Role of GDNF and its Cross-Talk with Other Growth Factors in the Dopaminergic System

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Role of GDNF and its Cross-Talk with Other Growth Factors in the Dopaminergic System
ROLE OF GDNF AND ITS CROSS-TALK WITH OTHER GROWTH FACTORS IN THE DOPAMINERGIC SYSTEM

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ACADEMIC DISSERTATION

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Helsinki 2012
“Tuleb taotleda suurimat, et saavutada suuremat”

F.Tuglas
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Selected Abbreviations

6-OHDA 6-hydroxydopamine
ARTN  artemin
BBB  blood-brain barrier
BDNF  brain-derived neurotrophic factor
CDNF  cerebral dopamine neurotrophic factor
CNS  central nervous system
CNTF  ciliary neurotrophic factor
CPu  caudate putamen
DA  dopamine
DAT  dopamine transporter
ERK  extracellular signal-regulated kinase
FGF  fibroblast growth factor
GDNF Hz  glial cell line-derived neurotrophic factor heterozygous mouse
GDNF  glial cell line-derived neurotrophic factor
GFAP  glial fibrillary acidic protein
GFL  GDNF family ligand
GFRα  GDNF family receptor alpha
ICV  intracerebroventricular
IGF-1  insulin-like growth factor 1
IL-6  interleukin 6
L-DOPA  L-3,4-dihydroxyphenylalanine, levodopa
LIF  leukemia inhibitory factor
M/+  heterozygous knock-in Ret-MEN2B mouse
M/M  homozygous knock-in Ret-MEN2B mouse
MANF  mesencephalic astrocyte-derived neurotrophic factor
MEN2A  multiple endocrine neoplasia type 2 A
MEN2B  multiple endocrine neoplasia type 2 B
MPTP  1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NCAM  neural cell adhesion molecule
NGF  nerve growth factor
NRP  neuropilin
NRTN  neurturin
NT-3  neurotrophin-3
NT-4  neurotrophin-4
NTF  neurotrophic factor
PD  Parkinson's disease
pERK  phosphorylated extracellular signal-regulated kinase
qRT-PCR  quantitative reverse transcriptase polymerase chain reaction
RECA-1  rat endothelial cell antibody-1
RET        rearranged during transfection
SN         substantia nigra
STR        striatum
TH         tyrosine hydroxylase
VEGF-A     vascular endothelial growth factor A
VEGF-C     vascular endothelial growth factor C
VEGFR      vascular endothelial growth factor receptor
Wt         wild type littermates
VTA        ventral tegmental area
List of original publications

This thesis is based on the following publications, which are referred in the text by Roman numerals and on unpublished results presented in the text.


*Equal contribution to the publication.

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Abstract

Parkinson’s Disease (PD) is a neurodegenerative movement disorder resulting from loss of dopaminergic (DA) neurons in substantia nigra (SN). Possible causative treatment strategies for PD include neurotrophic factors, which protect and in some cases restore the function of dopaminergic neurons. Glial cell line-derived neurotrophic factor (GDNF) family of neurotrophic factors have been to date the most promising candidates for treatment of PD, demonstrating both neuroprotective and neurorestorative properties.

We have investigated the role of GDNF in the rodent dopaminergic system and its possible crosstalk with other growth factors. We characterized the GDNF-induced gene expression changes by DNA microarray analysis in different neuronal systems, including in vitro cultured Neuro2A cells treated with GDNF, as well as midbrains from GDNF heterozygous (Hz) knockout mice. These microarray experiments, resulted in the identification of GDNF-induced genes, which were also confirmed by other methods. Further analysis of the dopaminergic system of GDNF Hz mice demonstrated about 40% reduction in GDNF levels, revealed increased intracellular dopamine concentrations and FosB/DeltaFosB expression in striatal areas. These animals did not show any significant changes in behavioural analysis of acute and repeated cocaine administration on locomotor activity, nor did they exhibit any changes in dopamine output following treatment with acute cocaine.

We further analysed the significance of GDNF receptor RET signalling in the dopaminergic system of MEN2B knock-in animals with constitutively active Ret. The MEN2B animals showed a robust increase in extracellular dopamine and its metabolite levels in striatum, increased tyrosine hydroxylase (TH) and dopamine transporter (DAT) protein levels by immunohistochemical staining and Western blotting, as well as increased Th mRNA levels in SN. MEN2B mice had increased number of DA neurons in SN by about 25% and they also exhibited increased sensitivity to the stimulatory effects of cocaine.

We also developed a semi-throughput in vitro micro-island assay for the quantification of neuronal survival and TH levels by computer-assisted methodology from limited amounts of tissue. This assay can be applied for the initial screening for dopaminotrophic molecules, as well as for chemical drug library screening. It is applicable to any neuronal system for the screening of neurotrophic molecules.

Since our microarray experiments revealed possible GDNF-VEGF-C crosstalk we further concentrated on studying the neurotrophic effects of VEGF-C. We showed that VEGF-C acts as a neurotrophic molecule for the DA neurons both in vitro and in vivo, however without additive effect when used together with GDNF. The neuroprotective effect for VEGF-C in vivo in rat 6-OHDA model of PD was demonstrated. The possible signalling mechanisms of VEGF-C in the nervous system were investigated - infusion of VEGF-C to rat brain induced ERK activation, however no direct activation of RET signalling in vitro was found. VEGF-C treatment of rat striatum lead to up-regulation of
VEGFR-1-3, indicating that VEGF-C can regulate the expression level of its own receptor. VEGF-C dopaminotrophic activity *in vivo* was further supported by increased vascular tissue in the neuroprotection experiments.
1. REVIEW OF THE LITERATURE

1.1 Brain dopaminergic system

Dopamine is one of the main monoaminergic neurotransmitters in the central nervous system. The mesodiencephalic dopaminergic (mDA) neurons – responsible for the major source of dopamine in the brain - reside in the ventral mesodiencephalon. mDA neurons can be further divided into anatomically and functionally different subgroups (Fig.1).

The majority of dopaminergic neuron cell bodies reside in the ventral midbrain consisting of substantia nigra pars compacta (SNpc, A9 region) with axons projecting to the dorsal striatum: consisting of caudate nucleus (medially) and putamen (laterally) which are divided by the internal capsule white matter and connected by ventrally lying fundus. These structures form the nigrostriatal dopaminergic system. The dorsal striatum receives afferent inputs from the cortex, from the substantia nigra and sends efferent input to both external and internal globus pallidus, SNpc and pars reticulata of SN (SNpr) via GABAergic neurons. Only 3-5% of the total neurons in the SN are dopaminergic neurons. Although their numbers are low, these neurons are strongly associated with motor function and voluntary movement (Kandel 2000; Chinta and Andersen 2005).

Medial to the nigrostriatal system lay the mesolimbical and mesocortical dopaminergic systems, which arise from

![Figure 1. Schematic presentation of human midbrain dopaminergic system. Author: Maarja Kotkas, modified by Anu Planken.](image-url)
the ventral tegmental area (VTA, A10) and project to nucleus accumbens/ olfactory bulb (also referred to as the ventral striatum) and cortex, respectively. These systems are thought to be responsible for behavioural processes such as mood, motivation, reward, addiction and stress (Kandel 2000; Chinta and Andersen 2005).

Dopaminergic neurons have also significant functions outside the nervous system, having central roles in cardiovascular, renal, enteric and hormonal functioning in both humans and rodents.

1.1.1 Development of the midbrain dopaminergic system

Dopaminergic cell development is complex and involves a series of processes and molecules. It can be further divided into early developmental events, such as regional specification, neuronal specification and differentiation. These initial events are influenced by both extrinsic and intrinsic signals. Extrinsic signals from fibroblast growth factor-8 (FGF-8), sonic hedgehog (Shh) and transforming growth factor-β2 and β3, along with Wnt1 and Wnt5a induce the action of internal transcription factors like Nurr1, engrailed-1 and -2, Pitx3, and members of the LIM homeobox family (Lmx1a and Lmx1b). The result is the generation of post-mitotic young neurons from mitotic cells, with fate of becoming fully differentiated mesencephalic dopamine neurons (Smits, Burbach et al. 2006; Smidt and Burbach 2007; Andressoo and Saarma 2008).

Later developmental events include the migration and path-finding of young mDA neurons, neurite outgrowth, guidance, pruning, synapse formation, connectivity and maintenance of the dopaminergic system. These events involve a wide variety of molecular clues, central players being ephrins, netrins, semaphorins, plexins, neuropilins and Slits and their cognate receptors (Smits, Burbach et al. 2006; Smidt and Burbach 2007; Andressoo and Saarma 2008). In addition, other molecules necessary for formation and maintenance of mDA tracts include: the neurotrophic factors – GDNF, neurturin (NRTN), mesencephalic astrocyte-derived neurotrophic factor (MANF), cerebral dopamine neurotrophic factor (CDNF); morphogens –Wnt-1 and -8, bone morphogenetic protein 4 and 7 (BMP-4, -7), Shh; transcription factors – engrailed-1/2; proteoglycans; leucine rich-repeat proteins and neurotransmitters (Smits, Burbach et al. 2006; Smidt and Burbach 2007; Andressoo and Saarma 2008; Van den Heuvel and Pasterkamp 2008).

The classic principle of neural development states that neurons are created in excess during embryogenesis, they compete for limited amounts of neurotrophic factors and the neurons which fail to receive neurotrophic support undergo embryonic or postnatal cell death. The developmental cell death of midbrain DA neurons is considered to be biphasic - the first phase of apoptosis in rats starts at embryonic day 20 (E20) and ends by postnatal day (P) 8-12 and the second peak is initiated at P14 and ceased by P28 (Burke 2004). Knowledge about the molecular mechanism of these two phases is limited, although the first phase is likely due to the limited supply of factors in the striatal target tissue, however no clear expla-
nation for the second phase exists (Burke 2004). Although there is some evidence that GDNF may be involved in the regulation of the number of nigral DA neurons, further studies on conditional knockout (cKO) animals are needed to clarify this point. In addition other factors, such as NRTN, CDNF and MANF may play important roles here.

1.2 Parkinson’s disease

1.2.1 Etiopathogenesis of Parkinson’s disease

Parkinson’s disease - characterized by progressive degeneration of the dopaminergic system - affects approximately 1% of the population over 60 years of age and about 5% of the population over 85. PD is primarily sporadic, however rare familial genetic forms exist in about 10% of PD cases. Candidate genes linked to genetic PD include α-synuclein, leucine rich repeat kinase 2 (LRRK2), Parkin, phosphatase and tensin homolog (PTEN)-induced novel kinase 1 (PINK1), DJ-1 and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) and several other genes (Moore, West et al. 2005). The aetiology of sporadic PD is unknown, possibly considered to result from gene-environment interactions. According to the „double hit hypothesis“ PD is unlikely caused by a single factor, but rather results from an interaction between multiple genetic mutations and/or the combination of a mutant gene and an environmental toxin (Olanow and Tatton 1999).

Early symptoms of PD include constipation and REM sleep behaviour disorder, which are considered to be the initial symptoms developing already 10 years before onset of disease. The anatomic substrate for this disorder lies within the brainstem. Olfactory dysfunction and impaired sense of smell are also considered to be the early signs of PD. These symptoms do not improve with dopamine replenishment therapy and thus could be considered to result from an insult to non-dopaminergic cells, likely to the noradrenergic, sympathetic and sensory neurons. The motor triad leading to diagnosis of PD includes resting tremor, rigidity and bradykinesia. In later disease stages also non-motoric symptoms of autonomic, cognitive and psychiatric disturbances develop. Advanced symptoms like L-dopa refractory motor symptoms, dementia, axial symptoms, imbalance, gait problems and dysarthria do not reflect dopaminergic pathology, but rather spreading of proteinaceous intracellular inclusions - Lewy Bodies (LB) beyond the nigrostriatal system, as there is a gradual loss of L-dopa response over the years. Dementia is present in 12 to 41% of PD cases and develops in majority of patients prior to death (Ahlskog 2005).

The neuro-pathology of PD is characterized by selective degeneration of dopaminergic neurons in the SNpc. Although the exact mechanism of neurodegeneration in PD is unknown, metabolic compromise, excitotoxicity and oxidative stress are considered to be the central lethal triplet responsible for causing apoptotic neuronal cell death (Olanow 1999; Alexi, Borlongan et al. 2000). The pathogenesis has been proposed to involve oxidative and nitrite stress, excitotoxicity, inflammation, mitochondrial dysfunction and altered proteolysis. These processes are part of interrelated cascades
that lead to neuron death through apoptosis. It remains unclear which process is the initial primary leading cause and which is the result of the progression of disease (Jenner and Olanow 2006). The hallmarks of illness are the presence of LB in surviving cells and occurrence of reactive microgliosis and to a lesser extent astrocystosis (Jenner and Olanow 2006). Although LB and α-synuclein (α-syn) pathology is present in many neurons, the extensive (80%) cell death is present only in SN dopaminergic and locus coruleus noradrenergic neurons (Jenner and Olanow 2006).

α-Syn is an intracellular protein widely expressed by the nervous system in presynaptic terminals and nuclear envelope, however the exact function of this protein is unknown. The monomeric forms of α-syn are considered to play a role in dopamine synthesis and content, as well as synaptic vesicle functioning and dopamine release (Venda, Cragg et al. 2010). The formation of the oligomeric and larger aggregated polymeric forms of α-syn are considered to be pathologic and are the main components of LB in PD. Protein misfolding and aggregation are normal processes in a protein’s life, however the dysbalance between the processes of protein synthesis, folding and clearance is considered to be central in the generation of pathogenic forms of α-syn (Lee, Desplats et al. 2010; Olanow and McNaught 2011). The imbalance of increased production and impaired clearance lead to a vicious cycle of protein accumulation, which interferes further with lysosomal and proteasomal clearance mechanisms. This causes accumulation of pathologic proteins and formation of LB inclusions, which impair cell defense mechanisms and critical cellular processes, and finally lead to neurodegeneration (Olanow and McNaught 2011).

Heiko Braak and colleagues suggest the “dual-hit hypothesis” as the pathogenic mechanism initiating PD, whereby a neurotropic pathogen (possibly viral) acts simultaneously (a) through the nasal route via anterograde transport through olfactory bulb, anterior olfactory nucleus to olfactory structures of the temporal lobe; and (b) via gastrointestinal route through submucosal axons of the Meissner’s plexus, followed by trans-synaptic transmission to the parasympathetic neurons of the vagal nerve and then through retrograde transport to the medulla, pons and further to midbrain (Braak, Del Tredici et al. 2003; Braak, Ghebremedhin et al. 2004; Hawkes, Del Tredici et al. 2009). This hypothesis of transport and spread of a neurotropic pathogen from peripheral organs to the CNS, is further supported by recent findings on PD patients transplanted with fetal mesencephalic grafts over a decade ago, where α-synuclein containing LBs and LB morphology had spread, via unknown mechanisms, to the previously healthy grafted embryonic dopaminergic neurons (Brundin, Li et al. 2008; Kordower, Chu et al. 2008; Olanow, Kordower et al. 2009; Olanow and Prusiner 2009). These findings suggest that the progression of the disease and synucleinopathy could be the result of a “prion-like” toxic mechanism, whereby the affected neurons of the host transfer the misfolded α-synuclein protein to unaffected healthy grafted neurons (Angot, Steiner et al. 2010; Olanow and McNaught 2011).
Six clinical stages for PD have been described by Braak. 1st and 2nd stage - the pre-symptomatic phase - are characterized by inclusion body pathology in medulla oblongata/pontine tegmentum and olfactory bulb/anterior olfactory nucleus. During the 3rd and 4th stages the SN and other nuclear gray areas in midbrain and forebrain are affected and symptoms appear and develop. During the 5th and 6th stage the mature neocortex is involved, and the disease manifests (Braak, Del Tredici et al. 2003; Braak, Ghebremedhin et al. 2004). A proportion of cases do not fit this scheme, as it is based only on detection of synucleinopathy and not on neuronal loss, although it is likely that α-synucleinopathy precedes cell death. Criticism on Braak’s staging scheme questions the correlation between the stages and clinical severity of the disease, as some Braak 6 stage patients have no motor symptoms at all and some stage 2 patients have intensive motor symptoms. Also synuclein pathology cannot be regarded as a prognostic factor, as some individuals present with synucleinopathy but no PD can be diagnosed. (Burke, Dauer et al. 2008)

1.2.2 Diagnosis of Parkinson’s disease
One of the major problems associated with PD is the lack of early efficient diagnosis. Current diagnostic methods allow for the detection of the disease only when the first symptoms are evident. By this stage about 70% of DA neurons in the SNpc have been lost. Moreover, first symptoms appear when the levels of striatal dopamine are reduced by at least 80%. Clinical diagnosis of PD is based on TRAP symptomatology – Tremor, Rigidity, Akinesia and Postural instability. Response to levodopa treatment is considered to confirm the clinical diagnosis of PD. Magnetic resonance imaging and positron emission tomography scans can assist in further diagnosis. The Unified Parkinson’s Disease Rating Scale (UPDRS) is utilized in assessment of PD clinical status, disability and impairment (Poewe 2009). The evaluation of safety and effect of a potential therapeutic substance in clinical trials of PD is based on the UPDRS score (Poewe 2009).

1.2.3 Therapeutic approaches to Parkinson’s disease
The therapeutic approaches to PD include symptomatic and disease modifying - neuroprotective and neurorestorative - treatments. Symptomatic therapy options include levodopa, dopamine agonist, combination therapies and surgical treatment (Savitt, Dawson et al. 2006). The disease modifying therapeutic approaches for PD have mainly focused on promoting neuronal survival and function, interference with neurotoxic mechanisms, as well as cellular replacement strategies (Table 1). A vast number of compounds have been tested including neurotrophic factors, anti-excitotoxins, immunosuppressants and -modulators, antioxidants, bioenergetic supplements and anti-apoptotic molecules (Meissner, Frasier et al. 2011). The most promising therapeutic targets for rescuing degenerating neurons are the neurotrophic factors. They influence cell survival, proliferation, differentiation, biochemical functioning, as well as neuronal plasticity (Peterson and Nutt 2008). Of particular relevance
in future PD therapies are GDNF family members: GDNF and NRTN (Airaksinen, Titievsky et al. 1999; Airaksinen and Sairma 2002). They have been shown to possess neuroprotective and neurorestorative effects on dopaminergic neurons, both in vitro and in vivo experimental models of PD (Lin, Doherty et al. 1993; Burke, Antonelli et al. 1998; Zurn, Widmer et al. 2001). Other promising candidates in the aspect of disease modifying options include MANF (Lindholm, Peranen et al. 2008; Lindholm and Saarma 2010), CDNF (Lindholm, Voutilainen et al. 2007; Lindholm and Saarma 2010) and vascular endothelial growth factor A (VEGF-A) (Yasuhara, Shingo et al. 2005), all having neurorestorative effects on DA neurons in animal models of PD.

Swedish company Neuro Nova has developed a novel drug candidate (sNN0031) for the treatment of moderate to severe stage Parkinson’s disease, that is designed to act on neural stem and progenitor cells in the brain. In animal models of Parkinson’s disease, treatment with sNN0031 restores motor function and improves neurochemical deficits. The product is comprised of the naturally occurring protein PDGF-BB (platelet-derived growth factor BB) formulated for intracerebroventricular (ICV) delivery (www.neuronova.com).

Another exciting strategy to treat PD is stem cell therapy, which holds promise to one day become an effective and routine approach in the clinic (Arenas 2010). Currently two approaches of stem cell therapy are being investigated for PD: the embryonic stem cells and adult stem cells. Embryonic stem cell therapy is limited due to ethical and tumorigenic risk issues, thus an alternative approach could be the use of adult-derived stem cells including bone-marrow derived hematopoietic mesenchymal cells, adipose, cord or placenta derived stem cells. The adult stem cells can be utilized in cell replacement therapies to enhance neurogenesis and they hold promise in the future replacement therapy for PD. They do not possess ethical problems, are autologous - thus no immunogenicity problems arise, they can be personalized for a specific patient and as low number of passages are used, no problems with tumour development exist (Arenas 2010).

The drug discovery field of disease-modifying therapy for Parkinson’s disease is progressing rapidly and extensive preclinical results are being generated, however technical, clinical safety and efficacy issues for these different approaches remain to be solved. Due to the heterogeneous nature of Parkinson’s disease, a single agent is unlikely to be clinically effective and rather a combination of multiple drugs, targeting different pathways of disease, will provide clinical benefit for the patients in the future.

1.2.4 Animal models of Parkinson’s disease

PD models have been developed to assist in understanding the pathogenic processes, molecular and biochemical pathways affected in PD and for testing possible therapeutic approaches. In principle these models should resemble the characteristic features seen in PD patients, including normal number of DA neurons at birth and onset of progressive neurodegeneration in late-adulthood, loss of DA neurons leading to symptoms of motor
dysfunction, including L-dopa responsive movement disorder – bradykinesia, rigidity and resting tremor (Beal 2001; Orth and Tabrizi 2003; Dawson, Ko et al. 2010). In addition Lewy body-like inclusions should be present. The model should be followable in a relatively short time for rapid screening and should be cost-effective (Beal 2001; Orth and Tabrizi 2003; Dawson, Ko et al. 2010). Since the exact aetiology of PD is unknown and the pathogenesis is likely to be multifactorial and heterogeneous, the establishment of disease models, displaying all features of clinical PD, has been difficult. The existing models of PD could be further classified into two broad categories: toxin-induced models and genetic animal models. Three main toxin-induced models have been described: 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) and rotenone. The correlation and resemblance of different animal toxin models to the clinical PD in humans depends on the location of the lesion, percentage of dopamine depletion in the striatum and the behavioural test which are used to evalu-
ate the extent of the lesion (Deumens, Blokland et al. 2002).

The 6-OHDA was the first described toxin model for PD. Injection of 6-OHDA to SN leads to selective accumulation of the toxin in DA neurons, resulting in dopaminergic cell death due to oxidative stress. In rats the lesion can be followed by behavioural analysis of rotational activity in response to amphetamine or apomorphine. Despite the visible motor deficit, there are no LB-like inclusions present (Beal 2001; Orth and Tabrizi 2003). Systemically administered 6-OHDA does not cross the BBB, thus stereotactical intracranial delivery of the toxin has to be utilized. Several different 6-OHDA lesions have been described based on the site of the lesion, the extent of nigrostriatal damage and dopamine depletion. In rats at least 80% DA depletion is required to overcome compensatory dopaminergic mechanisms which can complicate the interpretation of effects (Deumens, Blokland et al. 2002).

In general, three sites of lesion are mostly used, with both unilateral and bilateral injections. Injection of 6-OHDA to the medial forebrain bundle (MFB) – the most widely used model - causes almost total destruction of the SN and VTA neurons and leads to total depletion of DA in the CPu, super-sensitivity of postsynaptic DA receptors in the CPu and characteristic turning behaviour in response to D-amphetamine and apomorphine (Deumens, Blokland et al. 2002). This model resembles most the advanced stages of PD. More selective injection of 6-OHDA to the nigrostriatal part of the MFB produces a milder and clinically more relevant lesion, however achievement of this is technically demanding (Deumens, Blokland et al. 2002). The second approach is the injection of 6-OHDA to the SN of rats, which leads to 88% cell loss in the SN, however also 30% cells loss in VTA neurons occurs. Injection to this area is also technically difficult, due to the small size of the SN (Deumens, Blokland et al. 2002). The third site of injection, considered to be the best accessible and reproducible site, is the dorsal striatum. Injection to the ventrolateral or dorsomedial site of STR leads to either cell loss only in the SN or both SN and VTA, respectively (Deumens, Blokland et al. 2002). The partial 6-OHDA striatal toxin model represents milder and delayed degeneration of the SN neurons. Standardized injection to this site causes selective destruction of DA axons and terminals around the injection site, leading to protracted cell death of the accompanying DA cell bodies. This model closely resembles the early stages of PD in humans (Björklund, Rosenblad et al. 1997).

In general, the 6-OHDA striatal lesion is possibly the best model, as it is initiated by synaptic and retrograde degeneration of axons leading to neuronal cell death, recapitulating most the process of PD pathology in humans. Using higher doses of toxin or multiple injection sites, the extent and severity of the lesion can be regulated. In the case of toxin injection to SN or MFB the pathology does not correlate very well to the human PD, as the initial degeneration of neuronal cell bodies is rapid and severe, with axon degeneration being rather a secondary event. In conclusion, the 6-OHDA model does not resemble completely the pathology and symptomatology of human PD, due to the
Review of the Literature

acute non-progressive nature of insult, lack of LB pathology and tremor - the cardinal symptom in disease, as well as lack of other non-dopaminergic systems and brain areas - characteristic to late stages of disease (Schober 2004).

The most convenient and best described PD model is the MPTP toxin model. MPTP rapidly crosses the BBB and is converted to its active metabolite - 1-methyl-4-phenylpyridinium (MPP\(^+\)) by a catecholamine degrading enzyme - monoamine oxidase B. MPP\(^+\) is then transported into the cells by the dopamine transporter, where it inhibits the complex I of the mitochondrial respiratory chain or is taken up by the vesicular monoamine transporter to the synaptic vesicles. The MPTP model highly resembles the classical features of PD, but differs due to the acute onset of the process and due to the lack of LBs (Beal 2001; Orth and Tabrizi 2003). The MPTP model has been well established also in primate PD studies. The neurotoxic doses of MPTP in primates are up to 50-fold lower than in mice, due to differences in sensitivity. In primates the MPTP-induced lesion is long-lasting and permanent and can be treated with L-DOPA. The motor defects in primates resemble the PD symptoms in man, most prominently akinesia and rigidity and seldom hand tremor (Gerlach and Riederer 1996). Evaluation and comparison of the clinical defect in primate MPTP studies is a question of concern, as several different clinical rating scores (CRS) have been described for evaluation of the toxin model and therapy effect (Imbert, Bezard et al. 2000). In conclusion, controversy exists in regard to MPTP being a good model representing human PD, as in mice it does not induce nigral cell loss, but rather represents a model of dopaminergic dedifferentiation due to loss of dopaminergic phenotype. In addition the nature of the insult is acute and non-progressive, other non-dopaminergic systems are uninvolved and there is lack of LB pathology.

The third toxin model of PD is based on a naturally occurring compound - rotenone, used widely as a pesticide. Following intravenous infusion, rotenone is transported to the intracellular compartment, where it inhibits the mitochondrial complex I. This highlights the selective vulnerability of the nigral neurons to oxidative stress as rotenone inhibits the complex I mechanism uniformly in all brain cells (Beal 2001; Orth and Tabrizi 2003). Although it has been thought that rotenone is poorly taken up by the gastrointestinal tract, recently it was shown that intragastrical administration of rotenone causes PD-like symptomatology in mice. This process models the human pathological staging of PD and supports the alternative trans-synaptic toxin uptake mechanism, whereby enteric neurons facilitate the spread of environmental neurotoxic molecules and thus contribute to the development of PD (Pan-Montojo, Anichtchik et al. 2010). The rotenone model meets most of the criteria for an excellent model of PD, including motor deficits, selective degeneration of SN neurons, as well as presence of LB-like inclusions, however variability issues in individual rat susceptibility have been reported (Beal 2001; Orth and Tabrizi 2003).

An interesting model, in regard to the inflammatory aspect of PD pathology, is the lipopolysaccharide model (LPS). LPS
is an endotoxin derived from gram-negative bacteria, which induces stimulation of both peripheral immune cells and CNS glial cells (especially microglia) and leads to the release of immunoregulatory molecules and free radicals. The LPS model causes indirect inflammation-mediated neurodegeneration (Dutta, Zhang et al. 2008), which can be useful in modelling the human disease as PD is also accompanied by neuroinflammation.

Several animal models representing the genetic basis of PD have been developed, including the α-synuclein, DJ-1, PINK-1, Parkin, LRRK2 and mitopark mouse models (Burke 2007). Transgene over-expressing mutants have been generated for the autosomal-dominant genes α-synuclein and LRRK2, as well as knockout models for autosomal-recessive genetic forms: Parkin, DJ-1 and PINK-1. Although these models exhibit several useful features for studying PD pathology, such as presence of LB morphology in most of the models, they are limited due to lack of meaningful DA neurodegeneration, vague motor symptomatology, and slow progression of pathology (Dawson, Ko et al. 2010; Meissner, Frasier et al. 2011). The artificial MitoPark mouse model targets the mitochondrial dysfunction aspect in PD pathology and cell type-specific inactivation of mitochondrial transcription factor A leads to adult-onset, progressive, severe DA cell death accompanied by motor deficits responding to L-DOPA treatment (Ekstrand and Galter 2009). Recently a mouse model lacking the DJ-1 gene in all cells and RET receptor selectively in the DA neurons has been generated, showing accelerated selective age-associated loss of SN cell bodies, as compared to either single mutant alone (Aron, Klein et al. 2010).

In a more recent study LRRK2 overexpression together with A53T mutation in α-synuclein rendered significant neurodegeneration and cell loss in SNpc (Lin, Parisiadou et al. 2009). Recently, a model with targeted overexpression of human wild-type α-synuclein in the nigrostriatal system, using adeno-associated viral vectors, has been established. Overexpression of α-syn causes progressive degeneration of the nigral dopamine neurons and the development of axonal pathology in the striatum (Decressac, Ulusoy et al. 2011).

The genetic mouse models are the most versatile of the PD animal models as different systemic aspects can be studied, they exhibit α-synuclein pathology and some motor defects, but the aggregation of α-synuclein is independent of progressive neurodegeneration and there is no α-synuclein pathology present in the brainstem and nucleus vagus - the postulated early locations of PD pathology and spread (Dawson, Ko et al. 2010). In conclusion, no ideal animal model for PD exists, they all exhibit benefits and limitations and the selection of a suitable model should depend on the question under consideration.

1.3 Neurotrophic factors

Neurotrophic factors have significant physiological roles in neuronal survival, functioning and maintenance of the nervous system, from development to adulthood. They induce growth, differentiation and survival of neurons, as well as neuronal progenitor cells. Since the discovery of nerve growth factor (NGF) by Levi-
Montalcini more than 50 years ago (Levi-Montalcini and Cohen 1956; Levi-Montalcini 1987) the classical „target-derived“ neurotrophic factor hypothesis has been well established (Fig. 2). The concept states that during neural development neurons are over-produced in excess amounts, the presynaptic neurons compete for limited amounts of growth factors synthesized by post-synaptic targets, which determine the final number of neurons able to make synaptic connections in tissues, while the neurons not receiving neurotrophic support are removed by programmed cell death (Oppenheim 1991).

However, at present the NTF interactions are considered to be much more complex. A single NTF influences a variety of neuronal types and non-neuronal cells, certain types of neurons can be dependent on multiple different neurotrophic factors, different NTFs show overlapping patterns of activities through convergence of intracellular signal transduction pathways, or sharing of receptors and subunits (Korschning 1993). In CNS the trophic factors are mostly synthesized by neurons and glial cells, and they act in both autocrine and paracrine fashion. Traditionally the NTFs have been considered to be produced and released by the target cells, internalized through the nerve terminals via specific receptors and the ligand-receptor complex within the endosomes is transported retrogradely to the cell body where the signalling

**Figure 2.** Schematic presentation of the target-derived neurotrophic factor (NTF) hypothesis, stating that during development neurons are produced in excess amounts. Axons of developing neurons grow toward and compete for limited amounts of NTFs secreted by target tissues. The neurons which achieve support are able to make synaptic connections in tissues, while neurons which fail to receive support degenerate and are removed by apoptosis. Author: Maarja Kotkas.
mechanisms are initiated. The transport mechanisms seem more complex and also anterograde axonal transport has been described. Strictly retrograde signalling has been shown for NGF, other NTFs such as BDNF, IGF-I, FGF-2 and GDNF can be transported both retrogradely and anterogradely to and from target neurons, as well as through neurons by transcytosis, indicating that the same NTF can be utilized by more than one neuron or glial cell (von Bartheld, Wang et al. 2001). In addition, the availability and interconnections of different NTFs seem to be more complex, as recycling, storage and pools of factors have been described, in addition to de novo synthesis of NTFs upon need (von Bartheld, Wang et al. 2001).

Classically three families of neurotrophic factors have been characterized: the neurotrophins, GDNF family ligands (GFLs) and the neurotrophic cytokines. Recently a new class of potent neurotrophic factors has been discovered: the CDNF/MANF family of neurotrophic factors (Lindholm and Saarma 2010). However, other growth factors like members of the FGF family, IGF family, TGF-β family and VEGF-A, have been shown to possess neurotrophic properties.

Members of the neurotrophin family include the first characterized neurotrophic factor – NGF, BDNF and neurotrophins-3, -4 (NT-3,-4). They function in maintaining viability of sensory and sympathetic neurons of the PNS and several types of CNS neurons, like basal forebrain and striatal cholinergic, cortical, and retinal ganglion cells as well as precursor and neural crest cells during development (Huang and Reichardt 2001).

The neurotrophic factors have been characterized to have a central role in the immune system, have been shown to act also in the nervous system. The main neurotrophic cytokines are leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and interleukin-6 (IL-6). During development they regulate self-renewal of neural stem cells and determine the cell fate, participate in the developmental switch from neurogenesis to gliogenesis and in differentiation of astrocytes from neural progenitors (Bauer, Kerr et al. 2007). Neuropeptide cytokines regulate the survival of embryonic sensory neurons and most importantly postnatal spinal motor neurons. They have been shown to play a role in injury processes like peripheral nerve and spinal cord injury, autoimmune demyelinating disorders, as well as modulation of endogenous repair processes. They function both directly and indirectly by up-regulating the expression of neuropeptides and other neurotrophic factors (Bauer, Kerr et al. 2007).

The newest class of neurotrophic factors is the CDNF/MANF family. These two factors have been shown to have dual roles as extracellular NTFs and endoplasmatic reticulum resident cytoprotective proteins. Great interest has arisen due to their ability to promote the survival of midbrain dopamine neurons. MANF and CDNF have been shown to have neuroprotective and neurorestorative properties in animal models of Parkinson’s disease. These factors may function as target-derived NTFs for mDA neurons, but analysis of the respective knockout animals is needed to answer this question (Lindholm and Saarma; Lindholm, Voutilainen Review of the Literature

In adulthood the focus of NTFs is shifted from target-derived survival to the regulation of neuronal phenotype and function. In normal adult brain they have been shown to modulate long-term potentiation (LTP), learning and memory. NTFs function in the modulation of neuronal plasticity, influencing cell body size, dendritic arborization, terminal sprouting, gene expression and also in adult maintenance of neurons. They also function in neuroprotection and neural repair in response to axotomy, excitotoxicity, toxins and other types of injury and insults, such as ischemia, trauma and degenerative diseases (Sofroniew, Howe et al. 2001). As NTFs have been shown to influence neuroprotection, neurorestoration and biological functioning, there has been great interest in the therapeutic potential of these factors on various diseases.

1.4 GDNF family ligands

1.4.1 Expression and functions

GDNF family is comprised of four members: GDNF, NRTN, artemin (ARTN) and persephin (PSPN) (Airaksinen and Sarrma 2002; Smidt and Burbach 2007). The GFLs are distant members of the TGF-β superfamily, being characterized by seven conserved cysteins with identical spacing and homodimeric cysteine-knot proteins with similar spatial structure. They function as secreted proteins and are synthesized in the form of prepro-precursors. They bind to specific co-receptors known as GDNF-family receptor-α (GFRα) and signal through common transmembrane receptor tyrosine kinase RET (Rear-

Figure 3. Schematic presentation of the GDNF family of neurotrophic growth factors and receptors. Reprinted by permission from Macmillan Publishers Ltd. (Nature Reviews Neuroscience 3, 383-394) Copyright © 2002.
ranged during Transfection). The ligand specificity of GFLs is determined by binding to four different GFRα receptors: GFRα1 for GDNF, GFRα2 for NRTN, GFRα3 for ARTN and GFRα4 for PSPN. Crosstalk between the ligands and receptors has been shown for NRTN, ARTN and PSPN to GFRα1 and GDNF to GFRα2 and GFRα3 (Fig. 3).

GFLs have diverse expression patterns in the CNS and PNS. They function in the maintenance of sympathetic, parasympathetic, enteric, somatic sensory, cortical, motor and dopaminergic neuronal systems (Airaksinen and Saarma 2002).

In sympathetic ganglia (SG) RET receptor expression is widespread at embryonic day E11.5 and by birth RET, GFRα2 and GFRα3 expression is restricted to a subpopulation of sympathetic neurons. RET knockout (KO) mice exhibit 30% reduction in sympathetic ganglia size and misplacement of superior sympathetic ganglia (SCG), due to defective precursor migration to ganglionic site and increased apoptosis during development. ARTN and GFRα3 KO mice have reduced cervical and thoracic ganglia size and displacement. GFRα3 mutant mice have 50% cell loss in SCG neurons at birth due to defects in migration, proliferation and survival. NRTN and GFRα2 mutant mice have serious defects in the parasympathetic nervous system, but no significant changes in the sensory or sympathetic phenotype (Rossi, Luukko et al. 1999). Ret and GFRα3 mutant mice also have defects in neurite outgrowth (Ernsberger 2008).

GDNF is a target-derived survival factor for a subpopulation of motor neurons, as the GDNF deficient mice have 20-40% less spinal and cranial motor neurons. By E15 GDNF is expressed in all muscles. Most motoneurons in the spinal cord express the RET receptor. During development and postnatally GDNF regulates pool-specific cell migration, axonal outgrowth, branching and synaptic connectivity of motor neurons (Kanning, Kaplan et al. 2010). GDNF is secreted by both muscles and astrocytes. Overexpression of GDNF by astrocytes results in increased number of motoneurons due to rescue from programmed cell death (PCD) and this astrocyte-derived GDNF can promote long-term motor neuron survival following axotomy (Zhao, Alam et al. 2004).

GDNF signalling is required for migration and proliferation of parasympathetic neuronal precursors during early embryonic development. NRTN role in parasympathetic ganglia is to support postnatal target innervation and maintain their phenotype and trophic status. Thus there is a shift from GDNF to NRTN signalling in developing parasympathetic neurons. GDNF also promotes the migration, proliferation, survival and different...
tiation of enteric precursors. RET, GFRα1 and GDNF KO mice all lack enteric neurons in the gut distal to the stomach. NRTN supports enteric innervation and regulates neurotransmitter release in enteric neurons. A similar shift from GDNF to NRTN, as seen in parasympathetic neurons, occurs also in enteric neurons (Baloh, Enomoto et al. 2000; Airaksinen and Saarma 2002).

Due to the wide range of functions the GFLs have been implicated to play a role in motor neuron disease, sensory regeneration, neuropathic pain, ischaemia, epilepsy, addiction and Parkinson's disease (Airaksinen and Saarma 2002). Expression outside the nervous system has also been described, thus GFLs possess also non-neuronal properties, such as regulation of ureteric branching during kidney development and spermatogenesis (Sariola and Saarma 2003).

1.4.2 Signalling

The RET receptor is a single-pass transmembrane protein containing four cadherin-like domains and a cysteine-rich domain in the extracellular part, and an intracellular tyrosine kinase domain. RET is the only receptor tyrosine kinase, which does not bind its ligand directly, but requires a co-receptor - GFRα - for its activation. GFRα receptors are GPI-linked receptors located in lipid rafts on cell surface. They contain three globular cysteine-rich domains (except for GFRα4 having only two domains). Homodimeric ligands bind first to monomeric or dimeric GFRα receptors, this complex interacts in a Ca²⁺ dependent manner with the extracellular domains of two RET molecules, inducing the dimerization and phosphorylation of the intracellular tyrosine kinase domains which then transphosphorylate each other on crucial residues. On the intracellular part of RET at least six phosphorytrosine residues (Tyr687, Tyr905, Tyr981, Tyr1015, Tyr1062 and Tyr1096) act as docking sites for different proteins, triggering activation of intracellular signalling cascades like MAPK, PI3K/AKT, JUN, Src, PLCγ, SHP2, thereby contributing to neuronal survival, neuritogenesis and neurotransmission (Airaksinen and Saarma 2002; Runeberg-Roos and Saarma 2007).

Malfunctioning of the RET receptor leads to several inherited diseases. Inactivating mutations of RET cause Hirschsprung's disease, which is characterized by aganglionic megacolon. The activating mutations of RET lead to different types of multiple endocrine neoplasia (MEN)2 cancer syndromes, which are classified into three subgroups. The MEN2A syndrome is characterized by medullary thyroid carcinoma (MTC), phaeochromocytoma of adrenal medulla and hyperparathyroidism. This variant is mainly due to abnormal formation of S-S bridges between the extracellular domains of two RET molecules. The MEN2B syndrome presents with MTC, phaeochromocytoma accompanied by marfanoid habitus, thickening of the corneal nerve and ganglioneuromatosis of the buccal membrane and gastrointestinal (GI) tract. This syndrome is in most cases caused by a single germline mutation resulting in substitution of threonine for methionine at codon 918. These activating mutations in the intracellular kinase domain lead to activation of signalling cascades either as monomers or associated dimers. The third
syndrome - familial medullary thyroid carcinoma (FMTC) manifests with only MTC (Runeberg-Roos and Saarma 2007).

GFLs can also signal independently of RET using the neural cell adhesion molecule (NCAM) (Paratcha, Ledda et al. 2003) and this signalling is strictly GFRα1 dependent (Sjostrand and Ibáñez 2008). Alternative signalling by integrin β-1 and N-cadherin has been suggested, but the evidence is still indirect (Cao, Yu et al. 2008; Cao, Li et al. 2010). Recently it was shown that heparan sulfate proteoglycan syndecan-3 is a novel receptor for GDNF, neurturin, and artemin. GFL-syndecan-3 interaction mediates both cell spreading and neurite outgrowth with the involvement of Src kinase activation (Gerhardt, Cass et al. 1999). In addition, GDNF promotes migration of cortical neurons in a syndecan-3-dependent manner (Bespalov, Sidorova et al. 2011).

1.4.3 GFLs roles in the dopaminergic system

GDNF’s role as a neurotrophic factor for embryonic midbrain dopaminergic neurons in vitro was first described by Lin et al (Lin, Doherty et al. 1993). In vivo GDNF has been shown to support the DA neurons during the period of postnatal cell death and in adults it promotes survival, as well as regrowth and recovery following damage by MPP⁺ and 6-OHDA (Tomac, Lindqvist et al. 1995; Hou, Lin et al. 1996; Burke, Antonelli et al. 1998; Eggert, Schlegel et al. 1999; Gerhardt, Cass et al. 1999; Kramer, Goldman et al. 1999). Intracranial administration of GDNF to unlesioned animals causes behavioural effects of increased locomotor activity due to raised levels and turnover of brain monoamines including DA, as well as dose-dependent morphological changes due to increased cell size, axonal outgrowth and growth cone-like profiles (Table 2). Injection of GDNF into SN or cerebral ventricles is accompanied by side-effects, such as weight loss (Lapchak, Miller et al. 1996).

Extensive in vivo studies of GDNF effects on the dopaminergic system have been carried out on lesioned animals (Table 3). Many different approaches to investigate GDNF-mediated neuroprotection and neurorestoration in vivo have been utilized:

a) different lesion models including 6-OHDA, MPTP, partial lesions vs extensive lesions
b) timing of GDNF administration including before, during and following the lesions
c) different routes and sites of administration including intranigral, intrastriatal, intraventricular or via cerebrospinal fluid
d) different doses and means of administration, including single dose, single administration, different doses, continuous administration, mesencephalic grafts, lenti- and adeno-associated virus mediated administration or genetically engineered cells
e) different animal models including mice, rats and non-human primates

These in vivo experiments have established a clear conclusion that GDNF exhibits neuroprotective and neurorestorative effects in animal models of PD. GDNF induces behavioural improvement by prevention of lesion-induced motor deficits, as well as morphological changes.
Table 2. Experiments with intracranial delivery of GDNF to intact brain in animals.

<table>
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<tr>
<th>INTACT BRAIN</th>
<th>EFFECT</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Continuous expression by lentiviral vector</td>
<td>Initially: ↑DA turnover, ↑function In 6-weeks: ↑TH mRNA, ↑TH protein, ↓TH activity, normal DA</td>
<td>(Rosenblad, Georgievska et al. 2003; Georgievska, Jakobsson et al. 2004; Georgievska, Kirik et al. 2004)</td>
</tr>
<tr>
<td>Primates</td>
<td>↑motor function, ↑DA levels, release, ↑TH activity, ↑DA metabolites, ↑DA levels</td>
<td>(Gash, Zhang et al. 1995; Kordower, Emborg et al. 2000; Grondin, Cass et al. 2003; Eslamboli, Georgievska et al. 2005)</td>
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Expressed in protection and preservation of DA neuronal cell bodies and axon terminals, increased DA metabolism, as well as increased TH and dopamine levels in the striatum.

The in vivo neurorestorative and regenerative potential of GDNF – in case the factor is administered to the previously compromised midbrain - is of central clinical relevance (Tomac, Lindqvist et al. 1995; Kirik, Rosenblad et al. 2000; Kirik, Georgievska et al. 2001). In this paradigm GDNF influences the whole nigrostriatal system by restoring the phenotype of DA neurons. It functions by rescuing the TH-positive neurons, increasing cell body size, axonal sprouting and the number of axonal terminals, as well as by elevating tissue levels of DA and its metabolites, DA turnover and release. Positive results with GDNF were also gained on primate studies, where GDNF showed remarkable improvement in hemiparkinsonism in MPTP treated monkeys via different routes of administration (Gash, Zhang et al. 1995; Kordower, Emborg et al. 2000; Grondin, Cass et al. 2003; Eslamboli, Georgievska et al. 2005).
Table 3. Experiments with intracranial delivery of GDNF to lesioned brain.

<table>
<thead>
<tr>
<th>NEUROPROTECTIVE</th>
<th>EFFECT</th>
<th>6-OHDA</th>
<th>MPTP</th>
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<tbody>
<tr>
<td>GDNF to SN</td>
<td>↑TH+ cells, unchanged fiber density, unchanged motor behaviour</td>
<td>(Kearns and Gash 1995; Winkler, Sauer et al. 1996; Sullivan, Opacka-Juffry et al. 1998)</td>
<td></td>
</tr>
<tr>
<td>GDNF to STR</td>
<td>↑motor behaviour, ↑TH+ cells, ↑DA terminals, ↑DA levels</td>
<td>(Sauer, Rosenblad et al. 1995; Shults, Kimber et al. 1996; Kirik, Rosenblad et al. 2000a; Kirik, Rosenblad et al. 2000b; Rosenblad, Georgievskya et al. 2003; Lindholm, Voutilainen et al. 2007)</td>
<td>(Tomac, Lindqvist et al. 1995)</td>
</tr>
<tr>
<td>GDNF-secreting cells or grafts</td>
<td>↓rotational behaviour, ↑TH+ cells, ↑glial cells</td>
<td>(Sullivan, Pohl et al. 1998; Shingo, Date et al. 2002; Yasuhara, Shingo et al. 2005)</td>
<td></td>
</tr>
<tr>
<td>Primates to STR</td>
<td>↑motor improvement ↑TH+ cells, ↑fibers</td>
<td>(Eslamboli, Georgievskya et al. 2005)</td>
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<tr>
<th>NEURORESTORATIVE</th>
<th>EFFECT</th>
<th>6-OHDA</th>
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Table 3. cont.

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<tr>
<td>GDNF synthesizing transplant graft delivery</td>
<td>↑innervation ↑TH+ cells</td>
<td>(Granholm, Srivastava et al. 1997; Tang, Tien et al. 1998)</td>
<td></td>
</tr>
<tr>
<td>GDNF-secreting cells</td>
<td>↑TH+ cells ↓rotational behaviour ↑locomotor activity</td>
<td>(Lindner, Winn et al. 1995; Shingo, Date et al. 2002)</td>
<td></td>
</tr>
<tr>
<td>Primates: Gash 1996 to ICV Gerhardt to ICV Kordower to SN and STR Grondin to lateral ventricle and STR Gash 2005 to SN Xin to ICV Eberling to STR</td>
<td>↓Clinical rating score (CRS) ↑motor improvement ↑TH+ cells, fibers ↑metabolism in PET</td>
<td>(Gash, Zhang et al. 1996; Gerhardt, Cass et al. 1999; Kordower, Emborg et al. 2000; Grondin, Zhang et al. 2002; Gash, Zhang et al. 2005; Xin, Ai et al. 2008; Eberling, Kells et al. 2009)</td>
<td></td>
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The site of the lesion and administration of GDNF protein is of central importance in correlating the in vivo animal studies with possible clinical aspects. Two sites of administration for GDNF in brain have been mainly used: the intranigral and intrastriatal delivery. Application of GDNF to SN leads to efficient neuroprotection and neurorestoration of SN cell bodies, however degeneration of the nerve terminals is not prevented and fiber sprouting takes place around the nigral neuron cell bodies. Also functional recovery is not as evident, due to lack of regenerating axons. Application of GDNF to the SN also causes quite serious side-effects, as chronic infusion of GDNF to SN has been shown to cause significant weight loss in rats (Manfredsson, Tumer et al. 2009). Application of GDNF to the striatum leads to sparing of striatal fibers. In addition, protection of TH-positive nigral cell bodies and fiber sprouting also occurs inside the striatum and globus pallidus. In the case of striatal delivery functional recovery of motor behaviour is remarkable (Kirik, Georgievska et al. 2004).

Another aspect to be kept in mind, regarding the site of administration, is the anterograde-retrograde transport of GDNF in the dopaminergic system. Both ways of transport have been described (Kordower, Emborg et al. 2000; Ai, Markesbery et al. 2003; Georgievska, Jakobsen et al. 2004; Ciesielska, Mittermeyer et al. 2011).

1.4.4 Transgenic mouse models of GDNF and RET

To validate the physiological relevance of GDNF effects on the DA system, several transgenic models of GDNF signalling pathway have been generated.

Studies on GDNF, GFRα1 and RET KO animals have shown that DA neurons are not dependent on GDNF signalling during embryonic development, as these animals exhibit normal number of DA neurons in the ventral midbrain (Moore, Klein et al. 1996; Pichel, Shen et al. 1996; Sanchez, Silos-Santiago et al. 1996; Granholm, Mott et al. 1997; Granholm, Srivastava et al. 1997; Cacalano, Farinas et al. 1998; Enomoto, Araki et al. 1998). Postnatal-studies of the DA system from these KO animals are not possible, as they die after birth due to renal agenesis.

GDNF seems to function as a target-derived neurotrophic factor for the DA neurons postnatally during the natural cell death period (Burke, Antonelli et al. 1998; Burke 2006). This cell death is restricted to the first early phase of postnatal cell death and lasts till P7, after which the DA neurons become dependent on other factors (Oo, Kholodilov et al. 2003). The postnatal GDNF dependence of DA neurons has been further confirmed by transplantation studies using fetal ventral mesencephalon from GDNF null mice to wild type striatum, which impaired postnatal DA neuron survival (Granholm, Reyland et al. 2000). Further generation of mice selectively over-expressing GDNF in striatum showed that GDNF is indeed important for early postnatal cell death, as these animals have increased number of SN DA neurons following this period. However, this increase does not persist...
into adulthood and the number of SN DA neurons is not increased in adult animals, although there is increased number of VTA neurons (Oo, Kholodilov et al. 2003).

Studies on DA system of aged heterozygous GDNF animals, who exhibit about 50% decrease in the level of GDNF protein, have suggested a role for GDNF in long-term maintenance of DA system (Gerlai, McNamara et al. 2001; Airavaara, Mijatovic et al. 2006; Boger, Middaugh et al. 2006; Airavaara, Tuomainen et al. 2007).

Although GFRα1 receptor has been conditionally deleted during late enteric nervous system development, which lead to widespread death of enteric neurons and agangliosis (Uesaka, Jain et al. 2007), this receptor has not yet been deleted from the midbrain DA neurons. Kholodilov et al have generated mice, which selectively over-express GDNF in the striatum, cortex and hippocampus. These animals show increased number of SN DA neurons after the first phase of cell death, however not in adulthood (Kholodilov, Yarygina et al. 2004). Adult animals do not have increased dopaminergic innervation of striatum, but exhibit increased number of VTA neurons and increased innervations of cortex. They also show increased locomotor activity to amphetamine (Kholodilov, Yarygina et al. 2004). Results on the over-expression of GDNF in cells that naturally do not express GDNF should be very carefully interpreted. For example over-expressing GDNF under a testis specific promoter led to male infertility and testicular tumours in older age (Meng, Lindahl et al. 2000).

RET conditional knockout mice have provided additional information on the role of GDNF signalling pathway in the maintenance of the dopaminergic system. RET cKO mice created by Jain et al. (2006) showed no changes in DA cell number, size and in DA levels in the SN and VTA until 1 year of age and therefore the authors concluded that RET signalling is not critical for normal physiology of DA neurons in the SN and VTA in adult mice (Jain, Golden et al. 2006). However, the RET cKO mice generated by Kramer et al displayed loss of DA neurons in SN, nerve terminal degeneration in striatum and glial activation starting from one year of age. These data demonstrate that RET functions as a critical regulator of long-term maintenance in the nigrostriatal DA system (Kramer, Aron et al. 2007).

The results of these studies are not necessarily contradictory and possibly are the result of differences in animal’s age at the time of analysis. Further MPTP study on cKO RET mutants revealed no difference in survival of SN DA neurons compared to wild type animals. Impaired dopaminergic terminal and fiber regeneration, as well as decreased striatal DA concentrations appeared during the recovery period from toxin damage (Kowsky, Poppelmeyer et al. 2007). They concluded that RET does not influence the survival of DA neurons following MPTP insult, but facilitates the regeneration of axon terminals (Kowsky, Poppelmeyer et al. 2007).

Recently the Klein lab found that mice lacking DJ-1 and RET in the dopaminergic system have a rather significant loss of DA neurons in the SNpc, as compared to animals lacking RET in DA system (Aron, Klein et al. 2010).

The GDNF cKO mice created by Pascaul et al. (2008) with suppressed GDNF
expression starting from the age of 2 months, showed progressive decrease of brain TH levels and increased cell death in locus coruleus, SN and VTA area, with progressive hypokinetic symptoms resembling PD pathology (Pascual, Hidalgo-Figueroa et al. 2008). Authors concluded that GDNF has an indispensable role in maintenance of the adult DA system (Pascual, Hidalgo-Figueroa et al. 2008). These animals exhibited about 60% reduction in the GDNF mRNA and protein levels in the striatum, but had much more severe phenotype than GDNF Hz mice. This discrepancy can be explained by the fact that deletion of GDNF during embryogenesis may lead to compensatory developmental mechanisms by other factors or by non-RET signalling (Pascual, Hidalgo-Figueroa et al. 2008). The difference in results for these conditional GDNF KO mice as compared to the results of RET conditional KO might be due to the timing of the deletion, as the RET was deleted in cKO animals during embryogenesis as compared to two month old mice for the GDNF cKO. Another possible explanation for the differences between the phenotypes of RET and GDNF cKO mice is that the increased neuronal death might have been triggered by high levels of tamoxifen used to induce homologous recombination in GDNF cKO mice.

According to a commentary by Ibáñez, three possible explanations can be appointed for the differences. The first being alternative non-RET mediated signalling by GDNF, most likely through the NCAM receptor. The second, most likely explanation, is that due to absence of RET at embryonic stages, RET independent compensatory mechanisms are induced, which are normally suppressed by GDNF, rendering neurons dependent on GDNF. This question can be solved by generating RET cKO at later developmental stages or by selectively inactivating GDNF during embryonic development. The third option is that RET could, independently of GDNF, induce apoptotic dopaminergic cell death and GDNF is needed to prevent this apoptotic signalling by the receptor (Ibáñez 2008).

Studies in our laboratory show that different GDNF cKO mice have a modest loss of SNpc DA neurons (Jaan-Olle Andressoo - personal communication).

In addition, RET knock-in mutants have been generated where a specific tyrosine on RET receptor (tyrosine 1062) has been replaced by phenylalanine. These mice die around P27, are retarded in growth and display decreased number of enteric neurons, however small kidneys develop (Jijiwa, Fukuda et al. 2004). Other knock-in models for specific RET isoforms have also been generated, but only renal and enteric pathologies for these defects have been analysed (Jain, Encinas et al. 2006).

The knock-in MEN 2B mouse model has been generated by introducing a site-directed mutation, by substitution of the threonine in the RET receptor tyrosine kinase for methionine at position 918. These mice displayed C-cell hyperplasia and chromaffin cell hyperplasia progressing to pheochromocytoma, adrenal ganglioneuromas, enlargement of the sympathetic ganglia and male reproductive defect, but lack ganglioneuromas of the GI tract (Smith-Hicks, Sizer et al. 2000). Cancer syndrome does not manifest in these animals before the age of...
3-4 months, thus they can be utilized in studies evaluating the effects of constant over-activation of the GDNF signalling pathway.

1.4.5 GDNF and NRTN in clinical studies

Since the possible therapeutic potential of GDNF in several neurological disease paradigms has been well established in preclinical settings, the clinical trials on Parkinsonian patients have been undertaken. However, as GDNF is a relatively large protein with molecular weight of about 30 kilodaltons, it does not cross the blood-brain barrier. The problems with protein delivery to the affected brain region have resulted in poor results of all clinical trials performed to date. The first randomized clinical trial conducted by Amgen on patients with advanced PD concentrated on delivering the protein through an intra-cerebroventricular catheter directly to the cerebrospinal fluid in the ventricular space of the brain. However, no improvement in Parkinsonism was seen and several side-effects were registered, which lead to the conclusion that this route of delivery is ineffective since the factor does not reach the target tissues (Nutt, Burchiel et al. 2003). It is rather surprising that GDNF was delivered intraventricularly, since already at that time it was well established that GDNF is a basic protein with pI close to 10 and with affinity to heparan sulfates.

A trial by Gill et al used direct infusion of GDNF via a catheter and mini-pump to the posterior putamen of 5 patients. This open-label trial showed remarkably positive improvement in all the patients, which persisted for 2 years post-treatment (Gill, Patel et al. 2003). Very similar results were obtained in a study conducted under the direction of Dr. D. Gash in Kentucky, where in an open-label study 10 patients received GDNF unilaterally to striatum for six months (Slevin, Gerhardt et al. 2005). This success lead to the next randomized double-blind trial by Amgen, where majority of the study approach resembled that of Gills study and utilized similar infusion of GDNF directly to the brain of 17 PD patients, whereas 17 patients received placebo. The conclusions from this trial were again negative, as no significant clinical effect for GDNF over placebo was seen (Lang, Gill et al. 2006). Moreover, anti-GDNF antibodies developed in 7 patients. This is very surprising, as brain lacks the antibody-generating system. More recently it has become evident that in fact the pump connection in 7 patients was broken and therefore GDNF had leaked to the peripheral tissues of patients. Thus only 10 patients out of 17 received GDNF intrastriatally and therefore the results of this phase II trial were suggested for re-evaluation (Penn, Dalvi et al. 2006). To date these results have not been re-evaluated, however several new Phase II trials with GDNF are being initiated.

A phase I trial using the adeno-associated type-2-(AAV2)-NRTN infusion to putamen was performed on 12 PD patients. This trial demonstrated the safety of this approach in patients and a significant improvement in Parkinsonian condition was observed (Marks, Ostrem et al. 2008). This was followed by a phase II double-blinded trial on 58 patients (Marks, Bartus et al. 2010). After 12 months no statistically sig-
significant difference in the conditions of patients receiving NRTN gene therapy was observed. However, after 18 month treatment a small, but statistically significant benefit of NRTN was observed (Ceregene, press release 2010). Conclusions from this study regarding the site of administration to the putamen and the very modest clinical response led to the design of the next phase II clinical trial using AAV2-NRTN administration to putamen as well as substantia nigra. This study has been recently launched and is currently in the enrolment phase. Unfortunately the plan to deliver NRTN to the SN is not well justified. In human midbrain NRTN signals to RET via GFRα1 and not via its cognate receptor GFRα2. Therefore there is a strong risk that NRTN signalling, via GFRα1-RET, will similarly to GDNF induce a robust weight loss in patients (Manfredsson, Tumer et al. 2009). Recently published data on animals showed that properly scaled and targeted AAV2-NRTN to the SN is safe, effective and causes no weight loss (Bartus, Brown et al. 2011).

These trials have led to intensive discussion on the obstacles related to clinical trial design, including differences in cannula size, concentration of protein, infusion rate and statistical methodology for interpretation of the data (Hutchinson, Gurney et al. 2007; Matcham, McDermott et al. 2007).

Although studies on GDNF as a therapeutic protein in PD clinical trials have been halted by Amgen in 2005 and the concept of GDNF as a therapeutic molecule was for several years under consideration, scientists have continued to develop different drug delivery approaches to encompass the BBB obstacles. Possible alternative mechanism include small molecules which could pass this barrier, gene-therapy based on viral vectors, implantation of neural stem cells, implantation of growth factor producing cells, increasing endogenous GDNF expression in brain or receptors functioning as transporters of the protein through the BBB (Peterson and Nutt 2008; Vastag 2010).

In conclusion, GDNF and possibly also NRTN may have the potential as disease-modifying therapy for Parkinson’s disease. Animal studies on rodents and primates have well established the beneficial effects on dopaminergic neuron survival, neuroprotection as well as neurorestoration, however the clinical use of these factors to date has been delayed due to difficulties in delivery of the factors to the affected targets in human brain. Importantly, clinical trials with GDNF and NRTN are again under way and hopefully will provide promising results for disease modifying therapy of Parkinson’s disease.

1.4.6 GDNF role in addiction

Drug addiction can be viewed as a form of behavioural neuronal plasticity. All addictive drugs raise the extracellular levels of dopamine in the NAc and caudate/putamen. Cocaine increases dopamine levels by blocking the dopamine uptake by DAT. Morphine acts on the dopaminergic system through μ-opioid receptors on GABAergic interneurons which modulate the dopaminergic pathways and induce the release of dopamine from basal ganglia (Koob and Le Moal 2001). Repeated administration of addictive drugs leads
to biochemical and morphological alterations, including increased expression of TH, glutamate receptors and decreased levels of neurofilaments, in the dopamine neurons of the VTA and their target neurons in the NAc.

GDNF has been characterized as one of the NTFs having a central role in the neuroadaptive processes related to addiction (Carnicella and Ron 2009; Ghitza, Zhai et al. 2010). GDNF inhibits the rewarding effects of drugs by blocking the molecular and cellular changes. Infusion of GDNF to the VTA results in increased TH immunoreactivity in VTA and blocking of characteristic biochemical changes to drug administration, such as induction of protein kinase A subunit and ΔFosB transcription factor. GDNF infusion also causes behavioural effects by reducing animal’s sensitivity to the rewarding effects of cocaine (Messer, Eisch et al. 2000). Administration of GDNF to VTA decreases cocaine and alcohol self-administration (Green-Sadan, Kinor et al. 2003) and reduces place preference. In addition, other studies on GDNF using transplantation of GDNF over-expressing cells, delivery of nanoparticles with GDNF and continuous infusion via mini-pump to the striatum led to decreased self-administration of cocaine in rats (Carnicella and Ron 2009). Drugs of abuse also decrease the endogenous levels of GDNF. Downstream GDNF signalling pathways are affected as chronic administration of cocaine also decreases the levels of phosphorylated Ret in the VTA (Messer et al. 2000). It should be noted, however that other groups have not been able to reproduce these results.

Conversely intrastriatal infusion of GDNF blocking antibody and experiments in GDNF Hz mice showed enhanced response, increased sensitivity to the rewarding effects of cocaine and motivation to self-administrate and seek metamphetamine (Messer, Eisch et al. 2000; Airavaara, Planken et al. 2004; Yan, Yamada et al. 2007; Carnicella and Ron 2009). It is likely that physiological compensatory changes are induced in response to drug addiction, reward and altered drug-seeking behaviour, such as the increased striatal dopamine levels seen in GDNF Hz animals (Airavaara, Planken et al. 2004; Airavaara, doctoral dissertation 2006; Airavaara, Mijatovic et al. 2006; Airavaara, Tuomainen et al. 2007). The possible feedback loop for GDNF has been described, whereas the drugs of abuse decrease endogenous GDNF levels and signalling, resulting in increased rewarding effects and behavioural sensitivity to subsequent drug exposure (Messer, Eisch et al. 2000). It can be concluded that chronic drug exposure inhibits endogenous activity of GDNF on VTA leading to sensitized responses to subsequent drug administration (Bolanos and Nestler 2004). It has been postulated that GDNF and BDNF might have possible therapeutic potential in treating drug addiction (Carnicella and Ron 2009), but it is doubtful that a single neurotrophic factors, such as GDNF, could be used in treating drug addiction (especially in the case of multi-drug addiction), as the described effects seem to be drug-specific, brain site-specific and time-dependent (Ghitza, Zhai et al. 2010).
1.5 VEGF family of growth factors

Vascular endothelial growth factor (VEGF) family consists of seven members: VEGF-A, -B, -C, -D, -E, -F and placental growth factor (PIGF). VEGFs signal through receptor tyrosine kinases, VEGFR-1, -2 or -3. VEGF-A binds to and activates both VEGFR-1 and VEGFR-2, VEGF-C and VEGF-D signal through VEGFR-2 and VEGFR-3 (Fig. 4) (Mäkipää, Veikkola et al. 2001; Ferrara, Gerber et al. 2003; Tammela, Enholm et al. 2005). In addition, VEGFs use as co-receptors neuropilin (NRP) 1 and 2, molecules well characterized in repulsive axonal guidance processes as receptors for semaphorins (Carmeliet and Tessier-Lavigne 2005). NRP 1 is thought to be essential for vascular development, whereas NRP 2 is considered to be the co-receptor in lymphatic development. Functional interaction of VEGF-C with neuropilin receptors has been demonstrated (Kärpanen, Heckman et al. 2006). The direct signalling effects of neuropilins in endothelial

![Figure 4. VEGF family of growth factors and interaction with their receptors. VEGF, VEGF-B, PIGF and VEGF-E mediate vasculogenesis and angiogenesis, whereas VEGF-C and -D mediate lymphangiogenesis in physiological functioning, as well as during pathogenic processes. Reprinted by permission from the American Physiological Society (Physiological Reviews 82, 673-700) Copyright © 2002.](image-url)
cells are not completely clear, they have been thought to play a role in stabilization of the VEGFR-2 complex. VEGF family is one of the central players in the development and maintenance of the vascular system, by inducing vasculogenesis, sprouting of blood and lymphatic vessels, promoting proliferation and survival of endothelial cells and by increasing vascular permeability (Tammela, Enholm et al. 2005).

VEGF-A is essential during the development of the vascular system, by mediating both vasculogenesis and angiogenesis. It induces vascular permeability through extravasation of fluids and protein, which in turn activates the migration, proliferation and survival of endothelial cells. VEGF-A expression is up-regulated in case of injury and hypoxia, but it plays also a role in pathological angiogenesis, mediating the spread and metastasis of cancer cells. In endothelial cells VEGF-A signalling is mediated through both VEGFR-1 and -2. At least six isoforms of VEGF-A have been described, which are generated through alternative splicing (Tammela, Enholm et al. 2005). VEGF-A knockout animals die at E8-9 due to defects in vascular formation (Tammela, Enholm et al. 2005).

VEGF-C, the third member of the VEGF family, first described by Kari Alitalo’s group as a growth factor for lymphatic vessels, is 30% homologous to VEGF-A (Jussila and Alitalo 2002). VEGF-C is mainly expressed around developing lymphatic vessels and is recognized as the key factor in the development and regulation of the lymphatic system (Jussila and Alitalo 2002). It is synthesized as a preproprotein and binds to both VEGFR-2 and VEGFR-3. In lymphatic system it stimulates the migration of lymphatic endothelial cells and increases vascular permeability through VEGFR-3. At higher concentrations it also induces blood endothelial cell migration and angiogenesis through VEGFR-2 (Jussila and Alitalo 2002). It is up-regulated in response to proinflammatory cytokines, but not hypoxia. During early embryogenesis VEGF-C plays a role in the development of the blood vascular system and later in the generation of the lymphatic system. VEGF-C is produced as a precursor protein and different forms of VEGF-C are generated through proteolytic processing (Tammela, Enholm et al. 2005). Deletion of VEGF-C in mice leads to embryonic lethality, due to the absence of lymph nodes, whereas the blood vasculature appears normal (Karkkainen, Haido et al. 2004).

1.6 Neurovascular crosstalk

The nervous and vascular systems have distinct roles in the organism. The vessels supply and transport oxygen, nutrients and solutes to the tissues and the nerves are responsible for processing and transmitting information and stimuli. The nervous and vascular systems have been considered to be functionally and structurally different units of an organism, however lately studies have been published which indicate the existence of strong crosstalk between these systems and suggest the existence of shared growth factors (Carmeliet 2003; Carmeliet and Tessier-Lavigne 2005; Weinstein 2005; Raab and Plate 2007; le Noble, Klein et al. 2008). Several similarities in the construction of the
neuronal and vascular systems do exist. It is well established that vessels are often aligned alongside nerves in the organism. Recent evidence has indicated that nervous and vascular systems share common organizational principles and that this basis for patterning extends to the molecular level, including commonly shared growth factors and molecules involved in axonal guidance as well as angiogenesis. During development sympathetic nerves migrate along the vessels to their target (Honma, Araki et al. 2002), while sensory nerves determine the pattern of arterial differentiation and vessel branching (Miller 2002). Thus it is not clear whether neurogenesis or angiogenesis is the leading process during development. Most likely the collaboration between the two systems is bi-directional rather than unidirectional (Carmeliet 2003; Carmeliet and Tessier-Lavigne 2005; Eichmann, Le Noble et al. 2005; Weinstein 2005; Lazaro-vici, Marcinkiewicz et al. 2006; Larri-vee, Freitas et al. 2009).

The neuro-vascular crosstalk plays multiple roles in the development and homeostasis of both systems, from target-derived guidance, migration, patterning and remodelling to maintenance and survival. The deregulation in either of these systems, could lead to medically important diseases, among them neuropathological diseases such as Parkinson's and Alzheimer's disease. Thus understanding the mechanisms of neurovascular crosstalk is the basis to possible future therapeutic implications. It should be noted that molecular mechanism of neurovascular crosstalk and their implications to the pathogenesis of neurodegenerative diseases have not been studied extensively.

Growth factors known to be specific for the nervous system, have been found to have also effects on the vascular system, as well as factors known to be limited to the cardiovascular system have proven to be effective in neuronal development and maintenance (Raab and Plate 2007). The term „angioneurin“ has been suggested to define the molecules which affect both neural and vascular functioning, including effects related to regulation of angiogenesis, blood-brain barrier integrity, vascular perfusion, neuroprotection, neuroregeneration and synaptic plasticity (Zacchigna, Lambrechts et al. 2008). The angioneurins are promising therapeutic candidates for the treatment of vascular and neuronal diseases. Although traditionally neurological diseases have been considered to result from lesions within the neuronal systems, lately the role of vascular pathology in disease pathogenesis has been described for neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and dementia, as well as for non-degenerative pathologies such as stroke and epilepsy. Classical neurotrophic factors - NGF and BDNF can be characterized as „angioneurins“, as they have been shown to be essential during development and formation of the heart and vasculature. They regulate vasculogenesis and angiogenesis by autocrine and paracrine mechanisms (Zacchigna, Lambrechts et al. 2008; Caporali and Emanueli 2009).

Involved in the crosstalk of the neuronal and vascular systems, is also ARTN - a member of the GDNF family. ARTN is considered to be a vascular-derived
neurotrophic factor for the sympathetic neurons, innervating the central and peripheral blood vessels (Honma, Araki et al. 2002). It remains unclear, whether the stimulating effects of neurotrophic growth factors on vessel are direct, or rather through up-regulation of the level of classical angiogenic factors.

One central player in the crosstalk of angiogenesis, neurogenesis and pathogenesis is VEGF-A which has been well characterized as a classical angiogenic factor, but recently shown to have multiple roles in the nervous system in normal and pathological situations. VEGF-A has been shown to have pleiotrophic effects in the nervous system by enhancing perfusion and protection of endothelial cell survival, by stimulating endothelial cell neurogenic signalling, by promoting the survival and protecting from stress several types of adult neuronal cells. VEGF-A also increases neurogenesis, cognitive performance, memory and affects synaptic transmission, by stimulating neurite outgrowth and also by acting on non-neuronal cells (Zacchigna, Lambrechts et al. 2008). Thus in addition to the vasculogenic role, VEGF-A has direct effects on glial and neuronal cells in both central and peripheral nervous system.

Of specific interest is VEGF-A role on dopaminergic neuronal system. VEGF-A has been shown to be neuroprotective in both in vitro and in vivo models of Parkinson’s disease (Pitzer, Sortwell et al. 2003; Yasuhara, Shingo et al. 2004; Yasuhara, Shingo et al. 2005; Yasuhara, Shingo et al. 2005; Tian, Tang et al. 2007). The neuroprotection is considered to result from a direct impact on neuronal survival, as well as indirect angiogenic and glial proliferative effects. It is of remarkable interest that a neurorestorative effect in a rat model of Parkinson’s disease has been described for VEGF-A (Yasuhara, Shingo et al. 2005). VEGF-A immunoreactivity in the SN of Parkinsonian patients has been shown to be up-regulated whereas no change in the expression was seen in the striatum (Wada, Arai et al. 2006). VEGF-A levels in the serum, however, are not changed in PD patients (Infante, Mateo et al. 2007). VEGF-A expression by adeno-associated virus vector also increases the levels of GDNF in the striatum, which by activating additional RET receptors could result in rescuing more DA neurons (Tian, Tang et al. 2007). In addition, recent data on VEGF-B neurotrophic effects have been published, where stimulatory effects on neurogenesis and protection from motoneuron degeneration were demonstrated (Sun, Jin et al. 2006; Poesen, Lambrechts et al. 2008). In addition, VEGF-B was shown to be up-regulated in the rat cell culture model of PD and it also exhibited neuroprotective effects in this system (Falk, Zhang et al. 2009).

In conclusion, processes that play a role in the organism physiology and functioning are tightly interacting and crosstalk between different systems on the molecular and systemic level occurs constantly. The nervous and vascular systems converge in multiple pathways, share common growth factors and/or receptors and represent targets for possible therapeutic approaches. Interpretations of therapy response and possible side-effects should account for this aspect.
2. AIMS OF THE STUDY

The initial aim of this study was to describe the signalling pathways and target genes activated by GDNF in different neuronal systems and experimental conditions including \textit{in vitro}, GDNF KO mouse models, as well as following the over-activation of the RET receptor. The microarray analysis revealed that GDNF induced the expression of the VEGF-C growth factor, which lead to the studies on the neurovascular crosstalk and possible neurotrophic role of VEGF-C in the dopaminergic system of rodents, as well as during sympathetic development.

The specific aims of this study were:
1. To investigate which genes are regulated by GDNF \textit{in vitro} and \textit{in vivo} models.
2. To analyse the dopaminergic system of GDNF knockout heterozygous mice.
3. To analyse the effects of continuous RET activity in dopaminergic system of MEN2B knock-in mice.
4. To develop and validate a medium-throughput computer assisted \textit{in vitro} dopaminergic micro-island assay, in order to facilitate dopaminergic screening of growth factors and drug candidates.
5. To study the neurotrophic properties of VEGF-C on the dopaminergic system and possible cross-talk with GDNF.
3. MATERIALS AND METHODS

Most of the methods used in specific studies are described in detail in the original articles. The following tables list the methods used.

Table 4. List of methodology used in this study.

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<tr>
<td>Statistical analysis</td>
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3.1 Microarray analysis

Microarrays allow studying the transcription profile of thousands of genes in a single experiment. The oligonucleotide arrays are one of the most powerful tools for genomic research as changes in gene expression reflect cellular processes to environmental stimuli. The transcriptome changes, unlike the genomic changes, are highly dynamic and rapid. The investigations on expression site and extent allow to understand the activity and biological role of the transcribed mRNA and respective protein as well as possible regulatory mechanisms, cellular functions and biochemical pathways involved. The transcriptome analysis also helps to understand the molecular processes underlying disease and to evaluate the possible therapeutic applications on cells and organisms (Lockhart and Winzeler 2000).

The Affymetrix GeneChip technology utilizes oligonucleotides which have
been synthesized on slides by photolithography. Extracted RNA is transcribed to cDNA, which is transcribed to biotinylated cRNA. The fragmented cRNA is hybridized to the microarray slides and specific staining with biotin-conjugated antibodies is measured by a laser scanner. For each gene a set of eleven 25-mer oligonucleotide probes are present on the array, which provide several independent measurements for each transcript and are used to measure the level of transcript for a gene (Affymetrix Inc. www.affymetrix.com). Each of the 11 probes is paired to a mismatch probe containing a mutation, this serves as a control for hybridization specificity. The Affymetrix probe sets represent a series of perfect-match and mismatch oligonucleotide pairs, allowing evaluation of non-specific binding, normalization of background and performance of the probes, providing a robust measure of gene expression. Affymetrix array use feature size of about 8 μm², thus the 1.2 cm² slide area contains millions of probes. Algorithms evaluate the signal from each perfect-match and mismatch oligonucleotide probe and provide a single signal value for the probe set (Affymetrix Inc.).

Microarray analysis was performed from mouse Neuro2A-20 cells, treated for 48h with 100 ng/ml of GDNF or from pooled samples of 12-16 mouse midbrain punctures from GDNF Hz and wt animals. For Neuro2A analysis the cDNA synthesis was carried out with SuperChoice (Invitrogen), biotin labelled cRNA was generated from cDNA using IVT kit (Enzo, Farmingdale, NY). Fragmented cRNA was hybridized to Affymetrix MGU74Av2 chips in triplicate for both treatment groups. Chips were scanned using the GeneChip Scanner (Affymetrix). For GDNF Hz and wt samples One-Cycle Target Labelling kit (Affymetrix) containing the cDNA synthesis kit, IVT labelling kit and hybridization controls was used. Slides were scanned using the GeneChip 3000 scanner (Affymetrix). Microarray data was analysed according to previously published methodology (Consales, Volpicelli et al. 2007) using the robust multiarray average algorithm and normalized by the quantile-quantile regression method. Additionally data was prefiltered using the Li-and-Wong algorithm and call calculation for each probe using the MAS-5 algorithm. Statistical analysis was carried out using the SAM software (Consales, Volpicelli et al. 2007).

3.2 RNA and DNA methods

RNA was isolated using either the Trizol method (Donovan, Lin et al. 2000), Ambion RNAqueous Micro kit (IV) or Qiagen RNeasy mini kit (unpublished). In article II and unpublished microarray experiments, cDNA synthesis was performed using TaqMan reverse transcription reagents and quantitative RT-PCR (qRT-PCR) was performed by SYBR green using the ABI Prism 7000 SDS system (all by Applied Biosystems). In article IV the cDNA synthesis was done with the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics), qRT-PCR was performed using Lightcycler 480 SYBR Green system (Roche Diagnostics).
3.3 Cell culture methods

Cell culture for validation of microarray experiments was performed \textit{in vitro} from Neuro2A-20 cells constitutively expressing RET and stably transfected with GFRα1. Primary cultures were performed as described in detail in article III, from E13.5 mouse embryo midbrain floors or P1 SCG neurons. The exact growth factor concentrations used are listed in respective publications (III, IV).

3.4 Immunological methods

GDNF concentrations were measured by ELISA methodology, using the GDNF Emax immunoassay system (Promega) (I). Punctures of striatal tissues or embryonic dopaminergic cultures (IV) were lysed using lysis as described in (Runeberg-Roos, Virtanen et al. 2007). Lysed tissue protein extracts were processed for Western blotting as described in detail in articles II and IV.

TH immunofluorescence staining was performed as described (III, IV). RECA-1 immunohistochemistry was performed from free-floating cryosections using the reagents from the blood vessel staining kit, ECM 590 (Chemicon/Millipore) (IV).

3.5 \textit{In silico} methods

The primers were designed using either ABI Prism Primer Express software (Donovan, Lin et al.2000) or Roche Universal ProbeLibrary Assay Design Center (IV). The qRT-PCR data was validated using qRT-PCR with ABI Prism 7000 software (Applied Biosystems) (Donovan, Lin et al. 2000).

Table 5. List of growth factors used in this study.

<table>
<thead>
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<th>Growth factors</th>
<th>Source</th>
<th>Publications</th>
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<tr>
<td>NRTN</td>
<td>PeproTech Ltd.</td>
<td>III</td>
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<td>VEGF-C</td>
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<tr>
<td>HB-GAM</td>
<td>Prof. H. Rauvala</td>
<td>III</td>
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Table 6. List of antibodies used in this study.

<table>
<thead>
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<th>Antigen</th>
<th>Antibodies</th>
<th>Source</th>
<th>Publication</th>
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<tr>
<td>TH</td>
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<td>Millipore</td>
<td>II</td>
</tr>
<tr>
<td>DAT</td>
<td>monoclonal</td>
<td>Millipore</td>
<td>II</td>
</tr>
<tr>
<td>TH</td>
<td>polyclonal</td>
<td>Chemicon</td>
<td>III, IV</td>
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<td>p-ERK</td>
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<td>Sigma</td>
<td>IV</td>
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<td>ERK</td>
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<tr>
<td>RECA-1</td>
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<tr>
<td>c-RET</td>
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<tr>
<td>Phosphotyrosine</td>
<td>monoclonal</td>
<td>Upstate</td>
<td>IV</td>
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</table>
al. 2000) or Lightcycler 480 software (IV) followed by analysis with the comparative threshold cycle (CT) method 2-ΔΔCT (II, IV). Microarray raw data were initially analysed using MAS 5.0 software followed by data mining using Genespring software (Agilent Technologies/ Silicon Genetics). Statistical analysis of data is described in respective articles.

DA neuronal survival in culture was analysed by taking a picture of the whole micro-island area using a fluorescence stereo-microscope (Leica MZ FL III), at 3.2 x magnification with the GFP filter. Background normalization was performed in Adobe Photoshop software version 6.0.1, followed by quantification of cell number in Image Pro 5.1 software (Media Cybernetics) (III).

RET phosphorylation and pERK activation data were measured in AIDA Image Analyzer software (Raytest). Densitometry quantification was performed using the histogram profiles which were measured for all bands (IV).
4. RESULTS AND DISCUSSION

4.1 GDNF induced genes in cell culture

Microarray analysis of Neuro2A-20 cells treated with 100 ng/ml of GDNF for 48h was performed using Affymetrix mouse genome U74Av2 chips in three replicates for each group. Data analysis revealed gene expression changes over 1.6 fold for 39 genes, of which 25 were up-regulated and 14 down-regulated following GDNF treatment. Of these, 18 differentially regulated genes were selected for confirmation using qRT-PCR from independent experiments (Fig.5). The correlation between microarray data with qRT-PCR results was 94% with only one gene remaining unchanged according to qRT-PCR measurement. Thus the GDNF regulated genes detected by microarray analyses showed similar regulation as measured by qRT-PCR, although the exact level of fold change varied slightly between the two techniques. In regard to functional characterization of GDNF-induced genes, two distinctive processes can be distinguished among the up-regulated genes: the immunological and vascular processes. The genes of the immune system up-regulated by GDNF treatment include: interleukin 6 (IL-6) – the top most up-regulated gene, prostaglandin Endoperoxide synthase 1 (COX1), VEGF-C, endothelial protein C receptor, annexin A2, cytotoxic T lymphocyte-associated protein 2α, sphingosine-1-phosphate-phosphatase, T-cell death associated gene, cytotoxic T lymphocyte-associated protein 2β, S100 calcium binding protein A10 (calpactin), cytokine inducible SH2-containing protein, prostaglandin I receptor and urokinase plasminogen activator receptor. Genes of vascular processes up-regulated by GDNF included: VEGF-C, endothelial protein C receptor, annexin 2, tissue plasminogen activator, FGF-regulated protein, calpactin and urokinase plasminogen activator receptor.

Classically the immune system has been considered to function independently from the nervous system and the immunological processes have not been associated with normal brain functioning, rather characterized as part of pathogenic mechanisms activated in the brain following BBB disruption during pathological conditions in the CNS. There is growing evidence on the role of immunogenic proteins in normal functioning of the nervous system including stem cell renewal, cell fate decisions, neuronal differentiation and synaptic plasticity (McAllister and van de Water 2009). Molecular cross-talk between the immune and nervous systems has been demonstrated, including secretion of several overlapping factors, which are not restricted to either system. The factors displaying pleiotrophic roles in the immune and nervous system include NTFs (NGF, GDNF family ligands, neuroopoietic cytokines – IL-6, CNTF, LIF), cytokines and chemokines (Kerschensteiner, Meinl et al. 2009). The cytokines have been regarded to function in two distinct ways as: a) proinflammatory cytokines - which aggravate the pathogenic processes acting destructively on neuronal pathways and b) anti-inflammatory...
matory cytokines – which act neuroprotectively. However, this distinction might not hold true, as depending on the timing and context the same cytokines have been shown to exhibit either neuroprotective or neurodestructive effects. The inflammatory process in the CNS, in addition to the well-known neurotoxic effect, can also result in neuroprotection. The balance between protective and destructive factors is regarded to be the basis of neural-immune functioning and provides the outlook for possible future therapeutic implications (Kerschensteiner, Meinl et al. 2009; McAllister and van de Water 2009). In addition, there seems to be functional resemblance between the immune and neuronal system, as the immunological synapses, similarly to neuronal synapses, are asymmetric structures between two cells that secrete molecules to the effector cells and interestingly they share the same proteins and adhesion molecules that regulate specificity of synapse formation, cytoplasmic signalling and interactions (Yamada and Nelson 2007; McAllister and van de Water 2009).

We could further classify the genes up-regulated by GDNF in our study to be related to the vascular processes. These genes included: VEGF-C, endothelial protein C receptor, annexin 2, tissue plasminogen activator, FGF-regulated protein, calpactin and urokinase plasminogen activator receptor. Since no data existed on the effects of VEGF-C in the nervous system and very little was known about the regulation of the innervation of lymphatic vessels, we focused on VEGF-C and its possible link to the nervous system, which provided the basis for further

Results and Discussion

Figure 5. GDNF induced genes in Neuro2A cells. Comparison of the fold-change levels of 16 genes most regulated by GDNF, as measured by microarray and qRT-PCR analysis.
A similar experimental setup was performed using treatment of Neuro2A-20 cells with 100 ng/ml of NRTN, however this experiment did not result in any significant gene expression changes above 1.5 fold.

### 4.2 GDNF induced genes in animal models

In order to further characterize GDNF signalling using samples from *in vivo* GDNF models, we performed microarray analysis from pooled SN/VTA samples, isolated from adult GDNF Hz and wt mouse brains. The Affymetrix mouse genome 430 2.0 microarrays, with 39,000 transcripts, were tested with three pooled samples (n=7-8 per pool) for both the GDNF wt and Hz group. When setting the fold change cut-off at 1.5 we found very few significantly changed genes. In order to test the differences between our samples, we performed correlation analysis on the results obtained from the microarrays and found that two of the wt samples grouped together with the Hz samples and one wt sample differed remarkably from the other wt samples. We concluded, that the dissection and pooling of midbrain samples had been problematic in our experimental setup, which could have masked the real differences in gene expression and diluted the measurable effects of GDNF deletion in these animals. Despite these issues it was of interest for us to compare the gene expression levels of the least similar of the wt experiment to the mean of expression levels of the three Hz chips, in order to see if we could find any GDNF associated genes of scientific interest. This analysis resulted in 583 differentially regulated genes, with 358 up-regulated and 225 down-regulated genes. The results of this analysis were further classified into functional groups using the Gene Ontology (GO) classification (Table 7).

We found a long list of highly relevant changes, including genes related to signalling, apoptosis, dopaminergic and glutamatergic system, ion channel related genes, vesicular trafficking genes as well as growth factors. Remarkable was the fact that when comparing the resulting gene lists of the GDNF Hz animals to the Neuro 2A-20 results, we found none of the genes overlapping between the two experiments. On the other hand GDNF treatment and lack of GDNF are rather different experimental conditions, still one would expect at least some overlap between the results.

In addition, we studied the gene expression lists created by other groups who had been studying GDNF-induced gene expression by microarray analysis. Four different microarray studies on GDNF-induced gene expression have been published. Two studies have been performed on spermatogonial stem cells (Braydich-Stolle, Nolan et al. 2005; Schmidt, Avarbock et al. 2009). One study has been performed on embryonic rat midbrain dopaminergic cultures, using acute stimulation and treatment with 10 ng/ml of GDNF for 3h, utilizing the Affymetrix platform (Consales, Volpicelli et al. 2007). The fourth study was performed on embryonic and postnatal intact dorsal root ganglion neurons treated with 20 ng/ml of GDNF for 2h, with
Table 7. Functional classification of GDNF induced genes in GDNF Hz midbrain samples.

<table>
<thead>
<tr>
<th>DOWNREGULATED GENES</th>
<th>FC</th>
<th>UPREGULATED GENES / RECEPTORS</th>
<th>FC</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROWTH FACTORS/RECEPTORS</td>
<td></td>
<td>GROWTH FACTORS / RECEPTORS</td>
<td></td>
</tr>
<tr>
<td>brain-derived neurotrophic factor</td>
<td>2.1</td>
<td>ret proto/oncogene</td>
<td>1.9</td>
</tr>
<tr>
<td>decorin</td>
<td>1.6</td>
<td>growth hormone</td>
<td>2.7</td>
</tr>
<tr>
<td>kit oncogene (stem cell factor receptor)</td>
<td>1.6</td>
<td>neurotrophic growth inhibitory factor (metallothionein 3)</td>
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<tr>
<td>insulin-like growth factor I receptor</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>insulin-like growth factor II</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>neuregulin I (glial GF 2)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SIGNALLING</td>
<td></td>
<td>GROWTH FACTORS / RECEPTORS</td>
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</tr>
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<td>sprouty homolog 2</td>
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<td>guanine nucleotide binding protein, β2, related seq</td>
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</tr>
<tr>
<td>sprouty homolog 4</td>
<td>1.8</td>
<td>Rap1, GTPase-activating protein 1</td>
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</tr>
<tr>
<td>phosphatase and tensin homolog</td>
<td>2.1</td>
<td>regulator of G-protein signalling 10</td>
<td>1.6</td>
</tr>
<tr>
<td>SOCS2</td>
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<td>protein kinase, cAMP dependent, catalytic α</td>
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</tr>
<tr>
<td>protein phosphatase 3 - calcineurin A</td>
<td>2.2</td>
<td>protein kinase, cAMP dependent</td>
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</tr>
<tr>
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<td>regulatory, type Iβ</td>
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</tr>
<tr>
<td>protein phosphatase 1 – myosin phosphatase</td>
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<td>protein tyrosine phosphatase, receptor type S</td>
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</tr>
<tr>
<td>ubiquitin protein ligase E3A</td>
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<td>phosphatidylinositol glycan, class Q</td>
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<tr>
<td>ubiquitin-conjugating enzyme E2D 2</td>
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<td>MAPK 8 interacting protein 3</td>
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<tr>
<td>similar to ubiquitin specific protease 1</td>
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<td>protease, serine, 18</td>
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<td>ubiquitin carboxyl-terminal esterase L5</td>
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<td>serine/threonine kinase 39, STE20/SPS 1 homolog</td>
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</tr>
<tr>
<td>proteasome 16S subunit 12</td>
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<td></td>
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<td></td>
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<td>pleckstrin homology domain-containing family A member 3</td>
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<td>inositol triphosphate receptor type 2 gene</td>
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<td></td>
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<td>neurogranin (PK C substrate)</td>
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</tr>
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<td>cAMP-dependent protein kinase inhibitor regulator of G-protein signalling 16</td>
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<tr>
<td>serine/threonine kinase 18</td>
<td>1.5</td>
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<td></td>
</tr>
<tr>
<td>KH domain containing, signal transduction associated 3</td>
<td>1.5</td>
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<td>RAB 10</td>
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<td>GLUTAMATE SYSTEM</td>
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<td>DOPAMINE AND GLUTAMATE SYSTEM</td>
<td></td>
</tr>
<tr>
<td>GABA-A transporter 1</td>
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<td>engrailed 1</td>
<td>2.4</td>
</tr>
<tr>
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<td>dopamine receptor 2</td>
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</tr>
<tr>
<td>glutamate receptor, ionotropic, AMPA3 (α3)</td>
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<td>tyrosine hydroxylase</td>
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<td>dopamine transporter</td>
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<td>DA and cAMP regulated phosphoprotein (DARP32)</td>
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<tr>
<td></td>
<td></td>
<td>glutamate dehydrogenase</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GABA/B receptor 1</td>
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<tr>
<td></td>
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<td>glial fibrillary acidic protein</td>
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Table 7. cont.

<table>
<thead>
<tr>
<th>ION RELATED</th>
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<td>voltage/dependent Na⁺ channel β1</td>
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<td>subunit gene</td>
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<tr>
<td>solute carrier family 31 (copper transporter), member 1</td>
<td>supressor of K⁺ transport defekt 3</td>
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<td>solute carrier family 24 (Na/K/Ca exchanger), member 3</td>
<td>hyperpolarization activated cyclic nucleotide gated K⁺</td>
</tr>
<tr>
<td>ryanodine receptor 3 (i/c Ca store releaser)</td>
<td>plasmolipin</td>
</tr>
<tr>
<td>ATPase, H⁺ transporting lysosomal (vacuolar proton pump)</td>
<td>Ca channel, voltage dependent, subunit 4</td>
</tr>
<tr>
<td>K inwardly-rectifying channel, subfamily J, member 9</td>
<td>Cu/Zn superoxide dismutase</td>
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<tr>
<td>voltage-dependent anion channel 2</td>
<td>Ferritin heavy chain</td>
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<table>
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<th>VESICULAR TRANSPORT</th>
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<tr>
<td>caspase 8 associated protein 2</td>
<td>secretory carrier membrane protein 2 - SCAMP 2</td>
</tr>
<tr>
<td>Bcl2-associated athanogene 3</td>
<td>secretory carrier membrane protein 5 - SCAMP 5</td>
</tr>
<tr>
<td>Bcl2-interacting killer-like</td>
<td>EST, highly similar to transferrin lipocalin 2</td>
</tr>
<tr>
<td>cytochrome c oxidase, subunit XVII assembly protein homolog</td>
<td></td>
</tr>
</tbody>
</table>

The Affymetrix platform (Linnarsson, Mikaels et al. 2001). Quite surprisingly comparison of differentially regulated gene lists did not reveal any common pathways or genes between these and our studies. The above described different microarray analysis raised a number of questions: Why were there so few differentially regulated genes? Why were the gene expression fold change levels so low? Why were there so few common genes between different experimental setups of GDNF functioning?

Of possible explanations to the lack of common changes in gene expression, could be that all the experiments were conducted on different cell lines or neuronal systems, the used concentrations of GDNF protein varied and also the treatment times used were different. For example in our Neuro2A-20 cell culture experiment we used a 48 h time-point for GDNF treatment, expecting the results to reveal changes in genes which are involved in GDNF mediated cellular differentiation processes. Shorter treatment times would have most probably pinpointed immediate early genes and transcription factors associated with GDNF-related processes.

The above raised questions could also be explained by the highly complex nature of the nervous system. One issue is the heterogeneity of neuronal samples – cell culture samples and brain tissues contain a pheno- and genotypically diverse populations of cells, thus the gene expression changes in a specific subpopulations of cells might be masked by expression signals from accompanying cells. The heterogeneity causes a signal-to-noise problem in the detection of gene expression changes from a specific cell type (Henry, Zito et al. 2003; Mirnics and Pevsner 2004). This could be one reason for the lack of
overlap between our different experimental results. One possible solution to this aspect might be achieved by the single cell laser capture technique, where the specifically stained cells are laser capture punctured from surrounding tissue and the RNA is amplified and gene expression analysis performed on this preselected homogenous subgroup of cells. Another issue is that in mature nervous system de novo gene expression induction and repression is rarely seen, thus the changes are too small to be detected above the background noise (Henry, Zito et al. 2003; Mirnics and Pevsner 2004). This could explain the low levels of fold changes in our experiments. Another aspect is that neurons project to remote areas, thus the site of sampling is of critical importance. Although the changes in gene expression might take place in the cell body, the results of this change might be reflected in the effector regions (Henry, Zito et al. 2003; Mirnics and Pevsner 2004). Relevant to the GDNF Hz studies might also be the endogenous compensatory changes which have taken place following the 40-50% reduction in GDNF levels (Pas- cual, Hidalgo-Figueroa et al. 2008), thus the true changes regarding the GDNF effect might not have been reflected in our analysis.

In conclusion, changes in GDNF-induced gene expression seem to be highly dependent on the experimental setup including the concentration of the factor used, the type of cells under study and the duration of treatment. In addition, compensatory changes from the surrounding environment can mask the specific signal from certain cell types, as the magnitude of change in gene expression levels of neuronal systems does not seem to be very high. Our microarray gene expression analysis suggests that GDNF intervention could induce crosstalk on the molecular level between different physiological systems, including vascular and inflammatory systems.

4.3 Dopaminergic system of GDNF heterozygous mutant mice

We further studied the effects of depletion of one allele of GDNF gene on the dopaminergic system. Initially the GDNF levels were measured in ventral and dorsal striatums of GDNF Hz and wt animals using protein measurement by ELISA. The results showed a significant reduction by 44% (p-value-0.009) in the GDNF protein level in the dorsal striatum of GDNF Hz mice as compared to wt littermates. An 18% reduction was also present in ventral striatum, however this change was not significant (p-value-0.085).

The brain dopamine concentrations can be measured in two different ways. The first being measurement of post-mor tem striatal tissue concentration using high-performance liquid chromatography (HPLC) with electrochemical detection. This method is used to study the chemistry of a tissue on the intracellular level. The other option is to measure the in vivo extracellular concentration of a molecule using microdialysis. Using this method the concentrations can be measured over an extended period of time in living and moving animals. As the post-mor tem tissue concentrations of dopamine in GDNF Hz animals have been shown to be unaltered (Gerlai, McNamara et al. 2001) we
decided to monitor the in vivo extracellular dopamine levels using the microdialysis method. These studies revealed that the extracellular dialysate concentrations were significantly elevated in the GDNF Hz mice compared to wt by 2-fold in CPu and by 1.6-fold in the NAc. The dopamine metabolites DOPAC and homovanillic acid (HVA) showed no significant increase in GDNF Hz animals.

FosB/ΔfosB levels have been shown to be increased following repeated use of drug of abuse (Nestler 2005). In order to study the postsynaptic activity in GDNF Hz animals FosB immunoreactivity was measured by immunostaining. An increase in the level of FosB/ΔfosB nuclei was 5.7 fold in CPu and 2.2 in NAc core and 1.6 fold in NAc shell of the GDNF Hz mice compared to wt. This elevation could be linked to the increased extracellular dopamine levels in these regions and indicate sustained postsynaptic activation of these areas. No significant change was seen in behavioural experiments in basal locomotor activity.

The finding that extracellular dopamine levels are increased by two fold in GDNF Hz animals, who exhibit 44% reduction in GDNF levels, is unexpected. It is unlikely that the increased extracellular DA levels are the result of changes in dopamine synthesis, storage or metabolism as these changes should also have an effect on total tissue levels of DA, which remain unchanged in GDNF knockout and heterozygous embryos (Moore, Klein et al. 1996; Pichel, Shen et al. 1996; Sanchez, Silos-Santiago et al. 1996). Several mechanisms can be considered as the underlying cause for this increase in GDNF Hz animals. The most likely explanation is that elevation of extracellular DA could be a compensatory change, in order to enhance the synthesis of endogenous GDNF. Regulation of GDNF synthesis by dopamine through D1-receptor has been demonstrated (Ohta, Mizuta et al. 2000; Ohta, Kuno et al. 2003). Another possible explanation is that synapse formation in the GDNF Hz animals could be decreased and that increase in dopaminergic output is a compensatory effect for normal neuronal functioning. GDNF is considered to induce axon outgrowth, synapse formation and efficacy of dopaminergic neurons and it is likely that axon branching and synapse formation differ in GDNF Hz animals as compared to wt (Bourque and Trudeau 2000; Granholm, Reyland et al. 2000; Airaksinen and Saarma 2002). In conclusion, the results of elevated dopamine levels in striatal and limbic brain areas in animals with decreased levels of GDNF, indicate activation of neuronal networks and the importance of GDNF signalling in the dopaminergic system.

4.4 Dopaminergic system of MEN2B knock-in mice

In order to further study the RET signalling in vivo, the dopaminergic system of the adult MEN2B (M/M) animals was investigated. In these animals the Met919Thr mutation has been introduced, leading to continuous activation of
RET tyrosine kinase. These animals thus represent a model corresponding to the permanent activation of Ret by GFLs. The homozygous M/M animals develop C cell hyperplasia by the age of 6-10 months, but pheochromocytoma does not develop before 3 months of age. We used the animals at the age of 10-12 weeks, to avoid influence of the pathological abnormalities in the investigation of the dopaminergic system.

Initially the monoamine – dopamine, noradrenaline and serotonin – extracellular concentrations in wt, M/+ and M/M animals were measured by HPLC with electrochemical detection. A robust increase of 100% in DA levels in striatum of M/M homozygous mice was discovered, whereas the heterozygous M/+ animals showed about half increase (47%) as compared to wt animals. A similar significant increase in striatal DA metabolite levels was measured in M/M and M/+, as compared to wt animals: 148% and 108% for DOPAC and HVA respectively in M/M animals and 50% and 30% for DOPAC and HVA in M/+ animals. The cortical measurements showed increased DA levels of about 70% in both homozygous and heterozygous animals, whereas in the hypothalamus about 30% increase in DA levels was present only in M/M animals. No significant changes were detected in serotonin levels in any brain region and a 27% change in noradrenaline levels was visible only in the lower brainstem of M/M animals.

To understand the possible mechanisms of elevated DA levels, we further investigated the dopaminergic system of MEN2B animals on the protein level. Immunohistochemistry was performed on free-floating striatal sections from M/M, M/+ and wt animals. The striatal TH-protein optical density measurements showed increased levels of TH by about 75% in M/M and by 55% in the M/+ genotype, as compared to wt. Dopamine transporter (DAT) staining was increased similarly by about 50% in striatal sections for both M/+ and M/M genotypes as compared to wt. Further Western blotting for both TH and DAT levels was performed from striatal and SN/VTA punctures from all three genotypes. M/+ and M/M animals had a gene dose-dependent increase in TH protein levels in both striatal and SN/VTA samples. An increase was also visualized for DAT levels in striatal samples for both genotypes as compared to wt. Quantification of TH protein expression levels in SN/VTA Western blotting showed a 180% increase for M/M animals and 60% increase for M/+ animals as compared to wt.

Gene expression levels for Th and Dat were measured using quantitative RT-PCR from SN/VTA samples of the M/M and wt genotypes (Fig.6). A statistically significant increase of 2.5 fold in Th mRNA expression levels was detected for M/M animals as compared to wt. Although Dat mRNA expression change of 2.7 fold was also detected for M/M animals, no statistical significance was reached. The expression levels of other genes were studied in samples from M/M and wt SN/VTA, including Gdnf, Gfra1, Dopa decarboxylase and dopamine D1 receptor, however no change in expression was seen. Also the Gdnf mRNA expression levels were unchanged in striatum of MEN2B/MEN2B animals as compared to wt littermates.
The increase in DAT and TH mRNA and protein levels of MEN2B animals is in line with other studies, which have shown that exogenously administered GDNF leads to higher DA levels and DA turnover in vivo, as well as increases Th mRNA expression and stability in vitro (Hudson, Granholm et al. 1995; Martin, Miller et al. 1996; Gash, Zhang et al. 2005). Also GDNF has been shown to enhance the phosphorylation of TH in the striatum and SN of rats (Salvatore, Zhang et al. 2004). However, studies of chronic treatment of adult rats with GDNF have shown no changes in DA concentrations and rather decreased tyrosine hydroxylase mRNA and protein expression (Rosenblad, Georgievksa et al. 2003; Georgievksa, Kirik et al. 2004). The possible explanation for this discrepancy could be that in the above cases very high level of exogenous GDNF was expressed in the mature adult dopaminergic system and compensatory mechanisms can be activated to maintain the normal neuronal functioning, whereas in the MEN2B animals the continuous Ret signalling is permanent from early developmental stages.

Next, we hypothesized that the above described changes were due to increased DA cell number in the SN and VTA. TH immunochemical staining followed by stereological analysis was performed on dopaminergic neurons of SNpc and VTA. A statistically significant (26%) increase in TH-positive cell number was visualized in homozygous M/M animals in SNpc cells as compared to wt animals, but no statistically significant elevation in dopamine cell number was seen in heterozygous M/+ animals. No differences in TH-positive cell numbers for the three genotypes was found in VTA dopaminergic neurons, thus the RET-dependent increase in dopamine cell number seems to be confined to the nigral DA neurons. Further, we decided to investigate striatal DAT-positive varicosities for sprouting of dopaminergic neurons. However, although we could visualize an increase of 20% in the number of DAT-positive terminals in homozygous M/M animals as compared to wt, no increase was seen in heterozygous M/+ animals. Further behavioural experiments assessing locomotor activity were performed on MEN2B animals. Spontaneous locomotor activity in nonhabituated animals declined in all three genotypes during the 60 minutes, however, both M/M and M/+ animals showed similar decreased locomotor activity levels as compared to wt.

![Figure 6](image)

**Figure 6.** qRT PCR from wt and M/M animals for Th and Dat mRNA. Changes in gene expression levels for Th and Dat in M/M animals were measured as fold change of wt samples. The fold change level for Th was up-regulated by 2.5 fold (p=0.04) and for Dat by 2.7 fold (p=0.067). n=5-6 in each group. * p<0.05 students t-test.
Interestingly, no differences in the three genotypes appeared in habituated animals during 24 hours.

Thus our study on the dopaminergic system of MEN2B animals revealed a robust increase in DA and metabolite levels in the mesolimbic, mesocortical and nigrostriatal systems of these animals. This effect seems to be specific and confined to the dopaminergic system, as no significant elevation in serotonin or noradrenaline levels was seen in the MEN2B mice. The increased levels of DA can be partly explained by increased nigral dopaminergic cell numbers, however this was evident only in the homozygous animals. Thus it is likely that the above mentioned changes are mostly due to increased dopamine synthesis, which is further supported by increased TH mRNA and protein levels. GDNF and RET signalling is not considered to be important for prenatal development of nigral dopaminergic neurons (Marcos and Pachnis 1996; Moore, Klein et al. 1996; Pichel, Shen et al. 1996; Sanchez, Silos-Santiago et al. 1996; Enomoto, Araki et al. 1998), but rather to function during early stages of postnatal apoptotic cell death (Burke, Antonelli et al. 1998; Oo, Kholodilov et al. 2003; Kholodilov, Yarygina et al. 2004). Thus the increased number of dopaminergic neurons in MEN2B animals could result from rescuing of dopaminergic neurons and suppression of apoptosis by continuous activation of RET. Another possibility is that overexpression of RET during embryonic development causes developing neurons to acquire the dopaminergic phenotype.

Mijatovic et al. have further studied the mechanisms of increased DA levels in MEN2B animals and found this to result from enhanced synthesis of DA leading to increased storage and release from pre-synaptic terminal pools. Also higher re-uptake of DA has been shown in these animals, leading to unchanged basal extracellular DA levels (Mijatovic, Pattrikainen et al. 2008; Mijatovic 2009). In addition, neurotoxin PD model studies have been performed in MEN2B animals. The unilateral 6-OHDA injection to striatum showed significant resistance and lower TH-positive nigral neuronal loss in the MEN2B mice. The response to systemic MPTP was unaltered in the MEN2B animals as compared to wt, however this could have been due to utilization of the “milder” toxin model (Mijatovic, Piltonen et al. 2011). This study concluded that RET signalling is important for GDNF-induced neuroprotection of DA cell bodies but not for striatal dopaminergic nerve terminals (Mijatovic 2009; Mijatovic, Piltonen et al. 2011).

4.5 A new method to assay the survival of dopaminergic neurons in vitro

In order to initially assess the dopaminotrophic properties of a protein, factor or a chemical compound, quantification of the survival of rodent midbrain neurons in culture is often performed. However, all current techniques are limited due to the scarcity of material, assay unreliability and variability, low throughput and statistical power, arduous workflow, human error in quantification as well as difficulties in applications to KO studies. We have further enhanced the micro-island culturing technique, initially described by
Takeshima et al. (Takeshima, Shimoda et al. 1996), by creating a standardized template to form micro-islands of a defined size, followed by computer based quantification of cell survival using two different parameters – TH intensity level and TH-positive cell number. This method has been validated by quantification of survival of GDNF treated cultures, resulting in highly significant survival rate in the treatment group with \( p \)-value up to \( 10^{-10} \).

The quality of stained cultures enables the capture of high magnification images for quantification of neurite outgrowth, as demonstrated in publication III. The sensitivity of the method allows studies on additive effects of different factors, as demonstrated by co-treatment with GDNF/NRTN and GDNF/HB-GAM. Another benefit of the method is the possibility to culture embryonic midbrains from single KO or transgenic animals where the genotype is unknown at the time of isolation, resulting in 10 cultures per genotype. This has been impossible previously due to the limited amounts of sample material. We demonstrate the applicability of this method on RET KO animals, where surprisingly we observed an increase in TH levels but not in TH-positive cell number in the RET KO group. This indicates to-non-RET signalling, which affects TH expression levels but does not affect the number of surviving dopamine cells. Although the unchanged cell survival rate in RET KO has been demonstrated previously (Tara-viras, Marcos-Gutierrez et al. 1999), the increase in TH levels in non-RET signalling has not been described and warrants further studies.

In addition, the method is applicable on parallel functional studies of DA metabolism as demonstrated by DA uptake measurements followed by GDNF treatment. Finally, the method is applicable on other neuronal tissue cultures with limited amounts of material, as demonstrated by performing micro-island cultures from post natal day 1 SCG neurons.

This enhanced micro-island culture method with computer assisted image analysis aids in sensitive evaluation of the dopaminotrophic properties of proteins, chemical compounds or their combinations. Additionally, data from single embryos can be analysed and parallel functional studies performed. In case of automation, the method can be utilized for small scale screens of chemical libraries on primary dopaminergic neurons.

4.6 Neurotrophic effects of VEGF-C in the dopaminergic system

In order to study the neurotrophic effects of VEGF-C on the dopaminergic system, in vitro embryonic mesencephalic midbrain cultures from E13.5 mice were prepared. The cultures were plated using the above described micro-island method and treated with 100 ng/ml of VEGF-C, 10 ng/ml of GDNF or both factors simultaneously for 5 days. Quantification of TH-positive neurons revealed a significant increase of 13% in the VEGF-C treated and up to 40% increase in the GDNF treated wells, as compared to control buffer treatment (Fig.7). Thus VEGF-C effect on embryonic dopamine neurons seems to be about half of the effect of GDNF. However, no further additive effects were
detected for the simultaneous treatment with the two factors. This lack of an additive effect indicates that in embryonic dopamine neurons VEGF-C and GDNF affect the same population of neurons.

Gene expression levels for GDNF and VEGF-C signalling receptors and associated genes were measured by quantitative RT-PCR from both the embryonic cultures as well as from adult mouse SN samples. The mRNA from embryonic dopamine cultures and adult mouse midbrains expressed both Vegfr-1 and -2, but Vegfr-3 was virtually not expressed. The VEGF family co-receptors Nrp1 and Nrp2 mRNAs were expressed at relatively high levels in both embryonic and adult samples. In addition, the gene expression levels of Vegf-C, Th, Vegfr-1,-2 and -3 were measured by qRT-PCR from rat brain treated with VEGF-C (20 μg) for 8h. This treatment resulted in up-regulation of all Vegfr levels in SN by 1.5, 2.1 and 2.0-fold, respectively, for Vegfr-1,-2 and -3. A slight up-regulation of 1.6-fold was also detected for Vegf-C, whereas the Th expression remained unchanged. A similar result was seen in embryonic midbrain cultures treated with VEGF-C (100ng/ml) for 48h, where Vegfr-1 was up-regulated by 2.1 and Vegfr -3 by 1.7-fold. Vegfr-2 mRNA levels in this setup remained unchanged. VEGF-C binds preferably to VEGFR-3, however, binding to VEGFR-2 has been also demonstrated and is associated with the angiogenic effects of VEGF-C. VEGFR-3 is highly expressed during development, but in adult mice its expression is restricted to lymphatic endothelium. VEGFR-3 seems to function during the formation of new lymphatic vessels, as mature lymphatic vessels do not seem to depend on VEGFR-3 (Lohela, Bry et al. 2009). VEGR-2 is expressed on lymphatic and endothelial cells, but also on other cells including neurons (Tammela, Enholm et al. 2005). Our results indicate that VEGF-C can act via up-regulating the expression of its receptor in these neuronal systems.

We further decided to study the VEGF-C signalling in the nervous system, as crosstalk between RET and VEGFR-2 in a cell line has been described previously (Tufro, Teichman et al. 2007). Due to technical difficulties we could not perform the RET phosphorylation assay from embryonic DA cultures, thus we tested

**Figure 7.** VEGF-C effect on the survival of dopamine neurons in culture *in vitro*. Quantification of TH positive neuronal survival as percentage from untreated control samples. VEGF-C 100 ng/ml induced survival of 13% as compared to untreated samples. GDNF 10 ng/ml induced 40% more survival as compared to control. Simultaneous addition of 100 ng/ml of both VEGF-C and 10 ng/ml GDNF did not result in further significant neuronal survival as compared to GDNF alone (p=0.6). Means ±SEM are shown; n=32-68 in each group. * p<0.01 , ** p<0.001 treatment versus the control sample.
different cell lines (MG87, MN9D, Neuro 2A-20) for RET phosphorylation. However, in our hands 100 ng/ml of VEGF-C treatment for 10 minutes did not cause an increase in RET phosphorylation compared to untreated samples in any of the cell lines tested, although 100 ng/ml of GDNF induced a robust phosphorylation of RET. We then investigated the involvement of MAPK/ERK downstream signalling pathways in embryonic midbrain cultures treated with VEGF-C (100ng/ml) for 45 minutes, which resulted in slight phosphorylation of ERK as compared to the robust effect of GDNF treatment. This result was confirmed in samples from adult rat striatum treated with VEGF-C (20 μg/per rat) for 1h, which induced a significant 24% (p=0.024) increase in ERK phosphorylation as compared to contralateral vehicle injected striatum.

Further behavioural studies concentrated on the in vivo role of VEGF-C in a rat model of PD. The neuroprotection studies measured rotational behaviour when pre-treatment with VEGF-C, GDNF or a combination of the two factors was performed prior to the 6-OHDA lesion. A reduction in the ipsilateral rotations between the control group and the 30 μg VEGF-C treatment group was seen at 2 weeks (p=0.012) and the reduced rotational behaviour was highly significant (p=0.004) at 4 weeks post-treatment. At six weeks all groups treated with VEGF-C showed less rotation, however these results were not statistically significant. Our results show that VEGF-C can act as a neuroprotective molecule for nigral DA neurons in vivo.

The TH-positive cells were counted in the SN of the rats used in neuroprotection studies and the combination group receiving the simultaneous administration of VEGF-C and GDNF showed the most effective protection of nigral neurons by 97% as compared to 61% of the control group (p=0.004). GDNF treatment alone showed a neuroprotective effect of 84% (p=0.004) and VEGF-C treatment showed an effect of 70-73%, which was statistically not significant (p=0.056).

To quantify the nerve endings in the striatum of rats following 6-OHDA lesions optical density of TH-immunoreactivity can be measured. The optical density was higher in all the growth factor treatment groups – 56 to 58% for VEGF-C (p>0.05), 70% for GDNF (p=0.012) and 67% for the combination of VEGF-C and GDNF (p>0.05), as compared to 38% for the control group (IV).

We further evaluated whether the neuroprotective effects of VEGF-C could be mediated by indirect angiogenesis.

**Results and Discussion**

Figure 8. VEGF-C induces ERK phosphorylation in embryonic midbrain cultures. Activation of MEK/ERK pathway is very mild following VEGF-C treatment as seen by Western blotting. Treatment of E13 midbrain dopaminergic cultures with VEGF-C for 45 min resulted in slight phosphorylation of ERK, GDNF treatment induced robust phosphorylation of ERK.
in vivo was measured in the striatums of 6-OHDA-lesioned rats in the VEGF-C treatment groups, with 12% \( (p=0.041) \) more blood vessels in the VEGF-C 20 μg treatment group. In addition, the tube formation assay for VEGF-C was performed in HUVEC cells, which showed that VEGF-C (100ng/ml) could induce tube formation at 2h \( (p=0.001) \) whereas GDNF treatment results did not differ from the control samples.

Further immune markers staining for astrogliosis and microgliosis was measured from adult rat striatum injected with 20 μg VEGF-C, 3 days after administration. VEGF-C injection induced a robust increase in glial fibrillary acidic protein (GFAP) staining \( (p=0.003) \), however, no significant change in GFAP staining was seen in selected striatums from the neuroprotection experiments in 6-OHDA treated animals in comparison between the vehicle, VEGF-C and GDNF treatment, although prominent astrogliosis was seen around the needle in all treatment groups. Also the staining for microglial marker Iba-1 was measured in VEGF-C treated rats and prominent microglial activation was observed following 20 μg VEGF-C treatment \( (p=0.001) \). BBB disruption was studied from the VEGF-C injected rat brains. There was clearly less EBA-immunoreactive blood vessel staining around the needle cavity in VEGF-C treated striatums as compared to the control side \( (p=0.001) \), which is indicative of BBB disruption following VEGF-C treatment.

The lymphangiogenic properties of VEGF-C are well established, however this study was the first time the neurotrophic effects of VEGF-C in the dopaminergic system have been evaluated.

### 4.7 GDNF effects in the vascular system

Regarding the evaluation of vascular events related to GDNF signalling, we initially hypothesized that GDNF by up-regulating VEGF-C might affect the vasculo-lymphatic system and regulate the innervation of vasculature. We performed analysis of GDNF KO and wt animal vascular system on embryonic day 19 just before GDNF-deficient mice die. Initially paraffin sections of embryos were prepared and stained with a marker specific for the lymphatic vessels – Lyve, which specifically stains the lymphatic endothelial cells. We found 50% less lymphatic vessels in skin tissue of GDNF KO animals as compared to wt mice \( (n=4) \) (Planken et al. unpublished data). We further decided to study the branched lymphatic structures on whole-mount skin preparations of E19 GDNF and RET KO and wt animals. However, using this methodology we could not confirm the effect of a decrease in VEGFR-3 staining - another specific marker of lymphatic vessels. Neither did we see any change in PECAM staining, which is a panendothelial antibody staining the blood vessel endothelia (Planken et al. unpublished data). As the whole-mount preparations are more convenient for the visualization of three dimensional ramified network structures than two-dimensional sectioned tissue, we concluded that GDNF does not influence the development of lymphatic- and blood vessels during embryonic develop-
ment and these structures appear normal in GDNF KO animals.

### 4.8 Crosstalk of neuronal and vascular systems

This study on the mechanisms of possible crosstalk between the neuronal and vascular systems was initiated by the gene expression findings from the Neuro2A-20 study, demonstrating that GDNF up-regulates the main lymphatic growth factor VEGF-C. As the data regarding the investigation of vascular effects of GDNF did not provide any evidence of GDNF-mediated changes in blood or lymphatic vessels, we concentrated on the effects of VEGF-C in the nervous system. There is ample data on the neurotrophic effects of VEGF-A in various neuronal systems, however when we initiated our study, no paper studying the effects of VEGF-C on the nervous system had been published. The first paper studying the effect of VEGF-C on nervous system was published by Le Bras et al., demonstrating that VEGF-C acts as a trophic factor for neural progenitors in the vertebrate brain (Le Bras, Barallobre et al. 2006). We have further collaborated with Dr. Kirsi Sainio and Prof. Kari Alitalo groups investigating the effects of VEGF-C on the sympathetic nervous system, demonstrating that VEGF-C promotes the proliferation and survival of early sympathetic progenitors (Piltti doctoral dissertation 2009; Piltti, Planken et al. submitted). In this study we showed that the effects of VEGF-C on the induction of sympathetic progenitor cell (SPC) proliferation and survival are comparable to the effects of GDNF and ARTN. In addition, VEGF-C KO mice were shown to exhibit decreased size of sympathetic ganglia. In this system VEGF-C also activated ERK downstream signalling pathway, independent of VEGFR-3 and -2, as these receptors were not expressed in sympathetic progenitors. Interestingly we also observed that the SPCs isolated and cultured from RET KO and Hz animals failed to increase their proliferation in the presence of VEGF-C unlike the control samples. However, VEGF-C was unable to induce direct RET phosphorylation (Piltti, Planken et al. submitted).

Thus the results from these studies clearly demonstrate the neurotrophic properties of a well characterized lymphangiogenic factor -VEGF-C on different neuronal systems including the neural progenitors, developing sympathetic neurons as well as the dopaminergic neurons in embryonic and adult stage. In our study on the dopaminergic effects of VEGF-C we described VEGF-C to be a neurotrophic factor for embryonic dopaminergic neurons in vitro, as well as to act as the neurotrophic and neuroprotective protein in the adult rat neurotoxin model of PD. The exact signalling pathways which mediate the neurotrophic effects of VEGF-C in the neuronal systems are not clear. In embryonic dopamine cultures we observed the expression of Vegfr-1 and -2, but no expression of Vegfr-3, neither did the midbrain lysates isolated from adult mice express detectable levels of Vegfr-3. Following treatment of rat striatums with VEGF-C for 8h we could see up-regulation of all Vegfrs by up to 2 fold, which was also visualized in VEGF-C treated samples from embryonic midbrain cul-
tures where we could see up to 2-fold up-regulation of the Vegfr-1 and -3 mRNA, whereas Vegfr-2 remained unchanged in this setup. Also the VEGF family co-receptors neuropilins showed significant expression. However, both the embryonic DA cultures, as well as brain lysates, contain numerous other cells, thus the exact expression patterns in the dopamine cells cannot be confirmed. Our results on SPCs did not show any expression of Vegfrs-2 and -3, however up-regulation following VEGF-C treatment was not tested (Piltti submitted). Very low expression of Vegf receptors in dopaminergic neurons was also seen in VEGF-A dopamine study (Yasuhara, Shingo et al. 2004). Krum et al. found no expression of Vegfr-2 in normal brain, but noticed induction of expression of Vegfr-1 and -2 following infusion of vehicle or VEGF-A (Krum, Mani et al. 2002). Both our studies demonstrated downstream ERK/MAPK pathway activation, but failed to show any direct cross-talk on the level of RET phosphorylation by VEGF-C, as has been previously demonstrated for VEGF-A (Tufro, Teichman et al. 2007). Thus the results from SPCs proliferation assays in RET KO and Hz animals (where VEGF treatment, unlike in the control samples, failed to induce an increase in proliferation) (Piltti, Planken et al. submitted) needs further investigation.

The effects of VEGF-C in the nervous system might also be mediated by influences from other systems in the brain, such as the immune or vascular system. We found increased number of blood vessels in VEGF-C treated and 6-OHDA injured rat striatums. The increase in blood circulation might prove to be beneficial to neuronal survival and recovery as has been postulated for VEGF-A-treated 6-OHDA-lesioned animals (Yasuhara, Shingo et al. 2004; Yasuhara, Shingo et al. 2005; Yasuhara, Shingo et al. 2005). However, negative effects of possible extravasation and vessel leakage must be kept in mind for possible therapeutic applications. Another observation evoking concern, regarding possible therapeutic potential of VEGF-C, is the effect of BBB disruption which was demonstrated to be present in VEGF-C injected rat brain. In addition the VEGF-C treatment triggered microglial activation around the injection area. The mechanistic effects of microglial activation in this setting remain to be investigated, as both detrimental and beneficial effects have been described for this process.
5. CONCLUDING REMARKS

The current study has evaluated the role of GDNF in the midbrain dopaminergic system and investigated the crosstalk with other growth factors. This is the first study demonstrating the role of VEGF-C as a neurotrophic factor for midbrain dopaminergic neurons in the culture and in vivo. Some of the specific conclusions from the results are the following:

1. GDNF regulates gene expression in different neuronal systems, and up-regulates genes of the immune and vascular system. In Neuro 2A cells GDNF strongly up-regulates VEGF-C, a growth factor for lymphatic vessels.

2. Heterozygous GDNF knockout mice have increased intracellular dopamine concentrations and FosB/ΔfosB levels in the midbrain without significant behavioral changes.

3. GDNF receptor RET MEN2B mutation in knock-in mice leads to increased extracellular dopamine and its metabolite levels, increased number of nigrostriatal dopamine neurons and dopamine synthesis. This demonstrates that RET signaling controls the number of nigral dopamine neurons and regulates dopamine metabolism in the striatum.

4. An enhanced computer assisted micro-island method for culturing embryonic midbrain DA neurons was developed. This method can be utilized for the analysis of dopamine neuron survival from limited amounts of neuronal material, for studying the additive effects of growth factors and for drug screening purposes.

5. VEGF-C promotes neuronal survival of midbrain DA neurons in vitro and protects nigrostriatal dopamine neurons in rat 6-OHDA model of Parkinson’s disease.

6. VEGF-C increases the number of blood vessels in the midbrain of 6-OHDA-lesioned rats, but surprisingly also enhances astrogliosis and causes BBB leakage.
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