway (Paratcha et al., 2003).

### 4.4. Syndecan-3

Syndecan-3 is one of four members of the syndecan protein family present in mammals. Syndecans are transmembrane proteoglycans with a variable ectodomain to which one or more glycosaminoglycan (GAG) chains are covalently attached. Whereas syndecan-1 and -3 have both heparan sulfate (HS) and chondroitin sulfate (CS) chains, the serine-glycine motifs of syndecan-2 and -4 only bind the HS chains (Afratis et al., 2017). HS is composed of repeating disaccharides of N-acetyl-D-glucosamine and D-glucuronic acid. The HS chains are functionally highly important as they interact with multiple biological effector proteins like growth factors, chemokines, and extracellular matrix components. In addition, recent data demonstrate that also the core region located between the N-terminal HS chains and the transmembrane domain of syndecans can bind other proteins.
such as integrins, receptor tyrosine kinases, and phosphatases (Mitsou et al., 2017). Syndecan-3, for example, interacts in this manner with tissue factor pathway inhibitor (TFPI) in vascular and cancer cells (Tinholt et al., 2015). The transmembrane of syndecans is crucial for the dimer formation, as syndecans can apparently form both homo- and heterodimers on the cell surface.

The cytoplasmic domain of syndecan proteoglycans is rather short, and it is divided into three regions. The C1 area interacts with the cytoskeleton and modulates syndecan internalization, least conserved V region is responsible for syndecan-specific signaling, and the C2 region binds PDZ domain proteins via its hydrophobic tail and may be necessary for trafficking and exosome formation (Mitsou et al., 2017). When ligated by heparin-binding growth-associated molecule (HB-GAM), syndecan-3 can bind Src family kinases (c-Src and Fyn) via its V region, make (indirect) contact also with their substrates (cortactin, tubulin), and mediate neurite outgrowth and neuronal migration (Kinnunen et al., 1998; Hienola et al., 2006).

Syndecans participate in many essential cellular actions such as cell adhesion, proliferation, differentiation, and migration. Notably, syndecan-3 is the only member of the family expressed by the neural cells (Afratis et al., 2017). Recent work suggests an important role for syndecans in tissue regeneration (Chung et al., 2016). The expression of syndecans is significantly altered during cancer development and progression. Syndecan-3 is associated with pancreatic and prostate cancer, and it may play a role in tumor angiogenesis (Afratis et al., 2017).

Syndecan-3 was first identified as an alternative receptor for GDNF, NRTN, and ARTN by Bespalov and colleagues in 2011. The authors demonstrated that GFL binding to the HS chains of syndecan-3 led to the activation of the Src kinase and promoted cell spreading as well as neurite outgrowth of hippocampal neurons (Bespalov et al., 2011). One of the functions of syndecans is to present their binding partners to their cognate receptors, thereby promoting efficient signal transduction. Therefore, it is speculated that syndecan-3 could function as a co-receptor and deliver GDNF to GFRα1/RET or GFRα1/NCAM complex. Alternatively, GDNF-syndecan-3 complex could activate Src signaling pathway directly (Bespualov et al., 2011). Taken together, the interaction of GLPs and heparan sulfates is of high physiological relevance and potential therapeutic value, but further studies are needed to understand this phenomenon in more detail.

5. **Vps10p domain receptors (sortilins)**

The mammalian Vps10p domain receptor family includes sortilin, sorting protein related receptor with A-type repeats (SorLA), sortilin-related receptor CNS expressed 1 (SorCS1), SorCS2 and SorCS3 (Figure 10). The name vps10p is short for vacuolar protein sorting 10 protein identified in *Saccharomyces cerevisiae* (baker’s yeast) by Scott Emr and coworkers as a protein responsible for the correct sorting of
carboxypeptidase Y to vacuoles (Marcusson et al., 1994). Sortilins are type-1 transmembrane receptors that contain a highly conserved vps10p-domain (vps10p-D) at their N-termini, but differently from the protein encoded by the vps10 gene in yeast, there is just one copy of the domain instead of two (Willnow et al., 2008). Sortilin and SorLA were purified from human brain homogenate by receptor-associated protein affinity chromatography, while homologous SorCS1, SorCS2 and SorCS3 were identified by database mining (Jacobsen et al., 1996; Petersen et al., 1997; Hermey et al., 1999, 2004; Rezgaoui et al., 2001).

Sortilin serves as a prototype for the vps10p receptor family as its extracellular domain (ECD) comprises the vps10p-D solely. A crystal structure has been solved for the ECD of sortilin in complex with one of its ligands neurotensin. The analysis of the structural data revealed a novel protein fold made of three structural domains: a ten-bladed oval-shaped β-propeller structure with a conical tunnel inside serving as a ligand binding site, followed by two cysteine-rich domains (CRDs) that interact extensively with the β-propeller segment to stabilize its structure (Quistgaard et al., 2009).

Whereas sortilin most likely cannot change its conformation, SorCS1, SorCS2 and SorCS3 all contain a polycystic kidney disease (PKD) domain and a leucine-rich domain between their Vps10p-D and the transmembrane region (Figure 10). The presence of the leucine-rich repeat suggests a relatively flexible β-sandwich fold common to the E-set superfamily, and it is believed to be important in the formation of protein-protein interactions (Kobe and Kajava, 2001).

SorLa is a large mosaic receptor twice the size of other sortilins that are in the range of 100-130 kDa (Willnow et al., 2008). The extracellular domain of SorLA comprises the vps10p-D followed by epidermal growth factor (EGF) precursor homology domain, a cluster of eleven complement type repeats, and finally six fibronectin type III repeats (Jacobsen et al., 1996) (see Figure 10). The intracellular domain of sortilins is only 40-80 amino acids long and contains several motifs for interaction with cytosolic adaptor molecules that guide the complex trafficking of sortilins between the cell surface and various intracellular compartments (Glerup et al., 2014b).

All sortilins are abundantly expressed by the nervous system but can also be found in non-neuronal tissues, for example, sortilin and SorLa are expressed in both embryonic and adult kidney, lung, and liver. In addition, SorLA has been detected in an adult immune system (Glerup et al., 2014b). SorCS1 and SorCS2, which are mostly expressed in brain, can be found in adult liver, kidney, heart (SorCS1) as well as in adult lung and testis (SorCS2) (Rezgaoui et al., 2001; Hermey et al., 2004). The SorCS3 expression seems to be restricted to the nervous system as it has not been detected elsewhere (Oetjen et al., 2014). Interestingly, hippocampal expression of SorCS1 and SorCS3 but not SorCS2 can be regulated by neural activity, suggesting that they could participate in activity-dependent synaptic changes (Hermey et al., 2004).
During the last decade, sortilins have emerged as key regulators of intracellular protein sorting. Their interactions with neurotrophic factors and their receptors will be reviewed in section 1.5.2 of this thesis. Since sortilins are active both in the nervous system and several non-neuronal tissues, it is not surprising that many of them are linked to neuronal as well as metabolic disorders such as Alzheimer's disease (AD) and obesity. AD is a common neurodegenerative disease causing progressive dementia. The pathological symptoms often found in AD patients brains include neurofibrillary tangles and amyloid plaques. Amyloid plaques are formed when amyloid precursor protein (APP) is cleaved by β-secretase into aggregating neurotoxic Aβ peptide (reviewed by Willnow and Andersen, 2013). SorLA seems to retain APP in the intracellular compartments, thus minimizing the cleavage of the protein and subsequent formation of the toxic peptides (Andersen et al., 2005). Moreover, overexpression of SorCS1 leads to reduced Aβ levels, but the molecular mechanism behind this effect remains to be determined (Reitz et al., 2011).

Sorl1 encoding SorLA protein has also been linked with obesity in both humans and rodents by genome wide association studies (GWAS) (Schmidt et al., 2017). A recent study showed that SorLA knock-out mice were protected from diet-induced obesity due to enhanced thermogenesis in adipose tissue (Whittle et al., 2015). Moreover, soluble SorLA was able to repress thermogenesis by interacting with bone morphogenetic protein (BMP) receptors and inhibiting BMP/TGFβ signaling in adipocytes (Whittle et al., 2015).

Ubiquitously expressed sortilin interacts with a plethora of ligands, including neuropeptide neurotensin, prosaposin, lipoprotein lipase, and morphogen sonic hedgehog (Glerup et al., 2014b; Campbell et al., 2016). Sortilin levels are upregulated in several human cancer cells. Furthermore, in lung cancer cells sortilin is detected in exosomes in complex with TrkB and epidermal growth factor receptor
(EGFR), forming a so-called TES complex (Wilson et al., 2014). According to a recent model, sortilin may regulate the biogenesis of exosomes and secreted TES complex can activate ErbB signaling and modulate the secretion of angiogenic factors (Wilson et al., 2016). The function of SorCS1-3 is less studied, but GWAS suggest that SorCS1 is linked to diabetes and SorCS2 to bipolar disorder, schizophrenia and ADHD attention-deficit/hyperactivity disorder (Clee et al., 2006; Goodarzi et al., 2007; Baum et al., 2008; Ollila et al., 2009; Christoforou et al., 2011; Alemany et al., 2015).

Recent work by Glerup and co-workers confirmed and elucidated the role of SorCS2 in BDNF-dependent hippocampal plasticity (Glerup et al., 2016). In addition, SorCS2 seems to be essential for the proper development of inner ear hair cells (Forge et al., 2017). SorCS3 is detected in postsynaptic densities, where it interacts with a glutamate receptor sorting protein PICK1 (Breiderhoff et al., 2013). Faulty localization of PICK1 in SorCS3 knock-out animals is proposed to lead to altered synaptic plasticity by selective loss of LTD that in turn causes defects in spatial learning and fear memory (Breiderhoff et al., 2013).

5.1. Trafficking of sortilins

The Vps10p receptor family members are synthesized at ER as inactive precursor proteins that undergo proteolytic processing in the TGN to generate active mature proteins. The propeptide is believed to block the binding of possible ligands to the vps10p-D (Quistgaard et al., 2009). This idea may be correct for sortilin, but SorLA contains another binding site within its complement type repeats, and also SorCS3 precursor protein has been shown to interact with proNGF, indicating that these receptors are biologically active before the removal of the propeptide (Westergaard et al., 2005; Andersen et al., 2006).

As the Vps10p protein in yeast has a well-established role in sorting hydrolases towards vacuoles, it was proposed that the vertebrate homologues of Vps10p would have a similar function. Indeed, sortilin and SorLA have been shown to localize mostly intracellularly in perinuclear regions and in the TGN (Jacobsen et al., 2001; Nielsen et al., 2001). In neurons, sortilin and SorLA show a vesicle-like staining in the soma but are also detected in the axons, dendrites and the nerve terminals (Willnow et al., 2008). In the case of SorCS1, differently localized splice isoforms make the trafficking analysis rather complicated: while SorCS1a isoform is almost completely intracellular, other splice variants are localized at the cell surface (Nielsen et al., 2008). SorCS2 and SorCS3 can be found at the TGN as well as at plasma membrane. Sortilin, SorCS1, and SorCS3 can be secreted via the activity-dependent pathway, while SorLA and SorCS2 follow the constitutive secretory pathway (Hermey et al., 2004; Nykjaer et al., 2004; Schmidt et al., 2017). At the cell surface, all sortilins can be shed with different kinetics, and soluble forms are released to the extracellular space (Hermey et al., 2006). It is estimated that up to
of sortilin and SorLA can be found on the cell surface, but the proteins are quickly internalized through clathrin-coated pits to the endosomes.

Trafficking of the sorting receptors is assisted either directly or indirectly by adaptor proteins. Internalization of sortilin is mediated by its tyrosine-based motif (Nielsen et al., 2001), whereas the dileucine motif in the cytoplasmic tail of SorLA is recognized by an adaptor protein 2 (AP-2) (Nielsen et al., 2007). Internalized sortilin and SorLA are transported back to the TGN by retrograde sorting pathways, escaping lysosomal degradation and continue shuttling protein cargo between the TGN and endosomes (Nielsen et al., 2001, 2007; Schmidt et al., 2017). According to the current model, retrograde transport of sortilin and SorLA is assisted by the retromer complex, adaptor protein 1 (AP-1) and phosphofurin acidic cluster sorting protein (PACS1) (Nielsen et al., 2001, 2007; Schmidt et al., 2007). Anterograde transport of the two receptors from the TGN to endosomes is regulated in turn by Golgi-localizing, γ-adaptin ear homology domain, ARF-interacting proteins GGA1 and GGA1, which are monomeric clathrin adaptors, and AP-1 (Jacobsen et al., 2002; Schmidt et al., 2007; Herskowitz et al., 2012). The internalization and intracellular trafficking of SorCS proteins are not as well characterized as those of sortilin and SorLA. All three SorCS homologues contain tyrosine-based motifs similarly to sortilin, and AP-2 mediates the internalization of SorCS1 splice variants and SorCS3 (Nielsen et al., 2008; Oetjen et al., 2014). The adaptor proteins regulating the trafficking of SorCS2 have not been identified so far.

5.2. Interaction of sortilins and neurotrophic factors

Sortilins are perhaps best known for their ability to modulate trafficking and signaling of proneurotrophins. Historically, Anders Nykjaer and colleagues were the first to demonstrate that sortilin mediated proNGF induced neuronal cell death by forming a complex with the p75NTR receptor (Nykjaer et al., 2004). Upon ligand binding, sortilin most likely internalizes proNGF, as sortilin-deficient cells fail to uptake proNGF and do not cause cell death (Nykjaer et al., 2004). Next, proBDNF was shown to induce apoptosis in sympathetic neurons when bound to the same receptor complex (Teng et al., 2005). Interestingly, in addition to internalizing proBDNF from the cell surface in complex with p75NTR, sortilin can also regulate other aspects of BDNF cellular trafficking. First, sortilin binds to the pro-region of BDNF precursor protein and guides it to the regulated secretory pathway (Chen et al., 2005b). Intriguingly, sortilin fails to sort the BDNF Met66 variant to the activity-dependent pathway and these mutant mice display altered hippocampal structure and function and are more anxious compared to the wild-type animals (Chen et al., 2006). Thus, it is tempting to speculate that sortilin-BDNF interaction could serve as a molecular model to explain the effect of BDNF mutant in the etiology of anxiety and other neuropsychiatric diseases. Second, cytoplasmic region of sortilin has been implicated in the lysosomal sorting of mature BDNF (Evans et al., 2011). Last, sortilin can also indirectly modulate neurotrophin signaling by interacting with NTF.
receptors TrkA, TrkB and TrkC, facilitating their anterograde transport (Vaegter et al., 2011).

SorCS2 is the second member of Sortilins that mediates proneurotrophin signaling when co-expressed with p75NTR (Deinhardt et al., 2011; Glerup et al., 2014a). Importantly, SorCS2 processing gives rise to two variants with different functions in neurons and glia: single-chain SorCS2 regulates axon guidance in developing neurons, while two-chain SorCS2 variant conveys an apoptotic signal in peripheral glial cells (Glerup et al., 2014a). Moreover, in the hippocampus, SorCS2 can either weaken or strengthen the synapses, depending on the availability of coreceptors and ligands (Glerup et al., 2016). When in complex with p75, SorCS2 can modulate LTD by interacting with proBDNF or BDNF propeptide; activity-dependent interaction with TrkB results in translocation of the BDNF receptor to postsynaptic densities to maintain synaptic potentiation (LTP) (Glerup et al., 2016).

In contrast to the vast amount of work done characterizing the interaction of neurotrophins and their receptors with sortilins, just a few studies are describing the effects of the sorting receptor family on other neurotrophic factors. First, sortilin was shown to bind to the C-terminal site of CNTF and to internalize it from the cell surface. In addition, sortilin was able to promote CNTF receptor-mediated signal transduction independently of ligand binding (Larsen et al., 2010). Soon after, the results by Geng and colleagues indicated SorLA as a sorting receptor for proGDNF, targeting the ligand to the regulated secretory pathway without affecting GDNF signaling on the cell surface (Geng et al., 2011). A very recent study identified SorLA as a trafficking modulator for interleukin-6 (IL-6) and its receptor (IL-6R) in astrocytes (Larsen and Petersen, 2017).

Further studies are needed to elucidate whether sortilins can also regulate other neurotrophic factors or their receptors. Considering their expression pattern and essential role in the trafficking of secretory proteins, it is tempting to speculate that many interactions are yet to be discovered.
AIMS OF THE STUDY

Although the requirement of GDNF for normal development and homeostasis is well-known, the mechanisms regulating the trafficking of this neurotrophic factor are less studied. In addition, the possible biological function of GDNF precursor protein proGDNF has remained elusive.

The primary goal of this thesis focused on the characterization of cellular regulation of GDNF. The specific aims were to

1) Analyze the secretion mechanism of two GDNF splice isoforms (study I)
2) Determine the enzymes involved in the proteolytic cleavage of GDNF precursor protein proGDNF (study I)
3) Purify recombinant proGDNF and test its biological activity (study II)
4) Characterize the receptor binding properties of proGDNF (study II)
5) Investigate the interaction of a sorting receptor SorLA with GDNF and its receptors GFRα1 and RET (study III)
MATERIALS AND METHODS

Detailed information about materials and methods used in this study can be found in the original articles. Summary of the methods is presented in Table 1, and the methods that I have used or participated in are marked with a *.

Table 1. Methods used in this thesis.

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RESULTS AND DISCUSSION

1. Cellular regulation of two GDNF splice isoforms (I)

In this study, we characterized the expression, cellular localization, and secretion of two GDNF splice isoforms. In addition, we identified the enzymes responsible for the proteolytic cleavage of proGDNF into mature GDNF. Previously, it was known that alternative splicing of Gdnf gives rise to two splice isoforms, called pre-(α)pro-GDNF and pre-(β)pro-GDNF (Suter-Crazzolara and Unsicker, 1994; Trupp et al., 1995). Although additional human transcripts were described more recently (Airavaara et al., 2011), to date, these two remain the best-studied isoforms. The mRNAs of pre-(α)pro-GDNF and pre-(β)pro-GDNF share an identical signal sequence but differ in the length of the pro-region as alternative splicing results in the loss of 26 aa and substitution of 1 aa (Gly25Ala) in the β-form (Grimm et al., 1998) (I, Figure 1, A and B). The mature sequences are shown to be identical for both splice variants. GDNF is known to be secreted from the cells, but whether it is sorted to the constitutive or regulated secretory pathway, has remained unstudied.

1.1. Expression of pre-(α)pro-GDNF and pre-(β)pro-GDNF mRNAs in the brain and developing kidney

GDNF is widely expressed in the nervous system as well as in peripheral tissues. Previous studies have detected different levels of pre-(α)pro-GDNF and pre-(β)pro-GDNF mRNAs in the brain and outside the nervous system (Suter-Crazzolara and Unsicker, 1994; Trupp et al., 1995). Our results confirm the differential expression levels of the two GDNF splice isoforms. We found that pre-(α)pro-GDNF mRNA was the predominant form in embryonic and early postnatal mouse brain (I, Figure 1E) as well as in primary cultures of postnatal hippocampal neurons (I, Figure 1D). In the human adult brain, in contrast, pre-(β)pro-GDNF mRNA was expressed more abundantly (I, Figure 1C). The mRNAs of pre-(α)pro-GDNF and pre-(β)pro-GDNF were also detected in the developing kidney, but the levels decreased around birth (I, Figure 1F). Differently from what has been reported in the literature, we were not able to detect GDNF mRNA in mouse kidney after postnatal day 5 (P5) (Trupp et al., 1995).

1.2. Subcellular localization of pre-(α)pro-GDNF and pre-(β)pro-GDNF in cell lines and primary neurons

NTFs are small secretory proteins that are often expressed and secreted in response to neuronal activity. However, it is difficult to investigate the trafficking mechanisms in vivo, as the levels of endogenous NTFs are usually very low, and, to a large extent, the available antibodies fail to detect them specifically. To overcome
this problem, transiently overexpressed NTFs can be studied, or, alternatively, NTF constructs can be fused with different tags or fluorescent proteins to improve their detection. The drawback of adding tags is the possibility that they may interfere with protein trafficking and affect, for example, secretion either positively or negatively (Piccinini et al., 2013). The chance that a 27kDa green fluorescent protein (GFP) may disrupt the cellular regulation of a 30kDa GDNF dimer is even higher. When characterizing protein trafficking in cell lines, choice of the suitable expression vector is critical. Vectors with strong promoters (i.e., pAAV-MCS) can easily lead to the expression of high amounts of exogenous protein that can fill up the intracellular organelles and give a false view of the localization and processing of the protein.

In this study, we characterized the localization of transfected pre-(α)pro-GDNF and pre-(β)pro-GDNF in NGF-differentiated neuron-like PC6.3 cell line and primary cortical neurons by immunofluorescent staining. Both transiently overexpressed splice variants were detected in the Golgi complex and vesicle-like structures when analyzed either by confocal or immune electron microscope (I, Figures 2 and 3). Interestingly, quantification of splice variant localizations revealed that whereas (α)pro-GDNF was distributed more or less evenly between Golgi and the vesicles, the majority (~80%) of the protein encoded by pre-(β)pro-GDNF was found in vesicle-like structures of the PC6-3 cells (I, Figure 2B). In rat cortical neurons, the two splice isoforms co-localized partly with the Golgi marker GM130 (I, Figure 3B). Furthermore, they were detected both in axons and dendrites, and antibodies specific for either the mature region or the pro-segment of GDNF showed similar vesicle-like staining patterns (I, Figure 3, A and C). This finding suggests that GDNF precursor protein or the cleaved pro-region together with mature GDNF can be transported to the cell periphery in primary neurons (I, Figure 3C).

1.3. Processing and secretion of GDNF precursor proteins

GDNF is synthesized as a preproprotein. The signal sequence that is cleaved off after the protein synthesis targets the nascent polypeptide to the ER, but the role of the pro-region has not been studied extensively. Also, it is not known, which enzymes are involved in the proteolytic processing of proGDNF into mature GDNF. In the case of neurotrophins, members of the proprotein convertase family have been shown to cleave off the pro-segment of the proneurotrophins (Seidah et al., 1996a, 1996b). In addition, plasmin and selective matrix metalloproteinases are involved in the proteolytic processing of secreted proneurotrophins (Lee et al., 2001; Mizoguchi et al., 2011).

In order to determine, which enzymes are responsible for the cleavage of the GDNF precursor form, we expressed wild-type pre-(α)pro-GDNF and pre-(β)pro-GDNF cDNAs in CHO and NGF-differentiated PC6.3 cells in the presence or absence of furin endoproteinase inhibitor and GM6001, which is a broad-spectrum MMP inhibitor (I, Figure 7). In neuron-like PC6.3 cells, also the effect of α2 anti-plasmin was studied. WB analysis demonstrated that while GM6001 and anti-plasmin did
not prevent the cleavage of proGDNFs, furin inhibitor reduced the processing to some extent. To further analyze the role of furin and other members of the mammalian proprotein convertase (PC) family in the proteolytic processing of proGDNF, we utilized a Lovo cell-line deficient in endogenous furin activity (Takahashi et al., 1993b). Co-expression of human proGDNFs with members of the PC family proteins revealed that in addition to furin, PACE4, PC5A, PC7, and, to a lesser extent, PC5B can efficiently cleave pro-GDNF into mature GDNF (I, Figure 7E). Notably, different PCs are activated in various subcellular organelles, furin and PC7 can cleave proteins already in the TGN, but PACE4 and PC5A are secreted constitutively and can be detected from the cell surface (Seidah et al., 2008). Consequently, removal of the GDNF pro-domain can take place either in the TGN or upon secretion. Further studies are required to characterize the GDNF processing mechanisms in more detail.

To analyze whether GDNF can be secreted as a precursor form, we raised an anti-pro-GDNF peptide antibody that recognizes the pro-regions of both (α)pro-GDNF and (β)pro-GDNF (I, supplemental Figure S1). Next, pre-(α)pro-GDNF and pre-(β)pro-GDNF cDNAs were transiently expressed in CHO and PC6.3 cells, and subsequently detected by immunoblotting or immunofluorescence analysis (I, Figures 4 and 5). Using antibodies against the pro-region and the mature-part of GDNF, we were able to demonstrate that the two isoforms are secreted from cell lines in both pro- and mature forms (I, Figure 4). Furthermore, by mutating the putative furin cleavage site, we were able to prevent the cleavage of the precursor form substantially and increase the secretion of proGDNF variants (I, Figure 6, C and D). This finding is in accordance with a previous report where mutation of the furin cleavage site was shown to enhance the secretion of the unprocessed GDNF form (Oh-hashi et al., 2009). Notably, we cloned another GDNF mutant with six critical positively charged amino acids changed to alanines (named GDNF(6A) in study I). This mutated GDNF was efficiently secreted to the cell media and almost completely resistant to proteolytic cleavage (Figure 6, C and D). This construct was later used for the production of recombinant proGDNF characterized in study II.

1.4. GDNF splice isoforms respond differently to KCl-induced depolarization in neuronal-like cells

There are two main secretory pathways in mammalian cells, the constitutive pathway and the activity-dependent pathway. In the constitutive pathway, present in all mammalian cells, small secretory vesicles transport the cargo to the cell membrane and release it by default, ensuring a constant supply of the protein. The regulatory pathway is characterized by larger dense core vesicles (DCV) that are activated in response to stimuli (i.e., electrical activity) and require calcium to release the content of the secretory granules (Leßmann and Brigadski, 2009). The secretion mechanisms of neurotrophins have been studied in detail, and the results show that neurotrophins can utilize both secretory pathways. For instance, NGF and
NT4/5 are mainly localized to the vesicles of the constitutive pathway but can also be secreted along the regulated pathway (Leßmann and Brigadski, 2009). BDNF and NT-3, in turn, are secreted predominantly via the activity-dependent pathway in response to KCl-induced depolarization in neuronal cells (Mowla et al., 1999; Chen et al., 2004; Brigadski et al., 2005).

To investigate, which is the preferred secretory route of the proteins encoded by pre-(α)pro-GDNF and pre-(β)pro-GDNF, we utilized the NGF-differentiated PC6.3 cells and analyzed the secretion of proGDNFs upon depolarization by KCl. Western blot analysis of PC6.3 media showed that while the secretion of (α)pro-GDNF/GDNF did not change much after KCl treatment, the amount of (β)pro-GDNF/GDNF in the media was clearly increased (I, Figure 8A). This result was also confirmed by GDNF ELISA (I, Figure 8B). To assess whether the KCl-dependent secretion of (β)pro-GDNF/GDNF was dependent on calcium, we used a calcium chelator BAPTA-AM. The presence of BAPTA-AM prevented the secretion of KCl-induced (β)pro-GDNF/GDNF as well as pro-BDNF-EGFP/BDNF-EGFP (positive control) but did not affect the secretion of (α)pro-GDNF/GDNF (I, Figure 8B).

In addition to analyzing the media samples, we also studied the effect of KCl on the intracellular localization of the two GDNF splice variants. The results show that (β)pro-GDNF/GDNF were detected predominantly in the secretory vesicles positive for secretogranin II (SgII) and Rab3A, which are markers of the regulated secretory pathway (I, Figure 9, supplemental Figure S5). Moreover, the areas with strongest co-localization of (β)pro-GDNF/GDNF and SgII were found in the distal regions of the extensions (I, Figure 9A). In contrast, the co-localization of (α)pro-GDNF/GDNF with SgII was weak before and after KCl treatment and the two proteins co-localized most strongly in the proximal areas of cells (I, Figure 9, A and C). Taken together, these results suggest that the secretory mechanisms of proteins encoding pre-(α)pro-GDNF and pre-(β)pro-GDNF are different. The (β)pro-GDNF and corresponding GDNF are transported out of the neuronal cell primarily in dense core secretory granules of the regulated pathway, and the secretion can be upregulated calcium-dependently by KCl-induced depolarization. The secretion of (α)pro-GDNF and corresponding GDNF, in turn, seems to occur predominantly via the constitutive secretory pathway and is not significantly enhanced in response to depolarization. Our results differ from those of Wang et al., where (β)pro-GDNF was shown to accumulate in the Golgi and to be poorly secreted in comparison to the protein encoding pre-(α)pro-GDNF. The discrepancy could be explained by the fact that the other group studied C-terminally hemagglutinin (HA)-tagged cDNAs, whereas we used wild-type GDNF cDNAs. Even small tags may interfere with the proper sorting and trafficking of GDNF, especially when linked to the C-terminal region, which is known to be critical for binding GFRα receptors and activating RET (Eketjall et al., 1999; Baloh et al., 2000; Parkash et al., 2008).
2. The biological role and binding profile of proGDNF

As mentioned before, GDNF is synthesized as a precursor protein, but the possible function of the pro-form has remained unstudied. Earlier work indicates that the GDNF pro-region may be required for proper folding of the nascent polypeptide and it is involved, though not critical, for the secretion of GDNF protein (Grimm et al., 1998; Piccinini et al., 2013). We and others have shown, that proGDNF can be secreted, at least in cell lines and in primary astrocytes (Study I, Piccinini et al., 2013; Sun et al., 2014). Furthermore, it is possible to detect endogenous proGDNF precursor protein from the media of human glioblastoma cells (U87 MG) using modified GDNF-ELISA (II, Suppl. Fig. S3), but the amount is not sufficient for proper characterization of the protein.

To date, most information concerning the role of the neurotrophic factor precursors comes from the work on proneurotrophins. In contrast to the mature neurotrophins, proneurotrophins have been shown to elicit mainly apoptotic signals (Lee et al., 2001; Teng et al., 2005; Yano et al., 2009). Nevertheless, recent work suggests that the function of proneurotrophins can be regulated by the availability of signaling receptors and in the presence of TrkA, proNGF may also possess neurotrophic activity (Clewes et al., 2008; Masoudi et al., 2009; Ioannou and Fahnestock, 2017).

In the second study of my thesis, we focused on the characterization of the recombinant mammalian proGDNF mutant protein, resistant to proteolytic cleavage. Our primary goal was to assess whether proGDNF is a biologically active protein and determine its receptor binding profile.

2.1. Characterization of the proGDNF properties and purification

In study I, we characterized the processing of GDNF precursor protein and showed that members of the proprotein convertase family cleaved proGDNF into mature GDNF (see section 1.3 of Results and discussion). Moreover, in order to produce an uncleavable proGDNF mutant protein, we cloned a construct, where six basic amino acids located at the furin cleavage site and positions 11, 12 downstream of the furin site were mutated to alanines. Expression of this construct (called proGDNF6A in study II) resulted in enhanced secretion of GDNF pro-form, and, therefore, we chose to characterize it further. To confirm earlier findings, the expression of proGDNF6A and GDNF wt was compared in HEK293T cells and analyzed by immunoblotting using antibodies against the mature as well as the pro-domain of GDNF (II, Figure 1B). While GDNF wt was secreted primarily in the mature form, the molecular mass of the secreted proGDNF6A suggested that it was resistant to endogenous proteases and remained in the pro-form. Notably, when cells were kept in serum-containing medium (full medium), the secretion of both GDNF wt and proGDNF6A was enhanced. This result is in accordance with an earlier report where
GDNF stability was shown to be affected by the extracellular environment (Piccinini et al., 2013).

Next, we analyzed the heparin binding affinity of proGDNF6A. As the N-terminal region of the mature GDNF is proposed to be critical for binding heparin and heparan sulfates (Alfano et al., 2007; Bespalov et al., 2011), we hypothesized that proGDNF6A displays weaker binding to heparin. Indeed, proGDNF6A was eluted from the heparin affinity column with 0.4-0.7 M NaCl, while elution of GDNF wt that was used as a control, only started upon the addition of 0.7 M NaCl (II, Figure 1C). Since it has been demonstrated that mammalian GDNF is more stable than GDNF produced in the bacterial expression system (Piccinini et al., 2013), proGDNF6A was subsequently expressed in CHO cells using a patented QMCF Technology developed by Icosagen Ltd. Analysis of the CHO media showed that in addition to proGDNF6A, there was also some recombinant mature GDNF present, indicating that proGDNF6A underwent proteolytic processing to a small extent. Since we introduced a mutation of two arginines to alanines at position 11, 12 of the GDNF mature region, the recombinant mammalian GDNF was called GDNF2A to distinguish it from wt mature GDNF that was also used in this study. Using three chromatography methods (heparin affinity column, size-exclusion chromatography, and cation-exchange column), and taking advantage of the slightly different biochemical properties of proGDNF6A and GDNF2A, we managed to separate fractions that contained primarily proGDNF6A. The purity of the proteins was verified by reverse phase chromatography (data not shown) and SDS-PAGE stained with Coomassie Brilliant Blue (II, Figure 1D). Taken together, this is the first report on the purification of a recombinant, mainly cleavage-resistant proGDNF mutant protein, which also shows reduced heparin affinity.

2.2. Testing the biological activity of proGDNF6A *in vitro*

GDNF is a well-known survival factor for several neuronal populations, including a subset of sympathetic neurons. To assess, whether the recombinant proGDNF6A is biologically active, we tested its effect on postnatal rat superior cervical ganglion cultures in comparison with GDNF2A, and NGF, which is the most potent survival factor for these neurons. To our surprise, proGDNF6A displayed trophic activity, and supported the survival of the SCG sympathetic neurons dose-dependently, although it was not as efficient as GDNF2A (II, Figure 2A). Interestingly, we did not observe a significant additive effect when GDNF2A and proGDNF6A were applied together to primary neurons, suggesting that GDNF and proGDNF might signal via the same receptor pool that was saturated by the addition of exogenous ligands (II, Figure 2B).

Next, we tested the effect of GDNF2A and proGDNF6A in tissue culture. For that, we used mouse embryonic kidney explants. It has been reported that exogenous mature GDNF can induce extra ureteric budding as well as enlargement of the definitive ureteric tips (Sainio et al., 1997). After dissecting the explants, 5 nM
of recombinant proGDNF6A or GDNF2A was added to the medium, and the tissue cultures were maintained for 48 h. The presence of both proteins lead to the induction of extra ureteric buds in comparison to control tissues grown in the absence of GDNF (II, Figure 2C). Similar results were obtained when using 2.5 nM or 3.75 nM ligands (II, Suppl. Fig S1) Importantly, results of this assay also demonstrated the relative stability of recombinant proGDNF6A, as it was not cleaved during the 48 h culturing period (II, Figure 2D). Based on this result we can conclude that the observed morphogenic effect was the result of proGDNF signaling and not caused by the cleavage of the prodomain and subsequent activity of the mature GDNF.

These results indicate that the activity of the GDNF precursor form is very different from that of proneurotrophins, which are able to cause neuronal apoptosis. However, it does not rule out the possibility that proGDNF6A may possess dual activity, for instance, in the case of neuronal injury, or during aging.

2.3. Receptor binding properties of proGDNF6A

As proGDNF6A displayed trophic activity in the in vitro assays, we speculated that it could bind to GFRα1 and signal via RET. It is known from the literature that proNGF can interact with the TrkA receptor, although with slightly reduced affinity compared to the mature NGF (Clewes et al., 2008). To characterize the binding of proGDNF to GFRα1, we used two approaches. Results of the microscale thermophoresis (MST) analysis showed that proGDNF6A bound to GFRα1 with the $K_d \approx 90 \text{ nM}$ (II, Figure 3A). Taking into consideration that the experiment was conducted using soluble proteins, this affinity is similar to what has been reported for mature GDNF-GFRα1 interaction. Importantly, in the MST settings, we did not detect any binding of proGDNF6A to the RET ECD (II, Figure 3A) that is in line with earlier data showing that mature GDNF does not bind directly to RET either.

Next, we radiolabeled proGDNF6A and GDNF2A with $^{125}$I and characterized the binding abilities of the ligands in more detail in the cellular competition binding assays. Human GFRα1 and GFRα2 were transiently expressed on the surface of HEK cells, and radiolabeled proGDNF6A or GDNF2A were added to the cells in the presence or absence of unlabeled ligands. Results of the binding studies confirmed the initial result obtained with MST: proGDNF6A bound to GFRα1 with high affinity (II, Figure 3B). Furthermore, using GFRα1 mutant proteins deficient in GDNF binding, we were able to demonstrate that the proGDNF binding site is identical to that of mature GDNF (II, Figure 3C). This finding was next verified by showing that unlabeled GDNF efficiently displaced $^{125}$I-proGDNF6A binding from GFRα1 and vice versa (II, Figure 3D). All in all, this result is not very surprising. Compared to neurotrophins that all possess propeptides of almost the same length as the mature region, and may thus sterically hinder the binding sites located in the mature domain, the pro-segment of GDNF is less than half the length of the mature region. Therefore, it is not likely that it could physically block the C-terminal part (fingers)
of the mature GDNF that is required for GFRα1 binding (Baloh et al., 2000; Parkash et al., 2008).

Next, we wanted to study whether the iodinated ligands share similar affinity also to GFRα2. Unexpectedly, we were unable to detect binding of proGDNF6A to GFRα2, while mature GDNF2A clearly bound to the receptor (II, Figure 4, A and B). In line with the initial findings, unlabeled proGDNF displaced GDNF in cells expressing GFRα1 but not GFRα2, not even when the unlabeled protein was added in concentrations as high as 100 nM (II, Figure 4B).

Results from earlier studies show that the transmembrane RET receptor can stabilize the interaction of GDNF and its cognate GFRα1 receptor (Cik, 2000; Leppänen et al., 2004). Indeed, when we transfected HEK cells with the GFRα-receptors together with RET, the binding affinity of both proGDNF6A and GDNF2A to GFRα1 increased (II, Figure 4, C and D). The same effect was evidenced in the case of GDNF and GFRα2. Notably, proGDNF6A displayed a weak affinity to GFRα2 in the presence of RET and was also able to displace GDNF in binding to the complex comprising GFRα2 and RET (II, Figure 4, E and F). Together, these results suggest that the receptor binding properties of proGDNF6A are slightly different from the mature GDNF. While both ligands interact with GFRα1 in a similar manner, their affinity to GFRα2 differs significantly.

2.4. RET activation mechanism by proGDNF6A

A unifying characteristic feature of the proneurotrophins is their ability to bind to the p75NTR. As GFLs signal via a receptor complex consisting of a GPI-linked GFRα receptor and RET receptor tyrosine kinase, we wanted to test, whether GDNF precursor form could possibly bind to, or activate RET. Although the initial MST analysis indicated that proGDNF6A does not bind to the RET ECD, its ability to interact with a transmembrane receptor may be different. The analysis was carried out in fibroblasts stably expressing wt full-length RET, and we measured the ability of GDNF2A and proGDNF6A to phosphorylate RET in the presence and absence of soluble GFRα1. The results demonstrate that neither GDNF2A nor PROGDNF6A can activate RET alone. However, in the presence of soluble GFRα1, both ligands induced phosphorylation of RET (II, Figure 5A).

Next, we analyzed the time-dependence of RET activation in MG87RET cells transiently expressing the GFRα1 receptor. GDNF2A and proGDNF6A were added to serum-starved cells that were subsequently lysed 15, 30, 45, or 60 minutes later, and subjected to immunoprecipitation. In short, we did not see a difference in the phosphorylation pattern of the RET receptor, indicating that GDNF2A and proGDNF6A can activate RET to the same extent both in the presence of soluble and transmembrane GFRα1 (II, Figure 5B).

RET is known to contain several phosphorylation sites that lead to the activation of different signaling pathways (see Figure 9 of the literature review). To monitor, which pathway is induced by GDNF and proGDNF, we utilized a MAPK
activation detection system, developed and characterized by Sidorova and colleagues (Sidorova et al., 2010). In StrataαLuc cells, which express RET and GFRα1, GDNF2A and proGDNF6A showed similar efficiency in activating MAPK pathway (II, Figure 5C). In contrast, when the ligands were added to the NoStrataαLuc cells with soluble GFRα2, we observed a dose-dependent MAPK activation with GDNF2A, while proGDNF6A had a significantly weaker effect (II, Figure 5C). As this phenomenon might have been a result of a prolonged incubation time of the samples, leading to the degradation of proGDNF6A, we used an alternative method and analyzed the MG87RET cells, induced for 1 h with soluble ligands and co-receptors. In line with the results obtained using the MAPK-luciferase system, we saw that GDNF2A and proGDNF6A induced RET phosphorylation and consequent activation of the MAPK as well as AKT pathway in the presence of soluble GFRα1 (II, Figure 5D). However, proGDNF failed to activate RET and the downstream signaling molecules when applied with soluble GFRα2. Notably, proGDNF seemed to bind weakly also to a GFRα1 mutant protein lacking domain 1, as evidenced by a modest RET phosphorylation level. Domain 1 is proposed to be necessary for the stabilization of the ligand-receptor complex (Virtanen et al., 2005).

Taken together, these results suggest that proGDNF6A can activate RET and downstream MAPK and AKT signaling pathways in the presence of wt GFRα1. However, the interaction is not as stable as that of mature GDNF and GFRα1. Differently from mature GDNF, proGDNF6A is not able to induce RET phosphorylation together with soluble GFRα2. This finding may help to explain the results of the assays where biological activity of proGDNF6A was assessed (II, Figure 2 A-C). Neonatal SCG neurons express higher levels of GFRα2 than GFRα1 (Nishino et al., 1999), and may thus respond better to mature GDNF. The embryonic kidney explants, on the other hand, express solely GFRα1, which both ligands seem to activate to a similar extent.

### 2.5. Internalization of proGDNF6A

Finally, we compared the internalization rate of proGDNF and GDNF, using radiolabeled ligands and HEK293T cells transiently expressed with i) RET, ii) GFRα1, iii) RET+GFRα1, or iv) RET+GFRα1+SorLA. Iodinated proGDNF6A and wt recombinant GDNF were added to the cells and incubated on ice for 2 h. Subsequently, the plates were moved to a 37° C water bath, and the rate of the endocytosis was measured during a 40 minute period. The specificity of the ligand uptake was verified by the addition of unlabeled ligand in the longest time-point of the assay.

The results show that GDNF and proGDNF6A are not endocytosed by RET, but GFRα1 can mediate the ligand uptake in a very effective manner (II, Figure 6, A and B). The latter finding confirms the results presented in an earlier report (Vieira et al., 2003). Perhaps surprisingly, in this experimental set-up, the rate of ligand
internalization was not enhanced in cells expressing RET+GFRα1, or RET+GFRα1 +SorLA compared to cells expressing only GFRα1 (II, Figure 6, C and D).

Further analysis of the internalization is required to fully understand the kinetics of GDNF uptake, and the role of different GDNF receptors in this process. Regarding the comparison of GDNF and proGDNF6A internalization, we can conclude that both ligands are endocytosed possibly via a similar mechanism in vitro.

3. Interaction of SorLA with GDNF and its receptors

The vps10p-domain sorting receptor family members have emerged as important regulators of intracellular trafficking. The five receptors (sortilin, SorLA, SorCS1, SorCS2, and SorCS3) are abundantly expressed in the nervous system as well as non-neuronal tissues like kidney, lung, and heart (Willnow et al., 2008). In the NTF field, sortilin and SorCS2 have been identified as co-receptors for p75NTR and this receptor complex has been shown to mediate proneurotrophin signaling (Glerup et al., 2014b). In addition, a recent report identified SorLA as a trafficking regulator for interleukin-6 and its receptor in astrocytes (Larsen and Petersen, 2017). In this study, we analyzed the interaction of SorLA with GDNF and its receptors GFRα1 and RET and characterized the phenotype of SorLA-deficient mice.

3.1. Determination of the ligand-receptor binding site

Vps10p-domain receptors interact with their ligands mostly via the N-terminal vps10p-domain (Willnow et al., 2008). SorLA is the largest member of the receptor family, in addition to the vps10-p domain, its ECD contains an EGF precursor homology domain, a cluster of eleven complement type repeats, and six fibronectin type III repeats (Jacobsen et al., 1996) (III, Figure 1D). Using surface plasmon resonance (SPR) analysis, we compared the binding of all GFLs to the ECD of SorLA. The results revealed that SorLA bound with high affinity to GDNF but not to NRTN, ARTN or PSPN (III, Figure 1, A and B). It is known that GDNF is the only member of the GFLs with an N-terminal extension, and, to assess, whether this region was necessary for the binding, we produced a glutathione-S-transferase (GST) fusion peptide comprising GDNF propeptide and the N-terminal part of mature GDNF sequence (aa 20-115). This peptide did indeed bind to SorLA, although with lower affinity compared to mature GDNF (III, Figure 1C). Importantly, two other peptides encompassing the pro-region of GDNF or NRTN solely, failed to bind to SorLA, indicating that the N-terminal part is needed for the binding to take place. Thus, our results differ from a previous report claiming that SorLA interacts with the pro-region of (α)pro-GDNF (Geng et al., 2011).

To determine, whether the vps10p-domain is required for the interaction of SorLA and GDNF, the SPR analysis was conducted in the presence of excess SorLA
pro-peptide or small neuropeptide neurotensin, both of which are known to block the tunnel-like cavity of the vps10-p domain and prevent other ligands from binding. GDNF did not bind to SorLA in these assays, suggesting that the interaction of the two proteins is mediated by the N-terminal extension of GDNF and the vps10p-domain of SorLA (III, supplemental Figure S1A and S1B). As the vps10p-domain is highly conserved among the sorting receptor family, it would be interesting to test in future studies, whether other members of the vps10-p domain receptors can also interact with GDNF.

3.2. Internalization of GDNF by the SorLA-GFRα1 receptor complex

After characterizing the SorLA-GDNF binding by SPR and also in cells (III, supplemental Figure S1C), we were interested, whether SorLA can internalize GDNF from the cell surface. Using HEK293 cells, we showed that SorLA was able to internalize GDNF (III, Figure 1E). While the rate of endocytosis was not affected by the presence of RET receptor, cells expressing both SorLA and GFRα1 internalized GDNF more efficiently than SorLA alone (III, Figure 1E). To understand this phenomenon, SorLA and GFRα1 were co-immunoprecipitated (co-IP) and shown to form a complex both in the presence and absence of GDNF (III, Figure 2A). Subsequent SPR analysis revealed that the two receptors bind directly to each other with very high affinity (Kd ≈ 6 nM) (III, Figure 2B, and supplemental Figure S3A). Interestingly, SorLA was able to bind to GDNF and GFRα1 simultaneously with the same affinity as was seen in the individual binding experiments, indicating the possible formation of a ternary complex (III, supplemental Figure S3D).

After internalization, GDNF did not stay in complex with the two receptors, but was co-localized with an early endosome marker (EEA1) and degraded within an hour (III, Figure 1, F and G). Inhibition of lysosomes by leupeptin and pepstatin restored GDNF immunoreactivity (III, Figure 1G). The combined data demonstrate that extracellular GDNF is efficiently endocytosed by the complex of SorLA and GFRα1 and targeted to the lysosomes for degradation.

3.3. Regulation of GFRα1 by SorLA in cell lines and primary neurons

Next, we investigated whether SorLA affects GFRα1 trafficking. First, we compared the rate of internalization, and consequent cellular localization of GFRα1 expressed in the presence or absence of SorLA. In HEK293 cells expressing GFRα1 alone, we did not observe significant endocytosis of this GPI-linked receptor (III, Figure 2C). In the presence of SorLA, in contrast, GFRα1 was efficiently internalized in 45 minutes with and without GDNF (III, Figure 2C), and both receptors co-localized partly with the trans-Golgi marker TGN46 (III, Figure 2F). Furthermore, immunoelectron microscopy analysis revealed that SorLa and GFRα1 co-localized also in early endosomes and multivesicular bodies (MVBs) (III, Figure 2G). Second, we measured the stability of metabolically labeled GFRα1 and found that SorLA
prolonged the half-life of GFRα1 (III, Figure 2E; supplemental Figure S3, G and H). Together, these results indicate that differently from GDNF, SorLA does not target GFRα1 to lysosomes.

In addition to characterizing the interaction of SorLA and GFRα1, we were interested whether SorLA can also interact with other members of the GFRα receptor family. Co-IP analysis revealed that SorLA can indeed form a complex with GFRα2, GFRα3, and GFRα4, and trigger their internalization similarly to GFRα1 (III, supplemental Figure S4). Hence, SorLA seems to be a general sorting receptor of the GFRα protein family, and future studies will hopefully uncover the functional relevance of the other interactions. It would be interesting to assess, for instance, whether SorLA can modulate GFL signaling by regulating the availability of the GFRα receptors at the cell surface.

In addition to characterizing the sorting of GFRα1 in HEK293 cell line, we wanted to investigate whether SorLA has similar effects on the GDNF receptor in neurons. For this purpose, we utilized primary hippocampal (HC) neurons that express substantial levels of endogenous GFRα1 and SorLA, but not the RET receptor (III, Figure 5C). Comparison of GFRα1 levels and localization in HC cultures from wild-type or SorLA knock-out animals indicated that SorLA can indeed control the intracellular localization of GFRα1 in primary neurons. While the total levels of GFRα1 were not altered in KO neurons, the protein was detected primarily at the cell surface close to soma and the initial segment of filaments (III, Figure 5, F and E). In accordance, surface-localized GFRα1 was able to bind GDNF, but it failed to internalize the ligand efficiently in the absence of SorLA (III, Figure 5H). In conclusion, these results suggest that SorLA can mediate the endocytosis of GDNF/GFRα1 similarly both in HEK293 cells and in primary neurons.

### 3.4. Inhibition of GDNF signaling as a result of RET endocytosis by the SorLA-GFRα1 receptor complex

In order to study if SorLA can regulate the RET receptor tyrosine kinase regarding its trafficking and interaction with GFRα1, we started by performing co-IP analyses. The pull-down experiments demonstrated that SorLA does not inhibit the complex formation of GFRα1 and RET. On the contrary, in the presence of SorLA, the RET-GFRα1 interaction seemed to increase slightly (III, Figure 3A). Notably, in these experiments, GFRα1 pulled down only the immature 150 kDa form of RET that is detected intracellularly. Next, we showed that RET, in turn, had a positive effect on the SorLA-GFRα1 co-IP (III, Figure 3A).

To analyze whether SorLA is able to regulate the trafficking of RET, we used antibodies recognizing the ECD of RET and monitored the internalization of RET in HEK293 cells transfected with RET alone, RET and GFRα1, RET and SorLA, or RET with SorLA and GFRα1 (III, Figure 3, B and C). Interestingly, RET was efficiently internalized only in the presence of both SorLA and GFRα1 and subsequently detected in early endosomes (III, Figure 3D).
Results of the experiments described above suggested that SorLA could have an impact on GDNF signaling. To investigate the possible effects in more detail, we used a neuroblastoma cell line Sy5y, which expresses RET, GFRα1, and SorLA endogenously. We were able to demonstrate that the phosphorylation levels of GDNF-induced Erk and Akt increased as a result of using blocking antibodies for SorLA, and consequent inhibition of GDNF/GFRα1 endocytosis (III, Figure 4A). Moreover, by inhibiting endogenous SorLA by its propeptide, we saw an increase in the cell survival that was comparable to the effect seen upon adding exogenous GDNF to the cells (III, Figure 4C).

In addition to modulating cell survival, GDNF can also affect the proliferation and differentiation of SY5Y cells. We showed that SY5Y cells overexpressing SorLA proliferate more slowly (III, Figure 4F). Furthermore, the ability of GDNF to induce neurite outgrowth was significantly reduced in those cells. Importantly, the excess of SorLA did not block the retinoic acid-induced neurite outgrowth that utilizes a different signaling pathway (III, Figure 4G). Taken together, these results suggest that SorLA can inhibit the neurotrophic function of GDNF most likely by regulating the levels of the ligand and controlling the subcellular localization of the GDNF receptors.

3.5. Characterization of SorLA knock-out animals

SorLA is widely expressed in the CNS of both young and old mice (III, Figure 5A). Notably, it is also detected in the midbrain and striatum. Analysis of primary dopamine (DA) neuron cultures revealed that SorLA is expressed by the DA neurons as well as by the supporting cortical glial cells (III, Figure 6, A and B). In line with our previous findings, we were able to demonstrate that SorLA mediated the uptake of GDNF to glial cells (III, Figure 6D) and in SorLA KO animals, GFRα1 levels were increased on the cell surface of DA neurons (III, Figure 6G). We also showed that SorLA significantly inhibited GDNF-induced survival of DA neurons (III, Figure 6H).

To assess whether SorLA can regulate GDNF levels in vivo, we used the striatum and midbrain homogenates of wild-type and Sorl1 knock-out animals. Results of the ELISA analysis showed that GDNF levels were significantly increased both in striatum and midbrain of SorLA KO mice (III, Figure 6K). Previous work demonstrates that elevated expression of GDNF protein negatively regulates kidney morphogenesis (Kumar et al., 2015). Therefore, it was tempting to speculate that GDNF excess could potentially disturb also the normal functioning of the DA system. This question is relevant also in the light of a very recent publication where GDNF was proposed to modulate striatal DA homeostasis (Kopra et al., 2017).

The overall appearance of the midbrain DA system was indistinguishable in SorLA KO and wt mice (III, supplemental Figure S7A). Furthermore, in SNpc and VTA, the number of tyrosine hydroxylase (TH)-positive neurons, as well as the length of TH+ nerve fiber projecting to the striatum and nucleus accumbens,
respectively, were similar (III, Figure 7A-D). Nevertheless, we did observe a significant difference in the function of the nigrostriatal pathway, accompanied by reduced striatal TH protein levels, slightly reduced DA levels, and no response to amphetamine in SorLA-deficient animals (III, Figure 7E-J).

Next, SorLA KO mice were tested in different behavioral assays. The mice were characterized by hyperactivity, as they traveled almost twice the distance in comparison with the wt controls when placed in an open field for 40 minutes (III, Figure 7I). Moreover, animals lacking SorLA did not respond to amphetamine, most likely due to the perturbed activity of the dopamine transporter (DAT) in DA neurons. When tested in the elevated plus maze, SorLA knock-outs spent more time in the open arms than the control mice, displaying reduced anxiety (III, Figure 7K-M). Interestingly, analysis of GDNF heterozygote animals in the same paradigm revealed that these mice demonstrated increased anxiety, as evident from fewer entries and less time spent in the open arms (III, Figure 7, N and O). Together, these results suggest that SorLA is an important regulator of GDNF signaling in vivo. The characteristic features of the SorLA-deficient mice may reflect the effect of increased GDNF activity on the functionality of the DA system. Moreover, SorLA may be a novel target for future studies aiming to understand better the molecular mechanisms underlying anxiety and ADHD.
The goal of this thesis was to characterize the cellular regulation of GDNF, and to elucidate the putative function of GDNF precursor protein proGDNF. It is known that similarly to other NTFs, GDNF is secreted from the cells. We showed that the two GDNF splice isoforms seem to be regulated differently in terms of their cellular localization and secretory route (I). Our studies on GDNF processing demonstrate that several members of the PC family can cleave proGDNF into mature GDNF (I), interestingly, some of which are localized at the cell surface. Also, we found that both proGDNF variants can be secreted from cell lines (I, II).

Characterization of the recombinant proGDNF revealed that the precursor protein is biologically active and can elicit typical receptor tyrosine kinase downstream signaling pathways via the receptor complex comprising of GFRα1 and RET (II). Notably, proGDNF is not as effective as mature GDNF, and its receptor binding profile differs from that of the mature form when signaling via GFRα2. Importantly, this is the first time that biological functions of the GDNF precursor protein are reported. Further studies are required to investigate the role of proGDNF in aging and pathological conditions.

Finally, we identified SorLA as an alternative sorting receptor for GDNF and its receptors (III). Results of our studies propose an important role for SorLA in the regulation of GDNF signaling both in vitro and in vivo.

The main conclusions from this work are:

I. (β)pro-GDNF, but not (α)pro-GDNF, is secreted activity-dependently in neuronal-like PC6 cells

II. GDNF precursor protein proGDNF is proteolytically cleaved into mature GDNF by furin, PACE4, PC5A, PC5B and PC7, but not by matrix metalloproteinases or plasmin.

III. Recombinant proGDNF is biologically active; it can support the survival of sympathetic neurons and induce ureteric budding in embryonic kidney explants.

IV. ProGDNF can signal through GFRα1 and RET but, unlike mature GDNF, it does not signal via GFRα2.

V. SorLA is a novel sorting receptor for GDNF and a co-receptor for GFRα1. SorLA modulates GDNF signaling by targeting GDNF to lysosomal degradation, while internalized GFRα1 is recycled back to the cell membrane.

VI. SorLA knock-out mice are hyperactive and respond abnormally to amphetamine. These features are most likely caused by increased striatal GDNF levels leading to perturbed dopaminergic functionality.
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