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Novel neurotrophic treatments in rats and toxin sensitivity of genetically modified mice in the unilateral 6-OHDA model of Parkinson's disease

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ACADEMIC DISSERTATION

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"In another moment down went Alice after it,
never once considering how in the world
she was to get out again."

Lewis Carroll: Alice's adventures in Wonderland (1865)

Abstract

Abbreviations

List of original publications

Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by various motor and non-motor dysfunctions. The motor symptoms are mainly due to the death of dopaminergic (DAergic) neurons in the *substantia nigra*, which leads to loss of neurotransmitter dopamine (DA) in the striatum and thus, to impairments in controlling movements. The causes behind PD are rather poorly understood but are likely to arise from genetic and environmental factors and their interactions. Current therapies for PD can only alleviate the symptoms, and there is a great need for interventions that could slow down the progression of the disease. Neurotrophic factors (NTFs) are polypeptides that regulate the development and survival of neurons, and therefore are promising molecules for the treatment of neurodegenerative disorders. Glial cell line –derived neurotrophic factor (GDNF) is a potent NTF for DAergic neurons, and the discovery of novel NTFs could stimulate the development of regenerative therapies. A widely used tool in preclinical PD research to assess neurotrophic potential is the unilateral 6-hydroxydopamine (6-OHDA) –model in rodents. Furthermore, genetically modified mice can be useful in dissecting the mechanisms of neurodegeneration and identifying gene-environment –interactions.

The aims of this thesis were to study the neurotrophic potential of a structurally modified GDNF –variant and vascular endothelial growth factor C (VEGF-C) in the unilateral 6-OHDA –rat model of PD. Furthermore, the effects of 6-OHDA were assessed in two genetically modified mouse strains: one with a constitutively active GDNF -signalling receptor RET and the other expressing human A30P-mutated alpha-synuclein (aSyn).

We showed that the removal of first 38 amino acids from the protein sequence of GDNF increases its diffusion in the rat brain, but when compared with the full GDNF, is less effective in inducing behavioural recovery and protection of DAergic neurons. It is believed that GDNF is presented to its receptors by heparan sulphate proteoglycans of the extracellular matrix. The lack of heparin-binding domain may therefore lead to insufficient survival signalling.

VEGF-C was identified as a neurotrophic factor for midbrain DAergic neurons. Pretreatment with VEGF-C before 6-OHDA –lesioning could effectively attenuate behavioural deficits in rats despite modest effects on DAergic neuronal survival. VEGF-C was not effective when given one month after 6-OHDA –lesioning. Furthermore, gliosis and disruption of the blood-brain barrier were observed after VEGF-C –injection.

The studies on genetically modified mice showed that constitutive RET signalling can protect nigral DAergic neurons in MEN2B knock-in mice against 6-OHDA despite substantial depletion of striatal DA. The expression of A30P-aSyn did not sensitize the mice to the neurotoxic effects of 6-OHDA, but they exhibited an attenuated locomotor response to D-amphetamine and developed notable motor defects with age.

In conclusion, these studies provided new insights into the neuroprotective signalling of GDNF, lead to the identification of VEGF-C as a neurotrophic factor for DAergic neurons, and demonstrated that despite affecting the motor phenotype of mice, the expression A30P-aSyn does not sensitize the mice to the effects of 6-OHDA.

Abbreviations

[125I]IPCIT	[125I]- (–)-2β-Carboisopropoxy-3β-(4-iodophenyl)tropane
[125I]-PE2I	[125I]-N-(3-iodopro-2*E*-enyl)-2*β*-carbomethoxy-3*β*-(4′-methylphenyl)nortropane
[3H]BTCP	[3H]benzothiophenylcyclohexylpiperidine
5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine
6-OHDA	6-hydroxydopamine
A30P	Alanine to proline –switch at amino acid position 30
A53T	Alanine to threonine –switch at amino acid position 53
AADC	Aromatic L-amino acid decarboxylase
AAV	Adeno-associated virus
ABC	Avidin-biotin complex
AD	Alzheimer's disease
ADo	Autosomal dominant
ALS	Amyotrophic lateral sclerosis
AMPH	Amphetamine
ANOVA	Analysis of variance
APO	Apomorphine
AR	Autosomal recessive
ARTN	Artemin
AUC	Area under the curve
aSyn	Alpha-synuclein
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BHK	Baby hamster kidney
CDNF	Cerebral dopamine neurotrophic factor
COMT	Catechol-O-methyltransferase
CPu	Caudate and putamen
CT	Cylinder test
DA	Dopamine
DAB	3,3-diaminobenzidine
DAergic	Dopaminergic
DAT	Dopamine transporter
DOPAC	3,4-dihydroxyphenyl acetic acid
DRG	Dorsal root ganglion
E46K	Glutamic acid to lysine –switch at amino acid position 46
EBA	Endothelial barrier antigen
EPO	Erythropoietin
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FA	Forelimb akinesia
FGF-2	Fibroblast growth factor-2
GABA	Gamma-aminobutyric acid
GDF5	Growth/differentiation factor 5
GDNF	Glial cell-line derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFL	GDNF-family ligand
GFRα	GDNF-family receptor alpha
GP	Globus pallidus
GPI	Glycosyl phosphatidylinositol
GT	Genotype
HB-GAM	Heparin-binding growth-associated molecule
hMSC	Human mesenchymal stem cell
HPLC	High performance liquid chromatography

HRP	Horseradish peroxidase
HSV-1	*Herpes simplex* virus -1
HVA	Homovanillic acid
Iba1a	Ionized calcium binding adaptor molecule
ICV	Intracerebroventricular
IGF	Insulin-like growth factor
i.p.	Intraperitoneally
LB	Lewy body
L-DOPA	3,4-dihydroxyphenylalanine, levodopa
LTP	Long-term potentiation
MANF	Mesencephalic astrocyte-derived neurotrophic factor
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MEK	MAP-kinase-kinase
MEN2B	Multiple endocrine neoplasia type 2B
MFB	Medial forebrain bundle
mPrPh(A30P)asyn	Transgenic mouse strain with inserted human A30P aSyn gene
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
NCAM	Neural cell adhesion molecule
NGF	Nerve growth factor
NMDA	*N*-Methyl-D-aspartate
NRP	Neuropilin
NRTN	Neurturin
NPC	Neural progenitor cell
NT	Neurotrophin
NTF	Neurotrophic factor
ODC	Ocular dominance column
PB	Phosphate buffer
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PET	Positron emission tomography
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol3´-kinase
PLCγ	phospholipase gamma
PlGF	Placental growth factor
PSPN	Persephin
RET	Rearranged during transfection (receptor tyrosine kinase)
ROCK	Rho –associated protein kinase
SEM	Standard error of mean
SCG	Superior cervical ganglion
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
STR	Striatum
SVZ	Subventricular zone
TGF-β	Transforming growth factor-β
TH	Tyrosine hydroxylase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VTA	Ventral tegmental area
WT	Wild-type littermate mouse

List of original publications

This dissertation is based on the following original publications (I-IV):

I   Piltonen M., Bespalov M.M., Ervasti D., Matilainen T., Sidorova Y.A, Rauvala H.,
         Saarma M. and Männistö P.T (2009) Heparin-binding determinants of GDNF reduce
         its tissue distribution but are beneficial for the protection of nigral dopaminergic
         neurons. Exp Neurol 219(2):499-506

II   Piltonen M.*, Planken A.*, Leskelä O., Myöhänen T.T., Leppänen V-M., Auvinen P.,
         Andressoo J.-O., Alitalo K., Saarma M. and Männistö P.T. (2011) Vascular endothelial
         growth factor C acts as a neurotrophic factor for dopamine neurons in vitro and in
         vivo. Neuroscience 192:550-63
         *equal contribution

III   Mijatovic J., Piltonen M., Alberton P., Männistö P.T., Saarma M. and Piepponen T.P.
         (2011) Constitutive Ret signaling is protective for dopaminergic cell bodies but not for
         axonal terminals. Neurobiol Aging 32(8):1486-94

IV   Piltonen M.*, Savolainen M.*, Patrikainen S., Baekelandt V., Myöhänen T.T. and
         Männistö P.T. Comparison of motor performance, brain biochemistry and histology
         of two A30P α-synuclein transgenic mouse strains. (submitted)
         *equal contribution

1. Introduction

Parkinson's disease (PD) is a progressive, neurodegenerative disease which is characterized by the loss of dopaminergic (DAergic) neurons projecting from the *substantia nigra* (SN) to the *striatum* (Fahn, 2003). Deterioration of these neurons leads to the cardinal motor symptoms of PD: tremor at rest, rigidity, bradykinesia and postural instability. The motor symptoms begin to appear at a rather advanced stage of neurodegeneration but may be preceded by non-motor symptoms that also progress during the course of the disease. Non-motor symptoms include disorders of autonomic, sensory and neuropsychiatric functions. Another typical neuropathological hallmark of the disease, in addition to DAergic neurodegeneration, is the appearance of intracellular protein inclusions known as Lewy bodies. PD is usually diagnosed at 50-60 years of age, and it affects approximately 1% of the population over 70 years (Wirdefeldt et al., 2011).

The triggering causes of the disease are still largely unknown, although an estimated 5-10% of cases have a known genetic background. Several genes associated with PD have been identified, but the large majority of PD cases are sporadic (Bekris et al., 2010). Evidently, oxidative stress, mitochondrial dysfunctions and inflammation play a role in the pathology of the disease (Fahn, 2003). The cornerstone of treating the motor symptoms of PD is levodopa (L-DOPA), which is metabolized to dopamine (DA) in the remaining DAergic neurons in the brain. However, as the disease progresses, L-DOPA becomes less effective and causes motor complications such as wearing-off and dyskinesia. There is a great need for therapeutical interventions that could slow down the progression of the disease.

Neurotrophic factors (NTFs) are polypeptides that participate in the development and survival of neurons. Current classification recognizes three groups or families of NTFs: neurotrophins, glial cell line –derived neurotrophic factor (GDNF) family of ligands (GFLs), and neurokines (Bespalov and Saarma, 2007). Additionally, there are various other growth factors that serve their main function outside the nervous system, but also affect neurons. Because of their ability to support neurons, NTFs are studied as possible future therapeutics for various neurodegenerative disorders, including PD. One of the most widely used methods to model PD in experimental animals is to inject a catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA) unilaterally into the nigrostriatal pathway in rodents (Blum et al., 2001). 6-OHDA-induced neuronal death occurs mainly because of oxidative stress and mitochondrial dysfunction, both of which are also implicated in PD. A long list of NTFs have shown potential in protecting DAergic neurons from neurodegeneration and improving behavioural deficits in 6-OHDA-treated rodents, but so far these findings have not translated to the clinical level. GDNF and neurturin (NRTN) are so far the only NTFs that have been given to PD patients. Because of controversial outcomes, trials with GDNF have been discontinued, while NRTN trials are still ongoing. Despite yet inconclusive evidence of their clinical applicability, NTFs (or small molecules with similar effects), could provide a unique way of repairing the damaged the brain and protecting the remaining DAergic neurons.

2. Review of the literature

2.1 Parkinson's disease

PD is a progressive neurodegenerative disorder, which was first defined in detail by James Parkinson in *An Essay on the Shaking Palsy* in 1817. He describes cases that suffer from involuntary trembling of limbs, rigidity of muscles, as well as difficulties in executing movements and holding an upright posture. A number of other symptoms are listed as well, and it is recognized that the physical state, but not the senses or the intellect, of the patients deteriorates slowly but relentlessly in the course of time. The neuropathology and the proximate cause of the disease remained unsolved, and even today many questions concerning those aspects of the disease are still unanswered.

The prevalence of PD is approximately 1000/100,000 in population over 70 years of age, which makes it the second most common neurodegenerative disorder after Alzheimer's disease (AD), and the most common neurodegenerative movement disorder (Wirdefeldt et al., 2011). Since the prevalence increases with age, the number of cases is expected to rise notably in the ageing population during the coming decades (Hirtz et al., 2007). This, in turn, will add to the burden of medical costs.

The etiology of PD is complex, and is likely to arise both from genetic and environmental factors, as well as their interactions. There are altogether 18 genes/loci that are indicated in monogenic forms or as susceptibility factors for the disease, but so far only six of them have been well validated as causes of monogenic familial PD, accounting for 5-10% of cases (Table 1., Bekris et al., 2010). Common polymorphisms in the *SNCA* and *LRRK2* –genes have been identified as susceptibility factors as well. There is suggestive evidence that exposure to pesticides may increase the risk to develop PD, whereas coffee and smoking seem to be protective. All in all, there is very little conclusive data about various life-style or environment -related factors and their causal relationships with PD, which may partly be due to high variation in experimental setups (Wirdefeldt et al., 2011).

The clinical manifestation of PD is characterized by typical motor symptoms of parkinsonism (a neurological syndrome whose primary cause is PD, but can be related to other neurodegenerative disorders or caused by secondary environmental factors), which include bradykinesia, tremor at rest, rigidity, loss of postural reflexes, flexed posture and the freezing phenomenon. At least two of these symptoms need to be present when diagnosis of parkinsonism is done, one of them being either of the two first mentioned in the list (Hughes et al., 1992). In PD, the symptoms typically start asymmetrically and are significantly alleviated by L-DOPA -therapy (Fahn, 2003). In addition to the motor deficits, PD is accompanied by non-motor symptoms. These include various cognitive, neuropsychiatric, autonomic, sensory and sleep disturbances, which represent the outcome of neurodegeneration in other neurotransmitter systems than DAergic –namely serotonergic, noradrenergic or cholinergic. Many of the non-motor symptoms are already present before the motor defects appear (Park and Stacy, 2009).

Table 1. Well-validated genes associated with PD (modified from Bekris et al. 2010)

PARK locus	Gene	ADo/AR	Type of mutation
PARK1/PARK4	*SNCA* (alpha-synuclein)	ADo	missense mutations (A30P, A53T, E46K), duplication/triplication of gene
PARK2	*Parkin* (ubiquitin ligase)	AR	exonic deletions, missense and nonsense mutations, duplication
PARK6	*PINK 1* (kinase)	AR	missense and nonsense mutations, truncations
PARK7	*DJ-1* (antioxidant peptidase)	AR	missense mutations
PARK8	*LRRK2* (tyrosine kinase –like protein)	ADo	missense and nonsense mutations
PARK9	*ATP13A2* (membrane ATPase)	AR	missense mutations

ADo=autosomal dominant, AR=autosomal recessive

The neuropathological hallmark of PD is the degeneration of DAergic neurons in the *substantia nigra pars compacta* (SNpc), which subsequently leads to the loss of DA in the striatum. The loss of DA can be substantially compensated, since the motor symptoms start to appear only when about 80 % of striatal DA is lost, and 30-60 % of nigral DAergic neurons and 50-60 % of their striatal terminals have degenerated (Fahn, 2003, Cheng et al., 2010). However, neurodegeneration in the brain is not limited to the nigrostriatal DAergic system, but also affects other neurons (Jellinger, 1991). In addition, nigral cell loss is accompanied by gliosis, microgliosis and inflammation. Another typical neuropathological feature of PD is the appearance of dense intracellular aggregates called Lewy bodies (LB), in which α-synuclein (aSyn) is a major component. LBs are not only found in surviving nigral DAergic neurons, but the widespread pathology can be observed throughout the central, peripheral and enteric nervous system (Jellinger, 2012). aSyn is a presynaptically concentrated, predominantly unfolded protein that is prone to conformational changes (reviewed by Surguchov, 2008). Its main roles in the central nervous system are in maintenance of vesicle pools, transmitter release, lipid metabolism, regulation of proteasomal function and even neuroprotection. How aSyn becomes pathogenic still remains unsolved and is under vigorous studies. The prevailing concept is that the most toxic forms of aSyn are protofibrils and oligomers, formation of which may be induced by several factors, e.g. changes in the physicochemical environment or accumulation of the protein, mutations, and oxidative stress. Toxic aSyn-species can form pore-like structures on cell membranes, disrupt axonal transport and interfere with protein turnover. It is likely that Lewy bodies are an outcome of neuronal protection machinery that deposits harmful proteins into aggregates.

Medical treatment of the cardinal symptoms of PD is largely based on DA replacement therapy by L-DOPA, which is converted into DA by aromatic L-amino acid decarboxylase (AADC) (Fig.1). L-DOPA is always combined with AADC inhibitors (benserazide, carbidopa) and often with catechol-O-methyltransferase inhibitors (entacapone, tolcapone) to prevent L-

DOPA being metabolized into DA in the periphery before reaching its target in the brain. L-DOPA is the most effective therapy, but its efficacy is reduced as the disease progresses, which leads to motor complications such as involuntary movements and fluctuations in motor performance. Other drugs used are monoamine oxidase (MAO)-B –inhibitors (selegiline, rasagiline) that prevent DA metabolism in the brain, DA agonists (e.g. bromocriptine, pramipexole, ropinirole, rotigotine), and more seldomly anticholinergic drugs (e.g. biperiden) or amantadine. The available medicines alleviate the motor symptoms of the disease, but none of them can halt the progressive neurodegeneration.

Dopaminergic neuron   **Post-synaptic cell**
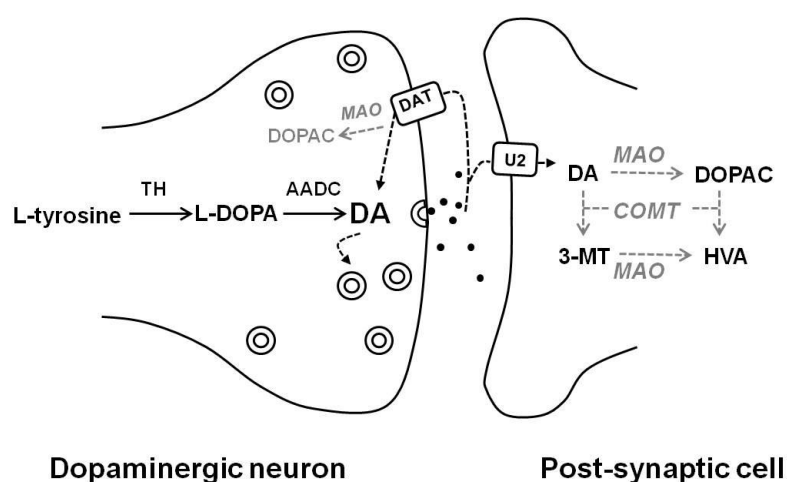
Figure 1. Synthesis and metabolism of dopamine (DA). Amino acid L-tyrosine is converted by the rate-limiting enzyme tyrosine hydroxylase (TH) into L-DOPA, which in turn is converted to DA by aromatic L-amino acid decarboxylase (AADC). Newly synthesized DA is packed into vesicles via the vesicular monoamine transporter 2. Once released into the synaptic cleft, DA is rapidly reuptaken to the neuron via the dopamine transporter (DAT) and restored into vesicles, or partly metabolized by the mitochondrial enzyme monoamine oxidase (MAO). DA is also partly transported into post-synaptic neurons or glial cells via uptake 2 (U2), and metabolized by MAO and catechol-O-methyltransferase (COMT).

2.2 6-hydroxydopamine –based rodent models of PD

Various approaches have been used in attempt to model PD in experimental animals (Kirik et al., 2002, Bezard and Przedborski, 2011, Blesa et al., 2012). These include (but are not limited to) 1) the use of neurotoxins such as 6-OHDA, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), metamphetamine, rotenone and paraquat, 2) induction of parkinsonian state with reserpine, which depletes nerve endings of DA, 3) induction of neurodegeneration through sustained neuroinflammation caused by lipopolysaccharide, 4) creation of genetically engineered mice carrying PD –related mutated genes and 5) overexperssion of aSyn in the nigrostriatal pathway via viral transfection. None of the current models can fully reproduce the range of neuropathological and behavioural defects seen in PD (see paragraph 2.1), and many of them are mainly focused only on DAergic neurodegeneration. Additionally, although they have been very useful in predicting the efficacy of symptomatic treatment, the translation

of effective neuroprotection into humans has been less successful. Nevertheless, all of these tools have provided us with important information regarding the molecular mechanisms and regulation of DAergic cell death.

There are extensive reviews covering various aspects, including molecular mechanisms of neurodegeneration and behavioural and neurobiological changes, of the 6-OHDA –rodent models of PD (Schwarting and Huston, 1996a, Schwarting and Huston, 1996b, Blum et al., 2001, Deumens et al., 2002). 6-OHDA, a hydroxylated analogue of DA, occurs naturally in very small amounts in rodent and human brain. It does not cross the blood-brain barrier (BBB), and therefore has to be injected straight into the brain in a stereotaxic operation to create a DAergic lesion. 6-OHDA is taken into catecholaminergic neurons via the DA transporter (DAT) and the noradrenaline transporter, although it has been shown that unspecific damage to other neurons can also occur especially with high concentrations of 6-OHDA. Although the toxic mechanisms of 6-OHDA are not fully understood, it is known that neural damage caused by the toxin is mainly due to two reasons. Firstly, rapid auto-oxidation and, to a lesser extent, deamination of the toxin by MAO-B generates reactive oxygen species such as hydrogen peroxide, hydroxyl and superoxide radicals and quinones. Their formation is likely to be enhanced by iron. Secondly, 6-OHDA can inhibit mitochondrial complex 1, which is a part of the oxidative phosphorylation system that produces adenosine triphosphate. These processes lead to DAergic neuronal death, which seems to happen both via apoptosis (programmed cell death) and necrosis. Importantly, both oxidative stress and mitochondrial dysfunction are implicated in PD.

6-OHDA is traditionally administered unilaterally (because of severe disability related with extensive bilateral lesions) into one of three locations: to the striatal terminal area, to the medial forebrain bundle (MFB) that contains the nigrostriatal (as well as mesolimbic) DAergic axons, or to the SN containing the DAergic cell somas (Deumens et al., 2002). The two latter ones represent a rapidly developing, acute lesion, which leads to extensive loss of DAergic neurons. The lesion is fully developed already in 3-5 days, assessed as decreased DA content in the striatum. Administration of 6-OHDA into the MFB usually results in a total lesion, in which at least 90 % of striatal DA and nigral tyrosine hydroxylase (TH)-positive neurons are lost. The extent of the lesion is better controlled and varied with intranigral administration, depending on the injection site and dose of the toxin. Therefore, it is possible to create partial lesions with intranigral 6-OHDA –injections. Striatal lesions are virtually always partial lesions, although again by careful choice of dose and site of 6-OHDA it is possible to create lesions of varying severity (Kirik et al., 1998). The most important difference between striatal lesions and those created closer to nigral cell somas is that striatal lesions cause a much slower, retrograde neurodegeneration that develops during several weeks. Neuronal death at the level of the SN is first observed about one week post lesioning, and is most rapid until 2 weeks post-lesion followed by a slower but progressive neurodegeneration up to at least 8 weeks post-lesion (Sauer and Oertel, 1994).

To assess the severity of the unilateral lesion, and the efficacy of potential therapeutic molecules in alleviating 6-OHDA –induced behavioural impairments, several behavioural tests can be employed (Deumens et al., 2002). One of the best known methods, drug-induced

rotational behaviour, was described by Ungerstedt and Arbuthnott (1970). In this test, the animal receives an injection of amphetamine to release DA or apomorphine to activate postsynaptic DA receptors. Due to imbalance of DAergic transmission between the brain hemispheres, this will cause the animal to exhibit rotational movement. Administration of amphetamine makes the animal turn ipsilaterally, i.e. towards the side of the lesion, because of greater DA release in the intact striatum. Apomorphine causes contralateral turning due to dominant activation of sensitized DA receptors in the lesioned striatum. Apomorphine can be reliably used only in animals with extensive striatal DA loss, because compensatory sensitization of the postsynaptic receptors is not seen with mild lesions. Generally, the bigger the imbalance in DAergic activity between the brain hemispheres after the 6-OHDA-treatment (i.e. the larger the lesion), the more the animals rotate. Especially the loss of TH-cells in the medial SN is associated with enhanced rotational behaviour (Olds et al., 2006).

It is also possible to measure locomotor activity without any drug administration, since 6-OHDA can cause a reduction in normal activity of the animals. Other commonly used tests that do not require a drug challenge are cylinder test, forelimb akinesia test and staircase test (Montoya et al., 1991, Olsson et al., 1995, Schallert et al., 2000). In the cylinder test, the animal is placed into a transparent plexiglass cylinder, and during the test period (usually 5 min) is scored for simultaneous and asymmetric front paw contacts with the cylinder. The forelimb akinesia test for rats measures the ability to initiate and execute movements. The rat is immobilized by the experimenter so, that only one front paw can be used and placed on a table surface. The rat is then moved in forehand direction and backhand direction along the table edge, and the number of adjusting paw steps, as well as the latency to initiate the steps, are measured. Staircase test measures skilled paw use and fine motorics of rats. In this test, rats are placed in an apparatus consisting of a starting chamber with an opening to a platform. After entering the platform, the rat will start picking up glucose pellets from descending stairs that are placed on both sides of the platform. Number of pellets retrieved and the lowest level of stairs reached are scored. In all of these tests without drug challenge, impairment in the use of front paw contralateral to the lesioned hemisphere is usually seen.

Despite being a valuable tool in PD research, the 6-OHDA-model has its limitations (Deumens et al., 2002). First, a unilateral model does not fully represent the bilateral clinical disease, but the use of a bilateral 6-OHDA-model brings about animal welfare problems (adipsia and aphagia are common). Second, most of the behavioural tests are developed to measure motor asymmetry. In addition, the brain can compensate for the loss of DAergic neurons. For example, with time the remaining TH-positive fibers in the lesioned striatum can undergo sprouting, which can lead to at least partial spontaneous recovery (Blanchard et al., 1996). This sprouting can even emerge from the non-lesioned side. Finally, variation is rather high in the 6-OHDA-model, not only between experimental subjects but also within one subject in the case of a bilateral lesion, and in the correlation between behavioural and morphological measures. Also, small lesions are difficult to quantify in terms of behavioural changes. Naturally, all the other models of PD have their limitations as well, and therefore it is more important to know which aspects of the disease are represented by each of them.

2.3 Potential therapeutic neurotrophic factors identified in the 6-OHDA – model of PD

Neurotrophic factors (NTFs) are proteins that participate in the regulation of various processes during the lifespan of a neuron. They regulate the proliferation of neuronal precursors and their differentiation, morphological maturation and neurite outgrowth as well as synaptogenesis and synaptic plasticity. According to a classical theory based on the early studies by Rita Levi-Montalcini and her co-workers starting in the 1940's, NTFs are secreted by a target tissue in very small amounts, which attracts neuronal processes to form connections with the target. Because of limited amounts of NTFs available, only the neurons that are able to form meaningful connections with the target stay nourished with the NTFs while those that failed to properly innervate the tissue die apoptotically. It is believed that NTFs can act in autocrine manner as well. NTFs are currently classified into three families: neurotrophins, GFLs, and neurokines (Bespalov and Saarma, 2007). The neurokines, or neuropoietic cytokines are a group of small proteins (interleukins 6, 11, 27, leukaemia inhibitory factor, ciliary neurotrophic factor, cardiotrophin 1, neuropoietin, cardiotrophin-like cytokine and B-cell stimulating factor 3) that have well-known functions outside the nervous system, but also have roles in the developing and adult brain and in brain injuries (Bauer et al., 2007). Additionally, there are many growth factors that are better known for their effects on tissues outside the nervous system, but can also act as neurotrophic factors. These include for example the vascular endothelial growth factor (VEGF) family proteins, erythropoietin (EPO) and insulin-like growth factor 1 (IGF1). The following paragraphs will focus on describing neurotrophins, GFLs, VEGF-ligands and some other selected proteins identified as NTFs for DAergic neurons with a special emphasis on their effects in the 6-OHDA –rodent models of PD.

2.3.1 Neurotrophins

Overview of neurotrophins and their signalling
Neurotrophins are the first family of NTFs described, and it includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4, in literature also referred to neurotrophin-5 or neurotrophin-4/5) (Huang and Reichardt, 2001, Skaper, 2008). Additional neurotrophins (6 and 7) have been identified in *Xenopus* and fish. In general, these proteins are active as homodimers, but the subunits may form heterodimers as well. They are all synthesized as proproteins, which are secreted and then cleaved by proteases to yield mature neurotrophins. The pro-neurotrophins are active molecules as well, but they have dramatically different actions from those of mature forms.

Neurotrophins signal through three transmembrane receptor tyrosine kinases: TrkA, TrkB and TrkC, which dimerize when they bind neurotrophins (Fig. 2.). The fourth receptor, p75[NTR] is structurally unrelated to Trk-receptors, and is rather a distant member of the tumour necrosis factor receptor family. It lacks a catalytic domain, but contains a particular death domain in its cytoplasmic part and acts through protein-protein interactions. Sortilin has also been recognized as an important receptor for neurotrophins (Nykjaer et al., 2004). Signalling of

these receptors can be modulated on many levels, and this complexity lies behind the plethora of effects that NTFs can have on various neurons and cells (Huang and Reichardt, 2001, Teng and Hempstead, 2004). An interesting feature of neurotrophins is that, while mature proteins activate Trk-receptors, p75^{NTR} and their combinations to induce either cell survival or apoptosis, pro-neurotrophins selectively activate p75^{NTR} in collaboration with sortilin to induce apoptosis (Lee et al., 2001, Nykjaer et al., 2004, Teng et al., 2005).

Signalling pathways that are activated by neurotrophins have been reviewed in detail, for example by Huang et al (2001). In summary, the activation of Trk receptors leads to phosphorylation of tyrosine residues in the cytoplasmic domains. A number of adaptor proteins connect Trk activation with intracellular signalling cascades, including MEK/MAPK (MAP-kinase-kinase/mitogen-activated protein kinase) mediating differentiation and survival of neurons, PI3K/Akt (phosphatidylinositol3´ -kinase/ Akt kinase) implicated in neuronal survival, and PLCγ (phospholipase gamma) mediating neurite outgrowth. Signalling through p75^{NTR} activates nuclear factor –κB to induce neuronal survival, or Jun-kinase and acidic sphingomyelinase (latter leading to formation of ceramide) to cause apoptosis. p75^{NTR} activation can also lead to modifications in cytoskeleton dynamics controlling neurite outgrowth.

Neuronal effects of neurotrophins

A variety of neurons and neuronal processes in the peripheral as well as in the central nervous system are responsive to neurotrophin signalling, as has been demonstrated in numerous cultured cell systems and animal models. Neuronal populations, whose survival depend at least partially on neurotrophin-Trk -activity include the sensory neurons of trigeminal ganglia, dorsal root ganglia (DRG), trigeminal mesencephalic nucleus, vestibular and cochlear ganglia, sympathetic ganglia, motor neurons, enteric neurons, cerebellar granule cells, retinal ganglion cells, cholinergic neurons and DAergic neurons (Hyman et al., 1991, Ruit et al., 1992, Yan et al., 1992, Avila et al., 1993, Ibanez et al., 1993, Lindholm et al., 1993, Morse et al., 1993, von Bartheld and Bothwell, 1993, Yan et al., 1993, Cohen et al., 1994, Cohen-Cory and Fraser, 1994, Ernfors et al., 1995, Huang et al., 1999, Chalazonitis et al., 2001). In addition, survival, proliferation or differentiation (Ghosh and Greenberg, 1995, Vicario-Abejon et al., 1995) is induced in precursors of oligodendrocytes, cortical and hippocampal neurons and in cultured neural crest cells (Kalcheim et al., 1992, Barres et al., 1994, Scarisbrick et al., 2000).

BDNF and NT-4 also have a specific and important function in guiding the formation of ocular dominance columns (ODCs) through TrkB. The formation of ODCs is inhibited when these molecules are scavenged by an immunoglobulin-bound TrkB (Cabelli et al., 1997). On the other hand, if BDNF or NT4 is applied in supraphysiological concentrations onto the developing visual cortex, the ODCs fail to form as well, which suggests that correct amounts of neurotrophins are critical for the process (Cabelli et al., 1995).

An important phenomenon regulated by neurotrophins is long term potentiation (LTP) to strengthen synaptic function, and synaptic plasticity (Lohof et al., 1993, Kang and Schuman, 1995). LTP is severely reduced in the hippocampi of BDNF and NT-4 knock-out mice, as well as in normal hippocampal slices treated with a BDNF/NT-4 scavenging decoy TrkB (Korte et

al., 1996, Chen et al., 1999, Xie et al., 2000). Of note is the role of BDNF and TrkB signalling in the effects of antidepressants. Treatment with antidepressants can upregulate the expression of BDNF and TrkB mRNA *in vivo*, and TrkB can be activated by the treatment even in the abscence of BDNF (Nibuya et al., 1995, Saarelainen et al., 2003, Rantamäki et al., 2011). Studies in TrkB –deficient mice have shown, that the receptor and its activation are required for antidepressant –induced behavioural changes (Saarelainen et al., 2003). It has been suggested that during antidepressant treatment, a developmental state –like plasticity is driven by activity-dependent expression of BDNF and that it is a pivotal element in the process that leads to optimal rewiring of neuronal networks by environmental guidance (such as psychotherapy) (Castren and Rantamäki, 2010).
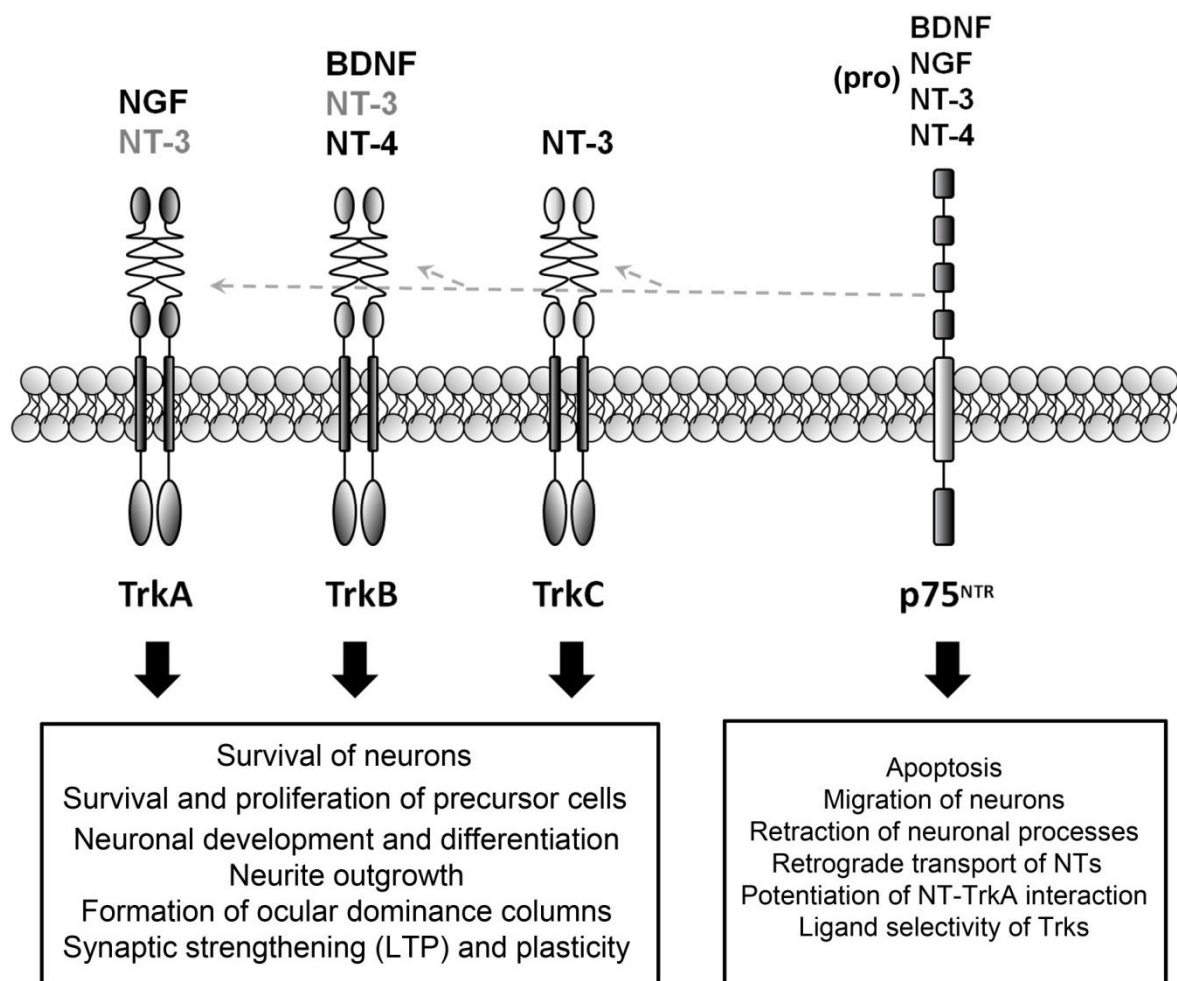
Figure 2. Neurotrophins, their receptors and effects on neurons. Neurotrophins act as dimers to activate the receptors, which also dimerize upon ligand binding. On Trk –receptors the intracellular tyrosine kinase domains are transphosphorylated. p75^{NTR} does not contain a kinase domain. The three Trk-receptors respond to specific neurotrophins (marked in black), whereas NT-3 is able to activate all of the receptors at least to some extent. p75^{NTR} is activated by all members of the neurotrophin family, as well as pro-neurotrophins, and it can interact with each of the Trk-receptors. Figure adapted from Chao, 2003 and Teng and Hempstead, 2004.

Roles of neurotrophins in the intact nigrostriatal DAergic system and associations with PD

Among the neurotrophin family members, BDNF, NT-3 and NT-4 seem to be strongly associated with DArgic function and the nigrostriatal pathway. mRNAs of BDNF and NT-4 as well as their receptors TrkB and TrkC are expressed on DAergic neurons (Seroogy and Gall, 1993, Seroogy et al., 1994, Numan and Seroogy, 1999). At least BDNF is retrogradely transported to the SN after intrastriatal infusions (Mufson et al., 1994). Although TrkA is normally not expressed on DAergic neurons, induction of TrkA via adeno-associated viral (AAV) vector in the rat brain leads to expression of the receptor as well as retrograde transport of exogenously applied NGF (Melchior et al., 2003).

Despite being expressed and having positive effects on DAergic neurons, neurotrophins do not seem to be crucially required for normal development of midbrain DAergic pathways (Ernfors et al., 1994, Klein, 1994, Liu et al., 1995). On the other hand, the role of a specific NTF in the development and maintenance of neurons may not be simple to assess in traditional knock-out animals, because compensatory mechanisms may obscure a relevant phenotype. In support of this, an injection of BDNF –neutralizing antibodies into the developing SN of normal mice leads to increased natural postnatal apoptosis of DAergic neurons (Oo et al., 2009). Loss of nigral TH-positive neurons is also observed in adult rats after an infusion of antisense oligonucleotides inhibiting the expression of BDNF (Porritt et al., 2005). Additionally, reduced expression levels of TrkB and C –receptors also lead to degeneration and morphological defects in nigrostriatal neurons (Zaman et al., 2004, von Bohlen und Halbach et al., 2005).

Under *in vitro* -conditions, BDNF, NT-3 and NT-4 increase the survival of mesencephalic DAergic neurons, whereas NGF is without effect (Hyman et al., 1994, Hynes et al., 1994, Spenger et al., 1995). When administered into intact rat brain, neurotrophins can boost the DAergic system. Even a single intranigral injection of BDNF can cause contralateral rotational behaviour in rats after administration of amphetamine, although the striatal DA concentrations are slightly decreased (Shults et al., 1994). Two-week intranigral and intrastriatal infusions of BDNF increases striatal DA turnover, and contralateral rotational behaviour is induced by amphetamine in rats (Altar et al., 1992). Similar augmentation was later seen with NT-3 and NT-4 (Altar et al., 1994a, Martin-Iverson et al., 1994). Furthermore, a BDNF-infusion into the SN increases the number of spontaneously active DAergic neurons, and elevates their firing rate (Shen et al., 1994).

Although it is not clear if neurotrophins play a role in the pathogenesis or compensatory changes in PD, alterations in serum levels and brain expression of neurotrophins have been detected in PD patients. Scalzo et al. (2010) reported that serum BDNF –levels are reduced in PD patients, most notably in the early stages of the disease. Thus, higher concentrations of BDNF were detected in patients with longer disease duration and more severe motor impairments. Similar changes in plasma levels and associations with disease severity were observed with NGF (Lorigados Pedre et al., 2002). It has also been demonstrated that amounts of BDNF protein and mRNA are reduced in the SN, caudate and putamen in PD (Mogi et al., 1999, Parain et al., 1999, Howells et al., 2000). Most of the DAergic neurons in the SN normally

express BDNF, which means that the nigral loss of BDNF partially results from the death of DAergic neurons. However, Howells et al. (2000) found reduced BDNF expression in the surviving DAergic cells in PD. TrkB-levels in the surviving neurons remain normal (Benisty et al., 1998). In contrast to all these reports describing lower levels of BDNF in PD brains, Salehi et al. (2009) found robustly elevated concentrations of BDNF in the cerebrospinal fluid of patients. They proposed that the source of excess BDNF is activated glia.

Effects of neurotrophins in the 6-OHDA –model of Parkinson's disease
The potential of neurotrophins (mainly BDNF) as dopaminotrophic factors has been explored in numerous studies. In addition, these studies have revealed that 6-OHDA -lesioning can increase the nigrostriatal expression of TrkB mRNA and slightly decrease TrkC –levels, whereas BDNF protein levels can either rise or decline in young and old rats, respectively (Zhou et al., 1996, Numan and Seroogy, 1999, Yurek and Fletcher-Turner, 2000, Yurek and Seroogy, 2000, Yurek and Fletcher-Turner, 2001). In one study, no changes were detected in nigral BDNF levels after 6-OHDA (Mocchetti et al., 2007).

BDNF
Spina et al. (1992) were the first ones to report the neuroprotective activity of BDNF against 6-OHDA in cultured rat mesencephalic DAergic neurons. In *in vivo* -setups, BDNF has been delivered as multiple doses or continuously via various applications such as secreting cells or viral transfection, results of which are summarized in Table 2. There are also experimental therapies that have beneficial effects in the 6-OHDA rat model and may partially act via upregulation of BDNF expression. These therapeutic candidates include a gamma-aminobutyric acid (GABA) receptor B -antagonist CGP 56999A and Leu-Ile –dipeptide (Nitta et al., 2004, Enna et al., 2006)

The outcomes of neuroprotection studies where recombinant BDNF was used are partially conflicting (Altar et al., 1994b, Shults et al., 1995), which may be explained by the differences in administration of both the NTF and 6-OHDA. BDNF -gene transfer either into the SN or STR significantly inhibits the development of rotational bias when 6-OHDA is administered weeks or months later, but these treatments have failed to protect TH-positive cells from degeneration (Klein et al., 1999). Another approach for long-term administration of BDNF, intrastriatally implanted BDNF-secreting rat primary fibroblasts, could almost completely prevent the loss of DAergic nerve terminals when a partial 6-OHDA –lesion was induced two weeks later (Levivier et al., 1995). It should be noted though, that more extensive lesions were employed in the studies utilizing viral transfection than in the study with secreting fibroblasts.

Neurorestoration experiments with BDNF are few. In two of them, BDNF-secreting transfected astrocytes were used, and a reduction in rotational behaviour was observed without significant protection of nigral TH-positive neurons (Yoshimoto et al., 1995). Also implanted BDNF-expressing and secreting human mesenchymal stem cells can prevent the augmentation of metamphetamine-induced rotational behaviour in rats with nigral 6-OHDA –lesions (Somoza et al., 2010). In contrast to the studies with transfected astrocytes, higher TH-immunoreactivity was seen in the striatum, accompanied by slightly enlarged TH-cell bodies

in the SN in BDNF-cell treated rats. This discrepancy may be due to a shorter interval between lesioning and treatment.

Collectively, there are several reports describing the functional efficacy of BDNF in the 6-OHDA rat model of PD. Although some groups have found increased survival or sprouting of DAergic neurons, surprisingly many papers describe a rather robust functional recovery without measurable neuroprotection. This is especially seen in neurorestoration experiments, none of which shows significant preservation of nigral DAergic cells. Thus, the recovery of motor functions may arise for example from enhanced functionality of surviving DAergic neurons, or some compensatory events in other neurotransmitter systems.

Table 2. Neuroprotective and neurorestorative effects of BDNF in the 6-OHDA –rat model of PD. Table includes summaries of behavioural changes, protection of striatal DA and nigral or striatal TH-immunoreactivity

Reference	BDNF	Time relation to 6-OHDA	6-OHDA	Behaviour	DA	TH	Other
(Shults et al., 1995)	3x22.5 µg,STR during 3 days	6-OHDA on day 2	1x25 µg, STR	APO ↓	n.m.	STR↑	
(Altar et al., 1994b)	12 µg/day, SN 14 d (minipump)	start 6 d before	0.2µg/h,STR (minipump), 8 d	AMPH ↓ APO ↑	STR 0	n.m.	DA-turnover ↑
(Klein et al., 1999)	AAV-BDNF, SN	6 months before	1x10.8 µg, SN	AMPH ↓ locomotor activity ↑	n.m.	SN 0	
(Sun et al., 2005)	HSV1-BDNF, 3xSTR	4 weeks before	4x7 µg, STR	APO ↓		SN 0	
(Levivier et al., 1995)	fibroblasts, 2xSTR	2 weeks before	1x8.75 µg, STR	n.m.	n.m.	n.m.	[3H]-mazindol (DAT) ↑
(Yoshimoto et al., 1995)	astrocytes, STR	15 days after	1x3.5 µg, SN	AMPH ↓	n.m.	STR 0 SN 0	
(Wang et al., 2002)	astrocytes, 3xSTR	20-24 d after	2x4µg, SN	APO ↓	n.m.	SN 0	TUNEL+ cells, SN↓
(Somoza et al., 2010)	hMSC, 2xSN	1 w after	3x1.33µg, SN	AMPH ↓	n.m.	STR↑	TH-cell size ↑

AAV= adeno-associated virus, AMPH= amphetamine-induced rotational behaviour, APO= apomorphine –induced rotational behaviour, DAT=dopamine transporter, hMSC= human mesenchymal stem cells, HSV1= herpes simplex virus 1, SN=substantia nigra, STR=striatum. Responses of BDNF-treated animals vs. lesion control animals: 0=no change, ↑= increased, ↓=decreased n.m.= not measured.

NT-3, NT-4 and NGF

Although NT-3 and NT-4 clearly affect DAergic neurons positively, their neuroprotective and neurorestorative potential has not been sufficiently studied in the 6-OHDA rat model of PD. In fact, the only report is by Altar et al. (1994b) who studied NT-3 in the same protocol they

used with BDNF (see Table 2). NT-3 was infused for two weeks intranigrally beginning six days before and then continuing for eight more days simultaneously with an intrastriatal 6-OHDA infusion. The behavioural effects were similar but less robust than with BDNF. Striatal DA levels or metabolism, however, were not affected.

Not surprisingly, reports concerning effects of NGF in 6-OHDA –models are few and inconclusive, since it generally does not have dopaminotrophic activity. However, Chaturvedi et al. (2006) have reported that amphetamine-induced ipsilateral rotations are fewer and striatal as well as nigral DA concentrations are higher in intranigrally lesioned rats that have received NGF into striatum three weeks post lesioning. They also found an increase in the survival of TH-positive neurons in the SN. In an earlier study, a six-week intracerebroventricular delivery of NGF was without effect in rats with nigral lesions, when the treatment was started four weeks after 6-OHDA delivery (Bergdall and Becker, 1994).

2.3.2 GDNF-family of ligands

Overview of GFLs and their signalling

Four proteins comprise the GFLs: GDNF, NRTN, artemin (ARTN) and persephin (PSPN). GDNF was the first member to be found in rat B49 glial cell line, and was reported to have potent neurotrophic effects on DAergic neurons (Lin et al., 1993). NRTN was found some years later as a trophic factor for sympathetic and sensory neurons (Kotzbauer et al., 1996). The last two members, PSPN and ARTN, were identified with homology cloning and database search (Baloh et al., 1998, Milbrandt et al., 1998). The GFLs are distant members of the transforming growth factor-β (TGF-β) superfamily, based on conserved locations of seven cystein recidues in the amino acid sequence. The proteins are expressed as precursors, preproproteins, which are proteolytically cleaved to mature proteins that form homodimers (Airaksinen and Saarma, 2002). At least GDNF is expressed in two alternatively spliced variants, in which the pro-sequences differ (Lonka-Nevalaita et al., 2010).

The GFLs use a complicated multicomponent signalling receptor system. It was first shown that the transmembrane receptor tyrosine kinase RET (rearranged during transfection) is a main signal transducer for GDNF, and that the presence of a cell-surface glycosyl phosphatidylinositol (GPI) –linked co-receptor is required for RET activation (Jing et al., 1996, Treanor et al., 1996, Trupp et al., 1996). Now there are altogether four GPI –linked co-receptors known to interact with RET: GDNF-family receptors (GFR) α1-4, which all preferentially bind only one of the family ligands (Fig.3) (Baloh et al., 1997, Jing et al., 1997, Baloh et al., 1998, Thompson et al., 1998, Worby et al., 1998, Masure et al., 2000, Scott and Ibanez, 2001, Sidorova et al., 2010). It is not fully understood, how and in which order the interactions between the ligands, RET and co-receptors are established, but in general the active signalling complex is a heterotetramer consisting of two units of RET and two units of co-receptors (Bespalov and Saarma, 2007). GFRα1, GFRα2 and GFRα4 exist in alternatively spliced isoforms, which may induce disparate signalling pathways and have distinct roles during development (Baloh et al., 1997, Lindahl et al., 2000, Dolatshad et al., 2002, Yoong et al., 2009). Soluble forms of GFRα1 and GFRα4 are found as well, and they can trigger signalling different from the membrane-bound form, or even independent of ligand binding (Paratcha et al., 2001, Mikaels-Edman et

al., 2003, Yang et al., 2007). Also RET is expressed in two isoforms, RET9 and RET51, which differ in their intracellular domains (Tahira et al., 1990). They do not interact with or fully compensate for each other, and they have distinct tissue distributions (Tahira et al., 1990, de Graaff et al., 2001, Tsui-Pierchala et al., 2002a, Tsui-Pierchala et al., 2002b, Yoong et al., 2005). To add more complexity to GFRα-RET –signalling, evidence exists that RET recruits different intracellular adaptor proteins outside lipid rafts than inside the rafts (Paratcha et al., 2001). This comprehensive study by Paratcha et al. (2001) describes a plethora of options for GDNF-GFRα1-RET –signalling. It was shown that GDNF-GFRα1 –complex can activate RET even if it is not located on the same cell, but in soluble form, bound to another cell, or immobilized in extracellular matrix. Also, RET is attracted to lipid rafts by both membrane-bound and soluble GDNF-GFRα1 –complexes, of which latter triggers sustained RET signalling. These findings offer an explanation to how GFL –signalling is organized in cells and tissues that express GFRα receptors but not RET. Various intracellular signalling pathways are activated by RET. These include MEK/MAPK, PI3K/Akt and PLCγ, which are also activated by neurotrophins (Airaksinen and Saarma, 2002).

In addition to GFRα-RET –receptor complexes, other signalling receptors have been identified for GFLs. In cells devoid of RET, GFRα1, -2 and -4 can associate with the neural cell-adhesion molecule (NCAM, specifically the NCAM-140 -isoform) and offer a high-affinity binding cite for the ligands (Paratcha et al., 2003). This activates the intracellular tyrosine kinases Fyn and FAK. Recently, a transmembrane heparan sulphate proteoglycan, syndecan-3, was described both as a signalling receptor and a presenting receptor for GFLs excluding persephin, which has the lowest affinity for heparin-like structures (Bespalov et al., 2011). The hypothetical model created on the basis of these experiments suggests that syndecan-3 concentrates GFLs, in the vicinity of their other signalling receptors. On the other hand, in the absence of the other receptors, syndecan-3 activates cytoplasmic Src-family kinase-signalling by immobilized (extracellular matrix-bound) GDNF. The discovery of syndecan-3 as a receptor for GDNF explains earlier findings demonstrating that in the absence of heparan sulphate structures, GDNF is unable to activate RET and therefore fails to induce typical morphological changes in midbrain cell cultures (Barnett et al., 2002). Finally, there is also interesting crosstalk between GDNF and Met receptor tyrosine kinase: in cultured kidney cells GDNF can indirectly and Src-dependently activate Met, and in GABAergic cells Met-activation seems to negatively regulate GDNF-GFRα1-signalling (Popsueva et al., 2003, Perrinjaquet et al., 2011). The emergence of these new signalling receptors has at least partially helped in explaining the more widespread expression of GFRα's in comparison to RET distribution.
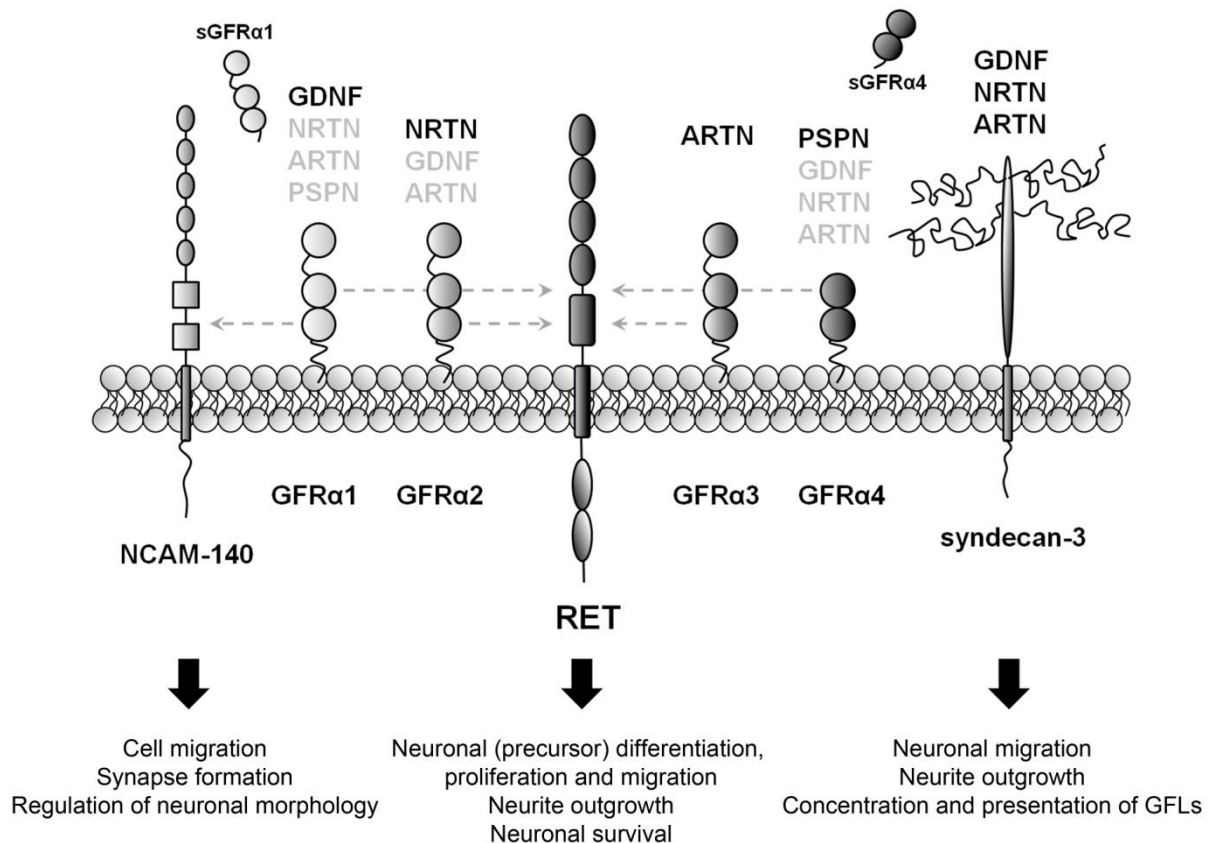
Figure 3. GDNF –family ligands, their receptors and effects on neurons. GFLs function as dimers to activate their receptors. GFRα –coreceptors are GPI –linked on the cell membrane (apart from the two soluble isoforms) and interact with RET to form heterotetramers and trigger intracellular signalling. GFRα1 can also interact with NCAM. Syndecan-3 is a heparan sulphate proteoglycan, the latest receptor identified for GFLs. The preferred ligand for each GFRα is marked in black. Figure adapted from Airaksinen and Saarma, 2002, Bespalov and Saarma, 2007 and Bespalov et al., 2011.

Also of note, it has been shown that the neurotrophic activity of GDNF on variety of cultured peripheral and central nervous system neurons requires the presence of TGF-β (Krieglstein et al., 1998). It is believed that TGF-β helps to recruit and cluster GFRα1 on the cell membrane, most likely to the lipid rafts (Peterziel et al., 2002). This co-operation is a relevant phenomenon also *in vivo*, as it was confirmed in the MPTP –mouse model of PD (Schober et al., 2007). However, this co-operation does not seem to be essential for the development of DAergic neurons, since GDNF/TGF-β2 double knock-out mice do not show DAergic neuronal loss at late embryonic stage (Rahhal et al., 2009). Instead, deficits are found in motor, sensory and sympathetic neurons. Furthermore, GDNF/TGF-β2 double heterozygous knock-out mice do not develop nigrostriatal degeneration with age (Heermann et al., 2010).

Neuronal populations and functions supported by GFLs
Phenotypes of GFL knock-out mice have been extensively reviewed earlier, (Airaksinen and Saarma, 2002) and are only summarized here. GDNF, GFRα1 and RET are all crucially needed during development, and full knock-out mice die after birth because of severe organic defects.

All of these signalling components are needed for normal development of kidneys and enteric nervous system (Schuchardt et al., 1994, Moore et al., 1996, Pichel et al., 1996, Sanchez et al., 1996, Enomoto et al., 1998). Enteric neurons below stomach are missing in all of the aforementioned knock-out mice. Also some parasympathetic ganglia (otic and sphenopalatine) are absent or the neurons are smaller in size and lesser in number (submandibular ganglion), and there is a loss of spinal and cranial motor neurons. Reduced soma size (DRG) and loss of sensory neurons (petrosal ganglion) is seen in mice lacking GDNF-GFRα1 -components, which manifests for example as respiratory difficulties in the heterozygous mice. Sympathetic neurons are affected as well by the absence of GDNF-GFRα1-RET-signalling, and especially in RET-deficient mice deficits are seen in practically all sympathetic neuronal populations. The creation of a conditional GDNF-null mouse revealed that if GDNF is abolished in the adult mouse nervous system, catecholaminergic neurons undergo extensive degeneration (Pascual et al., 2008). Such dramatic loss has never been found in traditional GDNF, GFRα1 or RET knock-out mice, which suggests that compensatory mechanisms take place in those animals, and that under normal physiological conditions GDNF is required for the maintenance of catecholaminergic neurons. Outside the nervous system, GFRα1 and GDNF are needed in the regulation of spermatogenesis (Meng et al., 2000).

NRTN and GFRα2 knock-out mice are viable, but the latter grow poorly. They exhibit subtle defects of sensory neurons (DRG), but NRTN-GFRα2-signalling seems to be especially important for the correct development and maintenance of parasympathetic target innervation. A switch in GFL dependence seems to occur during parasympathetic development: initially GDNF is needed for migration and proliferation of precursor cells. Cholinergic innervation is reduced for example in the intestine, pancreas, heart, salivary and lacrimal glands, and some parasympathetic ganglia or neurons are smaller in size in NRTN or GFRα2 –deficient mice. A moderate loss of enteric neurons is also seen. ARTN and GFRα3-deficient mice are viable, and the most striking phenotype of GFRα3 –knock-outs, similarly to RET -knock-outs, is severe sympathetic defect. ARTN-GFRα3-RET -signalling seems to be essentially required for migration and axon growth during the development of sympathetic innervation. Interestingly, also during the development of sympathetic neurons, the initially required ARTN –signalling (for proliferation of precursors, migration and initial neurite outgrowth), is switched to a later dependence on neurotrophins to maintain target innervation. PSPN and GFRα4-knock-out mice are viable and show no gross defects, although it has been reported that GFRα4 -deficient mice have impaired thyroid calcitonin production (Tomac et al., 2002, Lindfors et al., 2006).

Essentially, the aforementioned neural populations are supported by GFLs in *in vitro* conditions, but the list can be made even longer. For example, GDNF supports the survival and differentiation of cerebellar Purkinje cells, all of the ligands support the survival of GABAergic primary neurons and DAergic neurons (PSPN being less potent than the others), and all ligands but ARTN promote the survival of serotonergic primary neurons (Mount et al., 1995, Baloh et al., 1998, Horger et al., 1998, Åkerud et al., 2002, Ducray et al., 2006).

On top of the important functions of GFLs during development of different neural populations, they have further roles in adult nervous system. For example, GDNF seems to

reduce psychostimulant and alcohol reward, as reviewed by Ghitza et al., 2010. Heterozygous GDNF –knock-outs have impaired learning performance in the water maze paradigm, and GFRα2 –knock-out mice show memory deficits in several experimental setups (Gerlai et al., 2001, Võikar et al., 2004). GDNF is also associated with formation of synapses, enhancement of neurotransmitter release and thus, synaptic strengthening (Wang et al., 2001, Ledda et al., 2007).

GDNF and the nigrostriatal DAergic pathway

GDNF is one of the most potent NTFs for DAergic neurons and its signalling machinery is present on these neurons. In the adult brain, mRNAs of RET and GFRα1 are abundantly and GFRα2 to a lesser extent expressed on the SNpc DAergic cell bodies, and RET seems to be transported to striatal afferents of these cells as well (Nosrat et al., 1997, Widenfalk et al., 1997, Golden et al., 1998, Hirata and Kiuchi, 2007). GFRα1 is found in the striatum, where GFRα2 is virtually absent (Widenfalk et al., 1997, Kozlowski et al., 2004). It seems though, that striatal GFRα1 is not present on DAergic projections. Reports concerning the location of other GFL receptors in the nigrostriatal neurons were not found in literature search.

GDNF is retrogradely transported in the DAergic neurons, and its possible function as a target-derived NTF is supported by the expression of its mRNA in striatal medium sized neurons both during development and adulthood (Tomac et al., 1995, Bizon et al., 1999, Kawamoto et al., 2000, Oo et al., 2005). Although virtually no defects have been found in the nigrostriatal DAergic system in young GFL, GFRα- or RET-knock-out animals, there are interesting *in vivo* data pointing out that GDNF indeed is a target-derived NTF for the developing DAergic system. DAergic neurons in postnatal rodents undergo developmental neuronal death, peaking immediately after birth and again two weeks later. Oo et al. (2003) have shown that an intrastriatal injection of GDNF at the time of the first peak can significantly suppress natural cell death, and GDNF –neutralizing antibodies can augment it. In a further study, overexpression of GDNF in the striatum, cortex and hippocampus could also prevent DAergic neuronal death during the first peak of apoptosis, althoughthe effect did not persist to adulthood (Kholodilov et al., 2004). Instead, higher cell numbers were seen in the ventral tegmental area (VTA) in adult mice, which suggests that the fate of nigrostriatal and mesolimbic DAergic neurons can be differently regulated by GDNF. As already mentioned, GDNF –signalling is pivotal in maintaining catecholaminergic neurons in the adult nervous system (Pascual et al., 2008). Similarly, aged heterozygous GFRα1- or GDNF-deficient mice as well as conditional RET-knock-out mice show degenerative changes in their nigrostriatal DAergic neurons, which further emphasizes the role of GDNF in the maintenance of these neurons (Boger et al., 2006, Kramer et al., 2007, Zaman et al., 2008)

When administered into intact nigrostriatal pathway, GDNF can augment DAergic function. A single intrastriatal injection of GDNF is able to induce sprouting of TH-positive fibres, increase DA turnover in the striatum (usually accompanied by decreased DA levels) and elevate nigral DA concentrations (Hudson et al., 1995, Hebert et al., 1996, Cass et al., 1999). In a later study, similar administration of GDNF lead to augmented D-amphetamine and K^+ - stimulated DA release (Hebert et al., 1996). The dopaminotrophic actions also manifested as

increased spontaneous and D-amphetamine-induced locomotor activity in rats. These effects *in vivo* may be explained by specific findings *in vitro*: GDNF can increase DA uptake and the amount of DA released, upregulate the expression of TH and increase the phosphorylation of TH (Lin et al., 1993, Pothos et al., 1998, Xiao et al., 2002, Kobori et al., 2004).

Less is known of how the other GFLs affect the nigrostriatal DAergic system. However, similarly to GDNF, intranigral delivery of NRTN increases striatal DA turnover in intact rats, although DA levels get reduced (Cass and Peters, 2010a). Also, nigral DA concentrations were elevated, and D-amphetamine-induced DA overflow is increased. In aged rhesus monkeys, in which the DAergic system is already naturally compromised, expression of NRTN by AAV2-vector in the caudate-putamen (CPu) increases ^{18}F-fluorodopa uptake (CPu) and TH-immunoreactivity (CPu and SN) (Herzog et al., 2007).

Efficacy of GDNF in the 6-OHDA –model
The effects of GDNF have been extensively studied in the 6-OHDA –model of PD, utilizing various methods of delivery (Tables 3-7). Unquestionably, GDNF has potent effects on the injured DAergic system even as a single injection. Generally, the most consistent finding in neuroprotection experiments (i.e. GDNF is administered before 6-OHDA) is that the DAergic function and connections are most effectively spared if both GDNF and 6-OHDA are administered into the striatum (Shults et al., 1996, Aoi et al., 2000c, Kirik et al., 2000a, Aoi et al., 2001). Moreover, if GDNF is delivered into the SN in a partial lesion model, the DAergic cell bodies are robustly protected but it is not enough to maintain striatal innervation (Sauer et al., 1995, Kirik et al., 2000a, Rosenblad et al., 2000). GDNF also induces sprouting of TH-positive fibres locally at the administration site, and in the globus pallidus.

Some reports describe a higher preservation of striatal DA in cases when both the treatment and the toxin were given to the SN, but the striatal innervation was not studied by immunohistochemistry (Kearns et al., 1997, Sullivan et al., 1998, Fox et al., 2001). Therefore, it is not clear whether the higher amount of DA in the striatum is due to higher levels of DA in remaining nerve terminals or due to increased survival or sprouting of DAergic terminals. Neuroprotection studies including measurement of nigrostriatal DA are surprisingly few, but the findings are consistent. Also, studies with behavioural assessment are rather few, and the results vary although it seems that whenever GDNF and 6-OHDA are injected into the same location, at least drug-induced rotational bias is ameliorated (Shults et al., 1996, Sullivan et al., 1998, Aoi et al., 2000c, Kirik et al., 2000a, Aoi et al., 2001).

Results from the neurorestoration experiments (i.e. GDNF is given after 6-OHDA –lesioning) are perhaps less uniform, but some general conclusions can be drawn. Positive changes in striatal TH-immunoreactivity are seen again only if GDNF and 6-OHDA are both given intrastriatally (Aoi et al., 2000a, Aoi et al., 2000b, Aoi et al., 2001). In the studies by Kirik et al. (2001), this correlation was not seen, which could be explained by the substantial lesion that was employed. Also, it is curious that sequential administration of GDNF to any brain area in the partial lesion model seems to be rather ineffective in the neurorestoration paradigm: protection of nigral cell bodies or DA content is usually seen, but without positive striatal changes or reduction of rotational bias (Rosenblad et al., 1998, Rosenblad et al., 1999).

Intranigral GDNF combined with intranigral lesion generally has regenerative effects that are confined to the SN, but which is accompanied by reversal of rotational bias (Hoffer et al., 1994, Bowenkamp et al., 1995, Bowenkamp et al., 1997). Intracerebroventricular delivery of GDNF has similar effects as intranigral delivery in a partial lesion: recovery is seen only in the SN, and there are positive behavioural effects in PD rats (Lapchak et al., 1997b, Rosenblad et al., 1999, Kirik et al., 2001).

Regarding possible clinical applications, multiple or continuous intracerebral injections of trophic factors into the brain are a tedious and an inconvenient way to deliver the therapeutic protein. Therefore, it has been of interest to develop delivery systems that would provide long-term release or production of the NTFs. There appears to be three main approaches with GDNF: a) transfected cells that produce and release the protein, b) synthetic polymer materials that release growth factors and c) transfection of host cells by viral vectors for endogenous overexpression and release of the trophic factor. Results arising from experiments using these approaches are summarized in tables 5-7.

In general, sustained GDNF release by cell or polymer preparations has been effective in both neuroprotection and neurorestoration paradigms (Table 5). All of these experiments have been based on a partial 6-OHDA –lesion. In the neuroprotection studies, intrastriatally implanted GDNF-secreting neural stem cells, astrocytes and encapsulated baby hamster kidney cells (BHK) could significantly protect nigral TH-positive cells and their striatal fibers (Åkerud et al., 2001, Cunningham and Su, 2002, Yasuhara et al., 2005b). However, Ericson et al. (2005) reported that GDNF-producing astrocytes could only protect nigral TH-cell bodies but not striatal connections when implanted into the SN, and striatal implantation was completely inefficient. It is possible, that these astrocytes did not produce sufficient amounts of GDNF, although it was detected by immunohistochemistry in the brain tissue. All in all, these results are in line with those obtained with GDNF protein injections, although the local concentrations of GDNF most likely remain lower with sustained-release delivery systems. Importantly, no severe adverse effects were reported.

The results from neurorestoration experiments have been encouraging as well. GDNF-releasing human bone marrow cells, encapsulated BHK-cells, fibroblasts and polymeric microspheres could all induce behavioural recovery (i.e. reversal of rotational bias), and increase striatal DA or TH-innervation (Jollivet et al., 2004a, Jollivet et al., 2004b, Duan et al., 2005, Yasuhara et al., 2005b, Garbayo et al., 2009, Glavaski-Joksimovic et al., 2010). Nigral TH-cells were not rescued with polymeric microspheres, though it was not measured in the study by Garbayo et al. (2009). Still, it seems that the microspheres were capable of releasing enough GDNF to maintain sufficient striatal TH-density and/or DA release to attenuate rotational behaviour.

Table 3. Neuroprotection experiments with GDNF in the 6-OHDA –rat model of PD. Table includes summaries of behavioural changes, protection of striatal DA and nigral or striatal TH-immunoreactivity from experiments in which GDNF protein has been delivered as injections or via minipump before (or less than 24 h after) 6-OHDA. Table organized according to elevating GDNF dosage. GDNF and 6-OHDA –columns contain the dose and site of administration.

Reference	GDNF	Time between	6-OHDA	Behaviour	DA	TH	Other
(Kearns and Gash, 1995)	1x10 µg, SN	24 h	1x8 µg, SN	n.m.	n.m.	SN ↑	
	1x10 µg, SN	24 h	1x8 µg, STR	n.m.	n.m.	SN ↑	
(Sauer et al., 1995)	1x10 µg, SN	? Same day (after)	1x20 µg, STR	n.m.	n.m.	SN ↑ STR 0	
(Kearns et al., 1997)	1x10 µg, SN	0-24 h	1x8 µg, SN	n.m.	SN ↑ STR↑	SN ↑	Optimal time interval 6 h
(Fox et al., 2001)	1x10 µg, SN	6 h	1x8 µg, SN	n.m.	STR↑	SN ↑	Less robust in old rats
(Aoi et al., 2001)	1x10 µg, STR	24 h	1x20 µg, STR	APO ↓	n.m.	SN ↑ STR ↑	
(Kirik et al., 2000a)	1x25 µg, STR	6 h	1x20 µg, STR	AMPH ↓ APO ↓ FA ↓	n.m.	SN ↑ STR ↑	TH-fibres in GP ↑
	1x25 µg, SN	6 h	1x20 µg, STR	AMPH ↑ APO ↑ FA 0	n.m.	SN ↑ STR 0/-	TH-fibres in SN ↑
	1x25 µg, ICV	6 h	1x20 µg, STR	AMPH 0 APO 0 FA 0	n.m	SN 0 STR 0	
(Sullivan et al., 1998)	25 µg SN +50 µg ICV	0 h	1x8 µg, MFB	AMPH ↓	STR↑	SN ↑	PET scan: DAT ↑
(Shults et al., 1996)	4x0.1-10µg, STR in 8 d	4 d (+4d after)	1x25 µg, STR	AMPH ↓	n.m.	SN ↑ STR ↑	
(Sauer et al., 1995)	10 µg, SN every 2 d, 4 w	? Same day (after)	1x20 µg, STR	n.m.	n.m.	SN ↑ STR 0	
(Rosenblad et al., 2000)	SN, 14 d (minipump) + 7x5 µg in 18 d	0 h	3x7 µg, STR	FA 0 APO 0	n.m.	SN ↑	[3H]BTCP (DAT) 0 TH in GP ↑

AMPH= amphetamine-induced rotational behaviour, APO= apomorphine –induced rotational behaviour, DAT=dopamine transporter, FA= forelimb akinesia, GP= globus pallidus, ICV= intracerebroventriculartly, MFB= medial forebrain bundle, PET=positron emission tomography, SN=substantia nigra, STR=striatum. Responses of GDNF-treated animals vs. lesion control animals: 0=no change, ↑= increased, ↓=decreased n.m.= not measured.

Table 4. Neurorestoration experiments with GDNF in the 6-OHDA –rat model of PD. Table includes summaries of behavioural changes, protection of striatal DA and nigral or striatal TH-immunoreactivity from experiments in which GDNF protein has been delivered as injections or via minipump after 6-OHDA. Table organized according to elevating GDNF dosage. GDNF and 6-OHDA –columns contain the dose and site of administration.

Reference	6-OHDA	Time between	GDNF	Behaviour	DA	TH	Other
(Aoi et al., 2001)	1x20 µg, STR	4 w	1x10 µg, STR	APO ↓	n.m.	SN ↑ STR↑	
(Aoi et al., 2000b)	1x20 µg, STR	4 w	1x10 µg, STR	APO ↓	n.m.	SN ↑ STR↑	Transcient recovery
	1x20 µg, STR	4 w	1x10 µg ICV	APO ↓	n.m.	SN 0 STR↑	Transcient recovery
(Aoi et al., 2000a)	1x20 µg, STR	4 w	1x10 or 100 µg, STR	APO ↓	n.m.	SN ↑ STR↑	
(Hoffer et al., 1994)	1x9 µg MFB	4 w	1x0.1-100 µg, SN	APO ↓	SN ↑	n.m.	Only at highest dose
(Bowenkamp et al., 1995)	1x9 µg, MFB	4 w	1x100 µg, SN	APO ↓	n.m.	SN ↑ STR 0	TH fibres, TH-cell size in SN ↑
(Lapchak et al., 1997b)	1x20 µg, MFB	9 w	1x100/1000 µg SN	APO ↓	SN ↑ STR 0	SN ↑ STR↑	TH as activity
	1x20 µg, MFB	9 w +15,21 w	1-3x100/1000 µg ICV	APO 0 (vs. ctrl, ↓ vs.pre-injection)	n.m.	SN ↑ STR 0	TH as activity
(Bowenkamp et al., 1997)	1x9 µg, MFB	3 & 6 w	250 µg & 500 µg, ICV	Locomotor activity ↑	SN ↑	SN 0 STR 0	
(Winkler et al., 1996)	2x10 µg, STR	5 d	9x10 µg, SN every 4 d	AMPH 0 FA 0	n.m.	SN ↑ STR 0	
(Rosenblad et al., 1999)	1x20 µg, STR	1 d	8x5 µg, STR every 3 d, 3 w	AMPH 0	SN ↑ STR 0	n.m.	TH down-reg. in SN
	1x20 µg, STR	1 d	8x10 µg, ICV every 3 d, 3 w	AMPH 0	SN ↑ STR 0	n.m.	TH down-reg. in SN
(Rosenblad et al., 1998)	1x20 µg, STR	4 w	10x5 µg, STR every 2 d, 20 d	AMPH 0 FA ↓	n.m.	SN ↑	[3H]BTCP (DAT) ↑ lesion area↓
(Kirik et al., 2001)	3x7 µg, STR	2w	STR, 28 d (minipump)	FA ↓ Staircase 0 AMPH 0	n.m.	SN 0 STR 0	Transcient recovery of akinesia
	3x7 µg, STR	2w	ICV, 28 d (minipump)	FA ↓ Staircase 0 AMPH ↓	n.m.	SN ↑ STR 0	

AMPH= amphetamine-induced rotational behaviour, APO= apomorphine –induced rotational behaviour, DAT=dopamine transporter, FA= forelimb akinesia, ICV= intracerebroventriculartly, MFB= medial forebrain bundle, SN=substantia nigra, STR=striatum. Responses of GDNF-treated animals vs. lesion control animals: 0=no change, ↑= increased, ↓=decreased n.m.= not measured.

Table 5. Effects of sustained delivery of GDNF in neuroprotection and neurorestoration in the 6-OHDA –models of PD. Table includes summaries of behavioural changes, protection of striatal DA and nigral or striatal TH-immunoreactivity from experiments in which GDNF protein has been administered via sustained delivery systems before or after 6-OHDA –lesioning. GDNF delivery method and 6-OHDA –columns contain the dose and site of administration.

Reference	GDNF delivery method	Time relation to 6-OHDA	6-OHDA	Behaviour	DA	TH	Other
(Åkerud et al., 2001)	neural stem cells, 4xSTR	16 d before	1x4 µg, STR	AMPH ↓ APO ↓	n.m.	SN ↑	Mouse model
(Cunningham and Su, 2002)	astrocytes, 1xSN	6 d before	1x16 µg, STR	AMPH ↓	SN ↑ STR↑	SN ↑ STR↑	Mouse model
(Ericson et al., 2005)	astrocytes, 1xSTR	1 w before	7/3x7 ug, STR		n.m.	SN 0 STR 0	
	astrocytes, 1xSN	1 w before	7/3x7 ug, STR		n.m.	SN ↑ STR 0	
(Yasuhara et al., 2005b)	encapsulated BHK-cells 1xSTR	1 -0 w before	1x20 µg, STR	AMPH ↓	n.m.	SN ↑ STR↑	
(Gouhier et al., 2002)	polymeric micro-spheres, 2xSTR	0 h	1x16 µg, STR	AMPH ↓	n.m.	STR ↑	Behavioural effect during first weeks, [125I]-PE2I (DAT) ↑
(Yasuhara et al., 2005b)	encapsulated BHK-cells 1xSTR	1, 2, 4 w after	1x20 µg, STR	AMPH ↓	n.m.	SN ↑ STR↑	Effects more robust with early trans-plantation
(Glavaski-Joksimovic et al., 2010)	human bone marrow cells, 2xSTR	1 w after	1x16 µg, STR	AMPH ↓	n.m.	STR↑	
(Duan et al., 2005)	fibroblasts, 2xSTR	4 w after	2x7.5 µg, STR	APO ↓	STR↑	SN ↑	
(Jollivet et al., 2004a, Jollivet et al., 2004b)	polymeric micro-spheres, 2xSTR	2 w after	2x10 µg, STR	AMPH ↓	n.m.	SN 0 STR↑	
(Garbayo et al., 2009)	polymeric micro-spheres, 2xSTR	2 w after	2x10 µg, STR	AMPH ↓	n.m.	STR↑	

AMPH= amphetamine-induced rotational behaviour, APO= apomorphine –induced rotational behaviour, BHK-cells=baby hamster kidney cells, DAT=dopamine transporter, ICV= intracerebroventriculartly, MFB= medial forebrain bundle, SN=substantia nigra, STR=striatum. Responses of GDNF-treated animals vs. lesion control animals: 0=no change, ↑= increased, ↓=decreased n.m.= not measured.

There is extensive evidence, that GDNF -gene delivery is effective in protecting nigrostriatal DAergic neurons against 6-OHDA -toxicity in the partial lesion model. All reports describe an increase in the survival of TH-positive cells in the SN regardless of the vector used or the location of vector delivery. In only one study there was a lack of nigral recovery, but this was measured as TH mRNA, and could be attributed to the fact that long-term delivery of GDNF can downregulate TH in the nigrostriatal DAergic neurons (Rosenblad et al., 1999, Connor, 2001, Georgievska et al., 2004). Similarly to GDNF protein delivery, its gene expression in SN only is often not sufficient to protect striatal connections or prevent behavioural deficits (Connor et al., 1999, Kirik et al., 2000b). Still, Chen et al. (2003) and Connor et al. (2001) have reported of a higher striatal DA concentration or DAT –radioligand binding in comparison to control animals, but these effects were not accompanied by reversal of rotational bias. Conversely, Bensadoun et al. (2000) showed that in the abscence of striatal DAergic protection, apomorphine -induced rotations are reduced in mice. Surprisingly, striatal gene delivery is very efficient in improving behavioural measures, although striatal recovery is only seldom seen. This lack of striatal recovery, if measured as expression of TH, may not be a reliable measure because of its downregulation as already mentioned.

In the neurorestoration paradigm, the results of intrastriatal GDNF-vector delivery in partial lesion models are fairly consistent. Either striatal or nigral recovery, or both, of DA or TH-innervation is observed, and accompanied by behavioural improvements (Lapchak et al. 1997a, Wang et al., 2002, Zheng et al., 2005, Brizard et al., 2006). In the studies by Kozlowski et al. (2000), however, intrastriatal delivery of adenoviral GDNF was totally inefficient. Intranigral administration of vectors seems to have local dopaminotrophic actions that do not extend to the striatum, which is typical to the delivery of recombinant GDNF as well.

The variation in the experiments with viral vectors may be explained by several factors. Firstly, the amount of vector injected varies between the experiments. Secondly, the transfection efficacy of the viral vectors may differ, and thirdly, the vectors can preferentially transfect different cell populations. Whether it is essential to transfect nigrostriatal DAergic cells, or whether it is sufficient to transfect other cell populations around DAergic cells to offer trophic support from outside the nigrostriatal pathway, seems unsolved.

As a general conclusion, despite some discrepancies between studies utilizing various methods of delivery, GDNF has shown great potential as a therapeutic molecule for PD as assessed in the 6-OHDA –model. When a total lesion is employed, it is still possible to achieve at least local nigral recovery and behavioural recovery if GDNF is delivered into the SN. In a partial lesion model, GDNF –treatment is most effective when given intrastriatally. The observations, that behavioural recovery is possible without significant positive changes in striatal TH-innervation or DA content when compared to control treatment can be due to the "pharmacological effect" of GDNF, i.e. its ability to boost DA turnover and augment stimulated DA release.

Table 6. Neuroprotection experiments utilizing GDNF delivery via viral vectors in the 6-OHDA –models of PD. Table includes summaries of behavioural changes, protection of striatal DA and nigral or striatal TH-immunoreactivity from experiments in which GDNF –vectors have been administered before 6-OHDA. Table organized according to type of vector used. GDNF – vector and 6-OHDA –columns contain the dose and site of administration.

Reference	GDNF-vector	Time between	6-OHDA	Behaviour	DA	TH	Other
(Mandel et al., 1999)	AAV, SN	0 h	1x20 µg, STR	n.m.	n.m.	SN ↑	
(Mandel et al., 1997)	AAV, SN	3 w	1x20 µg, STR	n.m.	n.m.	SN ↑	
(Kirik et al., 2000b)	AAV, 2x SN	4 w	4x7 µg, STR	AMPH 0 CT bias 0 Staircase 0 FA 0	n.m.	SN ↑ STR 0	TH-fibers in SN ↑
	AAV, 3xSTR	4 w	4x7 µg, STR	AMPH ↓ CT bias ↓ Staircase ↑ FA 0	n.m.	SN ↑ STR ↑	TH-fibers in GP ↑
	AAV, 2xSN + 3xSTR	4 w	4x7 µg, STR	AMPH 0 CT bias 0 Staircase 0 FA 0	n.m.	SN ↑ STR 0	TH-fibers in SN ↑
(Choi-Lundberg et al., 1997)	Adenovirus, SN	7 d	1x16 µg, STR	n.m.	n.m.	SN ↑	
(Choi-Lundberg et al., 1998)	Adenovirus, STR	7 d	1x16 µg, STR	CT bias ↓ AMPH ↓	n.m.	SN ↑ STR 0	
(Connor et al., 1999)	Adenovirus, SN	7 d	1x16 µg, STR	AMPH ↑ CT bias 0	n.m.	SN ↑ STR 0	
	Adenovirus, STR	7 d	1x16 µg, STR	AMPH ↓ CT bias ↓	n.m.	SN ↑ STR ↑	STR c-fos after AMPH↑
(Connor et al., 2001)	Adenovirus, SN	7 d	1x16 µg, STR	AMPH 0	n.m.	SN 0 (mRNA)	[125I]IPCIT (DAT) ↑
	Adenovirus, STR	7 d	1x16 µg, STR	AMPH ↓	n.m.	SN ↑ (mRNA)	[125I]IPCIT (DAT) ↑
(Chen et al., 2003)	Adenovirus, SN	7 d	4x4 µg, STR	APO 0	STR↑	SN ↑	
(Do Thi et al., 2004)	Adenovirus, 6xSTR	7 d	20 µg, 3xSTR	AMPH ↓	n.m.	SN ↑ STR 0	GFAP-promoter
(Bensadoun et al., 2000)	Lentivirus, SN	2 w	1x4 µg, STR	APO ↓	STR 0	SN ↑	Mouse model
(Georgievska et al., 2002b)	Lentivirus, 2xSTR	3 w	1x20 µg, STR	AMPH 0	n.m.	SN ↑ STR 0	TH-fibers in GP ↑
(Georgievska et al., 2002a)	Lentivirus, 6xSTR	4 w	3x7 µg, STR	AMPH ↓ CT, FA 0 staircase 0		SN ↑ STR 0	TH-down-regulation

Table 6. continued

Reference	GDNF-vector	Time between	6-OHDA	Behaviour	DA	TH	Other
(Sun et al., 2005)	HSV-1, 3xSTR	4 w	4x7 µg, STR	APO ↓	n.m.	SN ↑	

AAV=adeno-associated virus, AMPH= amphetamine-induced rotational behaviour, APO= apomorphine –induced rotational behaviour, CT= cylinder test, DAT=dopamine transporter, FA= forelimb akinesia, GFAP=glial fibrillary acidic protein, GP= globus pallidus, HSV-1= *Herpes Simplex* 1, ICV= intracerebroventriculartly, MFB= medial forebrain bundle, SN=substantia nigra, STR=striatum. Responses of GDNF-treated animals vs. lesion control animals: 0=no change, ↑= increased, ↓=decreased n.m.= not measured.

Table 7. Neurorestoration experiments utilizing GDNF delivery via viral vectors in the 6-OHDA –rat model of PD. Table includes summaries of behavioural changes, protection of striatal DA and nigral or striatal TH-immunoreactivity from experiments in which GDNF –vectors have been administered after 6-OHDA. Table organized according to type of vector used. GDNF – vector and 6-OHDA –columns contain the dose and site of administration.

Reference	6-OHDA	Time between	GDNF-vector	Behaviour	DA	TH	Other
(Wang et al., 2002)	1x20 µg, STR	4 w	AAV, STR	APO ↓ CT bias ↓	STR↑	SN ↑ STR↑	
(Kozlowski et al., 2000)	1x16 µg, STR	7 d	Adenovirus, SN	AMPH ↓ CT bias 0	n.m.	SN ↑ STR 0	STR c-fos after AMPH↑
	1x16 µg, STR	7 d	Adenovirus, STR	AMPH 0 CT bias 0	n.m.	SN 0 STR 0	
(Zheng et al., 2005)	2x16 µg, STR	4 w	Adenovirus, 2xSTR	APO ↓	STR↑	SN ↑	
(Lapchak et al., 1997a)	1x20 µg, MFB	10 w	Adenovirus, SN	APO ↓ Locomotor activity ↑	SN ↑ STR 0	n.m.	
(Brizard et al., 2006)	1x3 µg, SN	4 w	Lentivirus, STR	Staircase ↑	n.m.	STR↑ SN 0	

AAV=adeno-associated virus, AMPH= amphetamine-induced rotational behaviour, APO= apomorphine –induced rotational behaviour, CT= cylinder test, DAT=dopamine transporter, SN=substantia nigra, STR=striatum. Responses of GDNF-treated animals vs. lesion control animals: 0=no change, ↑= increased, ↓=decreased n.m.= not measured.

Studies of NRTN and PSPN in the 6-OHDA –model of PD

Other GFLs than GDNF have been much less studied in preclinical models of PD. There is a handful of neuroprotection and neurorestoration studies each done with NRTN, in which various methods of delivery have been used in a partial lesion model in rats. There are no studies available concerning artemin, and only one report describing the effects of PSPN. In this one study, neural stem cells were engineered to express and secrete PSPN and grafted to four striatal locations in mice two weeks before striatal 6-OHDA (Åkerud et al., 2002). PSPN treatment could significantly prevent the death of TH-positive cells in the SN and reduce amphetamine-induced rotational behaviour.

Taken together, neuronal protection in the SN has been observed in all neuroprotection studies with NRTN, whereas maintenance of striatal innervation has not been as consistent. In both studies, in which NRTN protein was given as a single intrastriatal 5 µg injection 1-3 days before 6-OHDA, elevated *post mortem* striatal DA concentrations were observed (Oiwa et al., 2002, Cass and Peters, 2010b). Oiwa et al. (2002) also reported a reduction in amphetamine-induced rotations. In the study by Cass et al. (2010b) potassium- or amphetamine–induced DA release in the striatum was improved with NRTN as measured by microdialysis.

Rather surprisingly, when NRTN has been delivered intrastriatally for longer periods starting 14-15 days before 6-OHDA-lesioning, it has been ineffective in preserving striatal TH-fibres or DA, despite protection of nigral cells. Nevertheless, in two studies, utilizing NRTN-expressing neural stem cells or an AAV-vector –mediated delivery, reduced rotational behaviour was observed (Gasmi et al., 2007, Liu et al., 2007). The positive effect by AAV-NRTN (CERE-120) on nigral TH-cells was further confirmed by Bartus et al., 2011. Lentiviral expression of modified or wild-type (WT) NRTN did not lead to behavioural improvement (Fjord-Larsen et al., 2005). WT NRTN was without any protective effects in the studies, whereas the immunoglobulin signal peptide –conjugated modified NRTN could protect TH-positive cells, and was also retrogradely transported from the striatum to the SN. Of note, Gasmi et al. (2007) detected a significant protection of striatal vesicular monoamine transporter immunoreactivity by AAV-NRTN, which could mean that NRTN either downregulates TH or does not support the maintenance of TH-phenotype in nigrostriatal DAergic neurons. Indeed, Rosenblad et al. (1999) observed a reduction in the intensity of TH-expression in surviving nigral neurons in NRTN –treated and 6-OHDA –lesioned rats, and therefore TH may not be a reliable marker of DAergic cells and their projections in studies with NRTN.

Results of neurorestoration studies are rather inconsistent. Generally, a single administration of NRTN has been more effective than long-term delivery in the partial lesion model, as it was in the neuroprotection studies as well. Oiwa et al. (2002) reported of an amelioration of rotational bias accompanied by elevated striatal TH and DA levels after 5 µg of intrastriatal NRTN, although it was delivered as late as 12 weeks post lesioning. Horger et al. (1998) analysed only the survival of nigral DAergic cells, and found that an intranigral injection of 10 µg of NRTN one week after lesioning was sufficient to reduce neurodegeneration. Interestingly, in the same study, altogether 8x5 µg of NRTN given to the SN increased the survival of nigral neurons, but not their TH-phenotype. This is in line with Rosenblad et al. (1999) who used the same delivery regime as Horger et al. (1998), but delivered NRTN into the striatum or lateral ventricle. Only striatal delivery could significantly protect nigral cell bodies, but no behavioural or striatal recovery was detected. Striatal NRTN delivery via transduced bone marrow stromal cells three weeks after an extensive 6-OHDA lesion of the MFB and VTA, was ineffective (Ye et al., 2007).

In conclusion, NRTN and PSPN have been demonstrated to have positive therapeutic effects in 6-OHDA –treated rats although the correlations of behavioural, striatal and nigral recoveries are not very consistent with NRTN. Lack of reports concerning ARTN and PSPN may imply though, that there are a lot of unpublished negative data, which would not support a possible therapeutic role for these ligands in the treatment of PD. Also, it has been a matter

of discussion how NRTN induces the positive effects seen in the 6-OHDA –experiments, as there is virtually no GFRα2 in the striatum, and its expression is rather decreased, if changed at all by 6-OHDA –lesions (Marco et al., 2002, Kozlowski et al., 2004). However, since NRTN can also bind to GFRα1 (though not preferentially), it is probably at least one of the mediators of the effects.

2.3.3 CDNF/MANF –family of NTFs

Cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF), both of which are widely expressed in mammalian tissues, are so far the only known members of an evolutionarily conserved family of NTFs (Lindholm and Saarma, 2010). A structural peculiarity of these proteins is that they contain eight cysteine residues, which can be found in all the protein homologues in both vertebrates (CDNF and MANF) and invertebrates (only MANF homologue). Also unique to these proteins, the mammalian CDNF and MANF consist of an N-terminal saposin-like domain and a c-terminal domain with cystein bridges (Parkash et al., 2009). The signalling receptors for these NTFs have not yet been identified, but their roles as extracellularly functioning NTFs is supported by observations that both proteins seem to be secreted by transfected cells (Lindholm et al., 2007, Apostolou et al., 2008, Lindholm et al., 2008). However, MANF and CDNF are also retained in the endoplasmic reticulum (ER) (Apostolou et al., 2008). MANF gene and protein expressions are induced by ER stress, and it can protect cells against ER-stress –induced death, which may be a common mechanism of neuroprotection for the proteins (Lee et al., 2003, Mizobuchi et al., 2007, Apostolou et al., 2008, Tadimalla et al., 2008, Yu et al., 2010). Also, a recent article describes the ability of intracellular MANF to inhibit Bax-dependent apoptosis in neurons (Hellman et al., 2011).

MANF was the first member of the family to be discovered as a NTF, although it was already earlier known as "arginine-rich, mutated in early stage tumors" or arginine-rich protein, expressed by various tumours. MANF was detected in the culture medium of a rat astrocyte cell line, and was shown to protect DAergic neurons rather selectively (Petrova et al., 2003). Since then, it has been demonstrated that MANF expression is induced in neurons and glial cells in an experimental model of brain ischaemia (Lindholm et al., 2008, Yu et al., 2010). Furthermore, intracortical pretreatment with recombinant MANF or expression of MANF via AAV-vector can reduce the size of infarction and promote motor recovery in rats after middle cerebral artery occlusion (Airavaara et al., 2009, Airavaara et al., 2010). These studies describe a reduction in TUNEL –expressing neurons both in *in vitro* and *in vivo* conditions of hypoxia. Thus far, our knowledge of neuroprotective and neurorestorative actions of CDNF is limited to models of PD only.

Both MANF and CDNF have been studied in the partial 6-OHDA lesion model of PD in rats. The first report on the discovery of CDNF also describes robust neuroprotective and neurorestorative effects on both behavioural and morphological levels (Lindholm et al., 2007). The study showed that D-amphetamine –induced ipsilateral turning was significantly reduced already with 3 μg of CDNF, and that the nigrostriatal DAergic neurons were preserved at least to the same extent as with GDNF. In similar experimental setups as used for CDNF, MANF

could also effectively reverse the rotational bias (Voutilainen et al., 2009). In the neuroprotection experiment, TH-positive cells in the SNpc were effectively spared by MANF, but this protective effect was not seen in the striatal terminal area. In the neurorestoration setup, MANF was even more potent than GDNF in reducing rotations, but without significant effect on the survival of TH-positive cells. In support of CDNF being perhaps the more potent member of the family, at least when assessed in the 6-OHDA rat model of PD, the latest study in this field showed that a two-week minipump-infusion of CDNF could significantly reduce rotational behaviour in rats lesioned two weeks before the start of the infusion (Voutilainen et al., 2011). Also, the nigrostriatal DAergic cells and connections were well spared. MANF, however, was not effective in a similar experiment.

To date, the neuroprotective and neurorestorative effects of CDNF have been examined only in one study employing in MPTP mouse model of PD (Airavaara et al., 2011). The results of the study were, in all, the same as in the 6-OHDA rat model, and support the opportunity of exogenous CDNF as a novel neurotrophic treatment against DAergic neurodegeneration.

In the pursuance of the 6-OHDA studies, the distribution patterns of exogenously applied CDNF and MANF were studied (Voutilainen et al., 2009, Voutilainen et al., 2011). The distribution of CDNF after a single intrastriatal injection seems to largely resemble that of GDNF, including retrograde transport to the SN. MANF, on the other hand, seems to be relocated towards frontal cortical areas. Also, MANF is more readily distributed in the brain tissue at the early stages of a chronic infusion, when compared to CDNF and GDNF. The different distribution pattern of MANF may be beneficial if the molecule is to be considered for clinical use, since it has been speculated that one of the pitfalls in clinical trials with recombinant GDNF was its poor diffusion in the brain.

2.3.4 VEGF-family ligands

Evidence suggests that vascular defects and impaired blood circulation may significantly contribute to a variety of neurodegenerative diseases (Zlokovic, 2011). For example, the breakdown of the BBB leads to cerebral accumulation of substances that can cause oedema, direct neurotoxicity and generation of reactive oxygen species (eg. iron from red blood cells). Hypoperfusion and hypoxia lead to decreased protein synthesis, impaired activity of transporter proteins, failure in ion gradient maintenance and abnormal generation of action potentials. This may ultimately lead to oedema and lesions. Also, the metabolic profile in the vascular endothelium can change, and both toxic and inflammatory factors may be secreted to the brain tissue. Even normal aging is associated with disruption of the BBB and diminished brain blood circulatory functions. Of neurodegenerative diseases, cerebrovascular dysfunction is most extensively described in AD, but seems to play a part also in PD, amyotrophic lateral sclerosis (ALS), multiple sclerosis, and Huntington's disease. In this context, it may be of importance to study growth factors that affect the vascular system and have direct protective effects on neurons as possible therapeutic molecules for neurodegenerative disorders.

Overview of VEGF-family ligands and receptors

VEGF family is comprised of VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF), which are found in mammals, and VEGF-E coded by the *orf* virus, as well as VEGF-F from snake venom. Several of the family members are expressed in many isoforms due to alternative splicing or post-translational processing, which also alters their receptor specificity (Roy et al., 2006). There are three transmembrane tyrosine kinase receptors (VEGFR-1-3, Fig. 4), which upon binding the dimeric ligands generally form homodimers, but VEGFR-2 can also heterodimerize. Furthermore, VEGFR-1 and VEGF-2 are expressed as soluble monomeric forms, which can antagonise the effects of their ligands. VEGFR-1 also has a unique property of being up-regulated by hypoxia (Kendall and Thomas, 1993, Marti and Risau, 1998, Ebos et al., 2004, Tammela et al., 2005). VEGFs utilize neuropilins 1-2 (NRP1-2) as co-receptors. They are best known as receptors for class 3 semaphorins that have key roles in axonal guidance (Pellet-Many et al., 2008). Interaction with the co-receptors, as well as the formation of heterodimers from the tyrosine kinase receptors, leads to eg. increase in receptor affinity for ligands, augmented signal transduction or altered downstream signalling (Lohela et al., 2009). The principal effects of VEGF-family ligands are to induce and maintain angiogenesis and lymphangiogenesis during embryonal development and in adult during various physiological and pathological processes. These include wound healing, ocular neovascularisation, endometriosis, cardiovascular disease, and tumour (lymph)vascularisation and metastasis.

On the basis of their main physiological functions, the mammalian VEGF-family ligands can be roughly divided into angiogenic factors or modulators (VEGF-A, B and PlGF), and lymphangiogenic factors (VEGF-C and –D) (Lohela et al., 2009). VEGF-A is one of the most potent angiogenic factors with a crucial role in the embryonic development of blood vasculature. Both knock-out mice of VEGF-A and VEGFR-2 have an embryonic lethal phenotype (Shalaby et al., 1995, Carmeliet et al., 1996, Ferrara et al., 1996). VEGF-A stimulates proliferation, migration and survival of endothelial cells, induces vasodilatation and increases vascular permeability, mostly via VEGFR-2. In addition, it induces migration of hematopoietic stem cells and recruitment of inflammatory cells, probably via VEGFR-1. VEGF-A is also strongly induced in hypoxia, and it is expressed in various kinds of tumours and malignancies (Tammela et al., 2005, Roy et al., 2006). VEGF-B and PlGF, on the other hand, do not seem to be pivotal in embryonic vascular development. Instead, they seem to have angiogenic actions through VEGFR-1 under pathological conditions like inflammation and ischaemia, and especially the angiogenic effects of VEGF-B are rather weak under most circumstances (Lohela et al., 2009). VEGF-B has also been implicated in heart development and coronary vessel formation, and may even be considered as a survival factor for blood vessels (Bellomo et al., 2000, Zhang et al., 2009, Bry et al., 2010). A similar division can be made with the lymphangiogenic growth factors: VEGF-C is crucially required for the development of lymphatic vessels and the knock-out mice die as embryos, whereas this is not observed in VEGF-D knock-outs (Kärkkäinen et al., 2004, Baldwin et al., 2005, Koch et al., 2009). Both have active roles in the adult, also during inflammation and tumorigenesis. The growth of new lymphatic vessels is mediated through VEGFR-3, activation of which increases the mitogenesis, migration and survival of lymphatic endothelial cells. VEGF-C and D also cause vascular leakage and can induce angiogenesis via VEGFR-2 (Roy et al., 2006, Lohela et al., 2009).
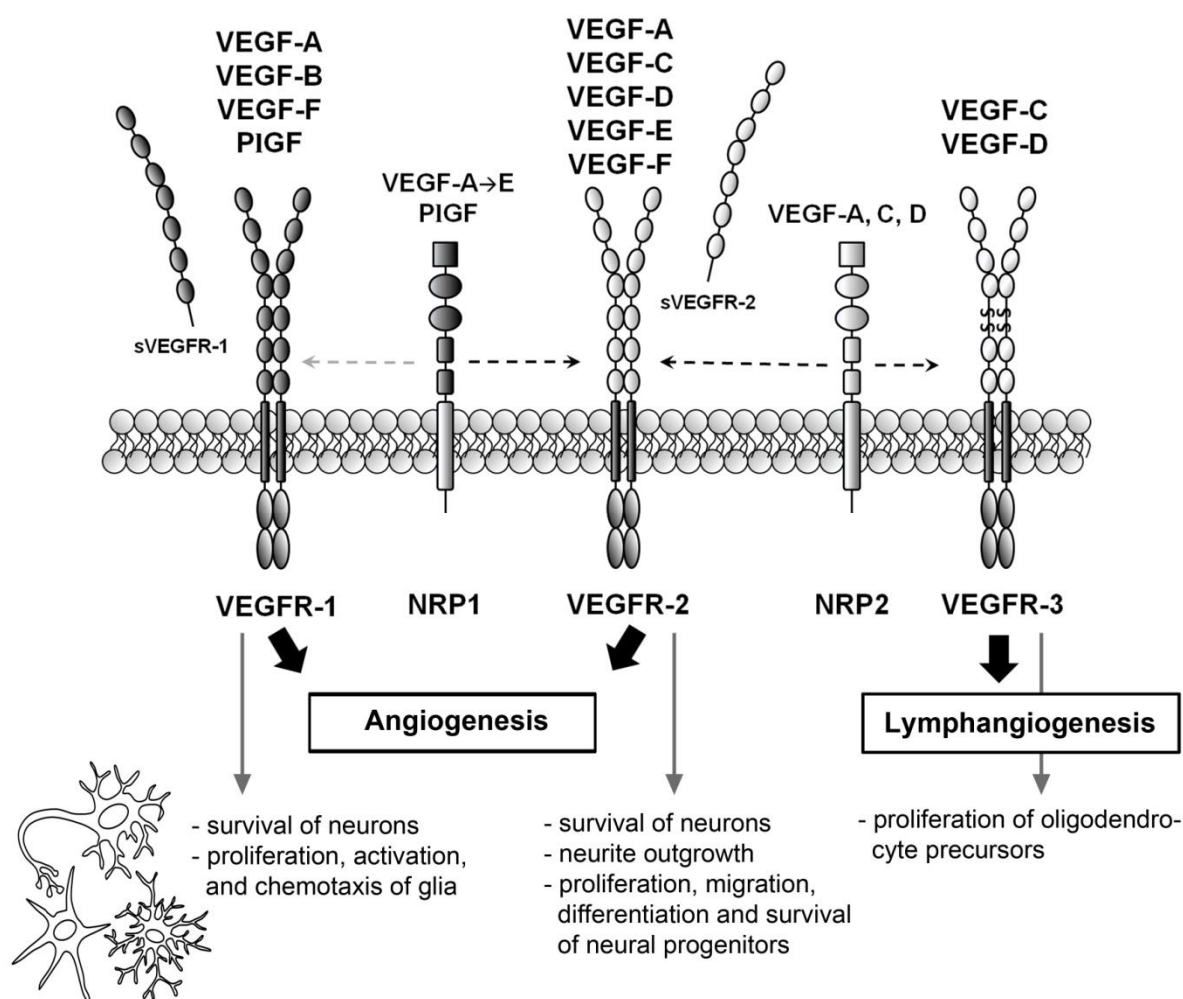
Figure 4. VEGF-receptors and their ligands. Dimeric ligands bind to their receptors, which also become dimerized upon ligand binding, and intracellular tyrosine kinase domains are transphosphorylated. VEGFR-1 and VEGFR-2 are mainly involved in regulation of angiogenesis, mediating proliferation, migration and survival of endothelial cells, vasodilatation and increase of vascular permeability. Their soluble forms act antagonistically. VEGFR-3 signalling increases the mitogenesis, migration and survival of lymphatic endothelial cells. NRP –coreceptors interact with VEGFRs to modulate the signalling, and VEGFR-2 can dimerize also with the other two VEGFRs. All three VEGFRs mediate specific effects in the nervous system, and also NRP1 likely has a role in VEGF-A –induced neurite outgrowth. Figure adapted from Ruiz de Almodovar et al., 2009 and Zachary, 2005.

Connections of VEGF-A to neurological disorders

VEGF-A is the only member of the growth factor family whose association to neurological disorders has been extensively studied, despite lack of compelling evidence of the involvement of VEGF-A –related genetic variations in these diseases. In AD, VEGF-A levels are elevated in the cerebrospinal fluid (Tarkowski et al., 2002) and it is co-precipitated in the amyloid plaques typically seen in AD brains (Yang et al., 2004, Ryu et al., 2009). Administration of VEGF-A seems beneficial in transgenic mouse models of AD (Spuch et al., 2010, Wang et al., 2011), and interestingly, β-amyloid can antagonize VEGFR-2 signalling *in vitro* (Patel et al., 2010). In PD

patients, the expression of VEGF-A, and in many cases also the expression of VEGFR-1, is increased in the SN, especially in reactive astrocytes (Wada et al., 2006). Concentration of VEGF-A in plasma is not changed in PD (Wada et al., 2006, Infante et al., 2007).

In ALS, changes in VEGF-A levels in plasma or cerebrospinal fluid have not been consistent, but a decrease in VEGF-A- and VEGFR-2-expressing motor neurons has been reported (Bogaert et al., 2006, Brockington et al., 2006). Also, a conclusive genetic link between VEGF-A and the human disease is lacking, although there are reports of VEGF-gene haplotypes associated with ALS (Bogaert et al., 2006, Ruiz de Almodovar et al., 2009). There is stronger evidence from animal studies showing a connection between VEGF-A and ALS, as reduced expression of VEGF-A via deletion of the hypoxia response element in the promoter of the gene brings up an ALS –like phenotype in mice (Oosthuyse et al., 2001). Moreover, cross-breeding these mice to another established mouse line modelling ALS ($SOD1^{G93A}$) leads to aggravation of motor symptoms and earlier death of the mice in comparison to either mouse line with a single gene mutation (Lambrechts et al., 2003). Also, administration or overexpression of VEGF-A delays the onset of symptoms and prolongs survival in rodent models of ALS (Azzouz et al., 2004, Zheng et al., 2004, Storkebaum et al., 2005, Wang et al., 2007). Finally, the ischaemic stroke is a condition in which it would be highly beneficial to restore blood supply to the oxygen-deprived areas of the brain as well as to provide neurotrophic support. VEGF-A and its receptors are upregulated in the ischaemic brain, but exogenous VEGF-A may not be a rational approach because it increases vascular permeability by disrupting the BBB (Rite et al., 2007, Beck and Plate, 2009). These effects may lead to increased brain aedema and leakage of unwanted molecules and cells into the brain, which in turn will compromise recovery.

Effects of VEGF-ligands on neurons and glia
The effects of VEGF-A on various kinds of neurons have been widely studied and reviewed (summary in Table 8). In general, studies have shown that VEGF-A can increase neuronal survival, proliferation and neurite outgrowth. In addition, VEGF-A also promotes the proliferation and migration of glial cells. Based on all this data, it is likely that the effects of VEGF-A on neurons are mediated both directly through receptors on the neurons, and indirectly via activation of astroglia and induction of neovascularisation.

Fairly little is known about the roles of other VEGF-family ligands in the nervous system so far. VEGF-B has been demonstrated to stimulate neurogenesis and to inhibit apoptosis in retinal ganglion cells and in the ischaemic brain. It also increases the survival of motor neurons and DRG sensory neurons as well as protects DAergic neurons against rotenone toxicity both *in vitro* and *in vivo* (Sun et al., 2006, Li et al., 2008, Poesen et al., 2008, Falk et al., 2009, Dhondt et al., 2011). VEGF-C seems to have a crucial function in the developing nervous system, and it is required for the proliferation of neural progenitor cells as well as oligodendrocyte precursor cells of the optic nerve (Le Bras et al., 2006). It is also implicated in adult neurogenesis in the hippocampus and subventricular zone after cerebral ischaemia (Shin et al., 2008). Recently, Chaballe et al. (2011) found that the proliferation of Schwann cells is reduced in primary cultures and in a model of Wallerian degeneration in PlGF knock-out

mice. PlGF can also protect cultured cortical neurons against ischaemic damage, prevent growth cone collapse in DRG and alleviate diabetic sensory deficits in mice (Cheng et al., 2004, Du et al., 2010, Murakami et al., 2011) .

VEGFR-1 and VEGFR-2 are expressed widely throughout the developing rat brain (Yang et al., 2003). In the adult rat brain, the expression levels are low, but the receptors have been found for example in cortical and hippocampal neurons (Sondell and Kanje, 2001, Yang et al., 2003, Yasuhara et al., 2004). Since VEGF-A also affects other neuronal populations (Table 8), the receptors are most likely present on those neurons as well. According to recent reports, also VEGFR-3 mRNA is extensively found throughout the developing and adult rat brain (Choi et al., 2010, Hou et al., 2011a, Hou et al., 2011b). An interesting feature of VEGFRs is that they are upregulated by hypoxic or mechanical injuries to the brain (Marti and Risau, 1998, Krum et al., 2002, Choi et al., 2007, Shin et al., 2008, Shin et al., 2010a, Shin et al., 2010b).

Generally, the neuronal effects of VEGF-A are mediated via VEGFR-2, and the effects on glial cells via VEGFR-1 (reviewed by Ruiz de Almodovar et al., 2009). The activation of VEGFR-2 in the neurons can induce several signalling cascades, including the MEK/MAPK, PI3K/Akt, PLCγ and Rho/ROCK (Rho/Rho –associated protein kinase) –pathways. Also, VEGF-A can inhibit the activation of caspase-3, although it is not known which receptor mediates this effect (Jin et al., 2001, Sanchez et al., 2010). Furthermore, VEGF-A (and PlGF) can block the chemorepulsion, growth cone collapse and apoptosis caused by Sema3A in a neuronal cell culture via interaction with NRP1 (Bagnard et al., 2001). The downstream signalling pathways of VEGFR-1 in glial cells include at least MEK/MAPK and PI3K/Akt –cascades (Mani et al., 2005).

VEGF-B, on the other hand, induces its protective effects on neurons through VEGFR-1 (Li et al., 2008, Dhondt et al., 2011). This activates MAPK/ERK -signalling, and leads to reduced expression of cell-death related proteins and genes (Li et al., 2008). Knowledge of VEGF-C signalling in the nervous system is still very limited, but since both VEGFR-2 and VEGFR-3 are expressed (and can be induced) in the brain, it is likely that both receptors are mediating the effects. Le Bras et al. (2006) have shown that at least the proliferative effect of VEGF-C on oligodendrocyte precursor cells is mediated via VEGFR-3.

Table 8. Effects of VEGF-A on neuronal and glial cells. (Modified from Zachary, 2005, Ruiz de Almodovar et al., 2009, Wittko et al., 2009, Ruiz de Almodovar et al., 2010, Erskine et al., 2011)

Cell type	Effect (conditions)
Central nervous system	
- *cortical neurons (+E)*	survival after hypoxia, neurite outgrowth
- *cholinergic neurons*	survival after NMDA stimulation
- *granule cells*	migration
- *DAergic neurons (+E)*	survival against 6-OHDA
- *hippocampal neurons (+P)*	survival after NMDA stimulation or hypoxia
- *motor neurons (+P)*	survival after hypoxia, hypoglycaemia and AR-polyglutamine toxicity
- *retinal ganglion cells*	neurite outgrowth, pathfinding at optic chiasm
- *astroglia*	proliferation, activation
- *primary microglial culture*	proliferation, migration
Peripheral nervous system	
- *DRG neurons*	survival, neurite outgrowth
- *SCG neurons*	survival, neurite outgrowth
- *Schwann cells (+P)*	survival, proliferation, migration
Neural progenitors / SCs	
- *cortical neuron precursors*	proliferation
- *SCG neural progenitors*	proliferation
- *SVZ neural progenitors*	proliferation, differentiation, migration
- *neural stem cells*	proliferation, migration, survival

+E=also embryonic; +P=also primary cultured neurons; DRG= dorsal root ganglion; NMDA= *N*-methyl-D-aspartate, SC=stem cell, SCG=superior cervical ganglion, SVZ=subventricular zone

Effects of VEGF-ligands in toxin-based models of PD

VEGF-A, and -B are the only ligands of the VEGF-family that have been studied in experimental models of PD, and all of them have shown neuroprotective or neurorestorative potential. The first report by Yasuhara et al. (2004) described the neuroprotective effects of encapsulated baby hamaster kidney (BHK) cells expressing VEGF-A against 6-OHDA -toxicity in cultured primary midbrain neurons and in rats. *In vitro*, VEGF-A reduced the number of apoptotic neurons and in the partially 6-OHDA –lesioned rats it significantly reduced amphetamine-induced rotational behaviour and protected TH-positive neurons and their projections. Later, the same group showed that low doses of VEGF-A are more efficient in neuroprotection (1 ng/ml *in vitro*, max. 11 ng/day secreted by capsules *in vivo*) than higher doses (10 or 100 ng/ml *in vitro*, max. 37 ng/day secreted by capsules *in vivo*) (Yasuhara et al., 2005c). A natural continuum after neuroprotection experiments was to examine the neurorestorative effects of VEGF-A in the same *in vitro* and *in vivo* models. Indeed, Yasuhara et al. (2005a) demonstrated, that VEGF-A can rescue DAergic neurons also when administered after 6-OHDA. Other approaches in delivering VEGF-A into the rat brain to promote recovery in PD rats have been successful as well. Tian et al (2007) reported a neuroprotective effect of VEGF-A-expression by AAV-vector in 6-OHDA treated rats. The most recent study with

rotenone-lesioned rats shows that grafted human umbilical cord mesenchymal stem cells expressing VEGF-A provide a more substantial functional and morphological recovery than control transplantation (Xiong et al., 2011). In addition, the differentiation of these cells into DAergic cells was enhanced by VEGF-A expression.

Based collectively on the mechanistic results from the aforementioned studies, it can be concluded that VEGF-A most likely produces neuroprotection and neurorestoration through several direct or indirect mechanisms. Firstly, VEGF-A induces the formation of blood vessels around the administration site, which may enhance neurovascular coupling. However, this phenomenon combined with increased vascular permeability and BBB breakage can be difficult to control. Yasuhara et al. (2005c) have even speculated, that higher doses of VEGF-A are less effective than low doses because of brain aedema that deteriorates recovery. Secondly, VEGF-A administration increases the amount of glial fibrillary acidic protein (GFAP)-positive astrocytes, which may provide neurotrophic support via secretion of other NTFs, such as GDNF as was shown by Tian et al. (2007). Thirdly, VEGF-A may directly influence the DAergic neurons via its receptors. However, Yasuhara et al. (2004) did not observe VEGFR-1 or VEGFR-2 immunoreactivity on DAergic neurons, but neuropilins were present.

Falk et al. (2009, 2011) have studied the neuroprotective potential of VEGF-B against rotenone toxicity in cultured rat midbrain cells and 6-OHDA -induced neurodegeneration in rats. Rotenone, like 6-OHDA, as an inhibitor of complex I of the mitochondrial respiratory chain also causes an increase in reactive oxygen species which leads to apoptosis in affected cells (Li et al., 2003). VEGF-B -expression is upregulated by rotenone treatment in the midbrain cell cultures, and exogenously applied VEGF-B protects the cells against the toxin. In the *in vivo* - model, amphetamine-induced rotations were significantly reduced in VEGF-B -treated animals, and the survival of nigrostriatal neurons was slightly increased. These studies, however, did not provide any mechanistic data. VEGF-B acts on VEGFR-1, which could lead to activation of astroglia and thus provide indirect neurotrophic support via increased expression of other trophic factors, as suggested for VEGF-A. However, Poesen et al. (2008) did not find significant astrogliosis after intracerebroventricular administration of VEGF-B, and therefore it still remains to be elucidated, whether glial responses might mediate at least some of the effects of VEGF-B. VEGFR-1 may also mediate direct positive effects on neuronal survival, although there is no definite evidence of the presence of the receptor on DAergic neurons. Another possible mediator is NRP1, but thus far there are no reports describing its role in the neuronal effects of VEGF-B. Finally, worth a mention is that VEGF-B, unlike VEGF-A, does not cause significant neovascularization, or vascular leakage when administered into the brain parenchyma. Therefore, the safety profile of VEGF-B is more promising.

2.3.5 Effects of other selected NTFs in the 6-OHDA –models of PD

Table 9 summarizes a list of selected growth factors or molecules associated with neural functions, that have shown therapeutic potential in the 6-OHDA model of PD. The list is by far not conclusive and could be made much longer if all single reports were to be collected. However, these examples show that a variety of growth factors that are often implicated in other physiological events can have positive effects on the DAergic system.

Fibroblast growth factor-2 (FGF-2) belongs to a large family of FGFs that have important functions during development, such as regulation of cell migration and formation of tissues and limbs (Grothe and Timmer, 2007). They are also implicated in neural development, protection and regeneration both in the central and peripheral nervous system. FGF2 and its tyrosine kinase receptors are expressed by the nigrostriatal DAergic neurons, and FGF2 is retrogradely transported in this pathway. Therefore, it is not surprising that exogenously applied FGF2 can protect midbrain DAergic neurons *in vitro* and *in vivo*. Additionally, 6-OHDA has been given to FGF2 –deficient and FGF2 –overexpressing mice (Timmer et al., 2007). Nigral TH-positive cells of the knock-out mice were moderately more prone to die after 6-OHDA insult, whereas the neurons of FGF2 –overexpressing mice were more resistant.

Growth/differentiation factor 5 (GDF5) is a member of the TGFβ –superfamily (reviewed by Sullivan and O'Keeffe, 2005). It is mostly implicated in skeletal and tendon development and joint morphogenesis for example through accelerating chondrocyte proliferation and differentiation. GDF5 and its receptors are expressed both in developing and adult rat brain. It has been shown that GDF5 can protect embryonic DAergic neurons and induce TH-positive phenotype *in vitro*. Although it is not clear how the effects of GDF5 on neuronal survival and behavioural deficits are mediated, it has been hypothesized that the effect falls directly upon neurons and at least is not mediated via astrocytes.

HB-GAM, also known as pleiotrophin has diverse roles in and outside the nervous system (Deuel et al., 2002). It is involved in the differentiation of glial cells, neurite outgrowth, synaptic plasticity, angiogenesis and mitogenesis of several cell types in the periphery. It signals through receptor tyrosine phosphatase β/ζ as well as syndecan-3, which both are expressed by SN DAergic neurons (Marchionini et al., 2007, Ferrario et al., 2008, Taravini et al., 2011). Interestingly, the latter has been shown to be a receptor for GFLs as well (Bespalov et al., 2011). HB-GAM has also been implicated in the development of DAergic neurons, and there is a slight increase in HB-GAM –expressing cells in the SN of PD patients (Marchionini et al., 2007). *In vitro*, HB-GAM can increase the survival of TH-positive primary neurons, and potentiate the effects of GDNF in the same experimental system (Hida et al., 2003). All these findings suggest that HB-GAM may have beneficial effects in preclinical models of PD. So far, the most successful attempts have been to deliver HB-GAM via viral vectors for sustained expression (Taravini et al., 2011, Gombash et al., 2012). Nigral TH-positive neurons were protected both in the neuroprotection and neurorestoration paradigms. Behavioural measures were improved in the neuroprotection experiments by Gombash et al. (2012) but only when a less substantial partial lesion was employed. Unfortunately, striatal TH-immunoreactivity was not measured in that experiment, so it is not known, whether reduced paw use asymmetry in the cylinder test was related to improvements in DAergic innervation of the striatum.

EPO is a hormone regulating the production of red blood cells in the bone marrow, and is clinically used in the treatment of anaemia. EPO and its receptor are also found in neurons and glial cells, and it seems to have a role in neuronal development and recovery from brain injury, such as ischaemia (Sanchez et al., 2009, Jerndal et al., 2010). It was shown that EPO can protect DAergic MN9D neurons against 6-OHDA toxicity *in vitro* by activating the PI3K/Akt – pathway, a common signalling cascade for NTFs (Signore et al., 2006). In the 6-OHDA -model, EPO is active only when given into the brain, not systemically (Xue et al., 2007). Surprisingly, intrastriatal AAV-EPO significantly elevates the amount of red blood cells in rats, which raises concern about the safety of AAV-EPO (Xue et al., 2010). It was proposed that the use of a mutated EPO without hematopoietic effects could be a solution to avoid serious side effects related to excess production of red blood cells, such as thrombosis.

IGF1 is a polypeptide mediating the effects of the growth hormone, and it has growth-promoting effects in various tissues through regulation of cell growth and development. Both IGF1 and its receptor are abundantly expressed in the brain (Bondy and Cheng, 2004). It is known that IGF1 can activate PI3K/Akt –signalling in the brain. Since the same signalling cascade is involved in neuronal protection, it is one possible candidate in mediating such effects of IGF1 as well. In the 6-OHDA –model, it was confirmed that the positive effects are mediated specifically by IGF –signalling, as the effects could be blocked with an IGF-receptor antagonist (Quesada and Micevych, 2004). A naturally occurring tripeptide, GPE (glycine-proline-glutamate), cleaved from the N-terminal of IGF1 exists. It can also prevent DAergic neurodegeneration and attenuate behavioural deficits in the 6-OHDA -model (Guan et al., 2000, Krishnamurthi et al., 2004). It is yet unknown how these effects are mediated, since the tripeptide does not activate the IGF1 receptor.

In conclusion, there is vast group of molecules that primarily affect other processes in the body, but can be considered as NTFs for the midbrain DAergic neurons. Firstly, this is indicative of a complex regulation and perhaps a plethora of compensatory mechanisms involved in the development, maintenance and protection of DAergic neurons under normal and physiological conditions. Secondly, although it is not always clear how these molecules induce their protective effects, their existence may stimulate new ideas to develop novel therapies for neurodegenerative brain diseases. In addition, they could be useful as conjunction therapy with other NTFs to offer neurotrophic support via different mechanisms of action.

Table 9. Neuroprotective and neurorestorative effects of selected growth factors in the 6-OHDA –rat model of PD. Table includes summaries of behavioural changes, protection of striatal DA and nigral or striatal TH-immunoreactivity

Reference	Growth factor, dose & site	Time relation to 6-OHDA	6-OHDA dose & site	Behaviour	DA	TH	Other
(Shults et al., 2000)	FGF-2 –expr. fibroblasts 2xSTR	2 w before	1x25 µg, STR	AMPH ↓ APO ↓	n.m.	SN ↑ STR↑	
(Sullivan et al., 1999)	GDF5, 50 µg STR or SN	0 h (just before)	1x8 µg, MFB	AMPH ↓	STR 0	SN ↑	
	GDF5, 50 µg SN+STR, SN+ICV	0 h (just before)	1x8 µg, MFB	AMPH ↓	STR↑	SN ↑	ICV-delivery ineffective
(Hurley et al., 2004)	GDF5, 25 µg STR or SN	1 w after	1x20 µg, STR	AMPH ↓		SN ↑ STR 0	
	GDF5, 25 µg STR or SN	2 w after	1x20 µg, STR	AMPH ↓ (SN only)		SN 0 STR 0	
(Gombash et al., 2012)	AAV-HB-GAM, 2xSTR	4 w before	2x10 µg, STR	AMPH 0 CT 0	n.m.	SN ↑ STR↑	
	AAV-HB-GAM, 1xSTR	4 w before	1x15 µg, STR	CT ↑	n.m.	SN ↑	
(Taravini et al., 2011)	Adenoviral HB-GAM, SN	7 d after	1x20 µg, STR	n.m.	n.m.	SN ↑ STR↑	
(Signore et al., 2006)	EPO 1x1-20U, STR	30 min before	1x3 µg, STR	body asymmetry↓ APO ↓	STR↑	SN ↑ STR↑	mouse model
(Xue et al., 2007)	EPO 1x20U, STR	1 d before	1x15 µg, STR	AMPH ↓ staircase ↑	n.m.	SN ↑ STR↑	inflammation in SN ↓
(Kadota et al., 2009)	EPO 100IU/d, for 7 d, ICV (minipump)	30 min after	1x20 µg, STR	AMPH ↓	n.m.	SN ↑	neurogenesis in the SVZ
(Xue et al., 2010)	AAV-EPO, STR	3 w before	1x15 µg, STR	AMPH ↓ CT bias ↓	n.m.	SN ↑ STR↑	anterograde transport to SN
(Quesada and Micevych, 2004)	IGF1, ICV or i.p. 7d (minipump)	0 h (just before)	1x8 µg, MFB	CT bias ↓	n.m.	SN ↑ STR↑	ICV more effective
(Ebert et al., 2008)	IGF1-expr. NPCs, 2xSTR	1 w after	3x7 µg, STR	AMPH ↓ CT bias 0	n.m.	SN ↑ STR 0	

AAV=adeno-associated virus, AMPH= amphetamine-induced rotational behaviour, APO= apomorphine –induced rotational behaviour, CT= cylinder, EPO= erythropoietin, GDF5= growth/differentiation factor 5, ICV= intracerebroventriculartly, IGF= insulin-like growth factor, i.p.= intraperitoneally, MFB= medial forebrain bundle, NPC= neural progenitor cell, SN=substantia nigra, STR=striatum, SVZ= subverntricular zone. Responses of treated animals vs. lesion control animals: 0=no change, ↑= increased, ↓=decreased n.m.= not measured.

2.3.6 General remarks regarding NTF therapy for PD

Because of the ability of NTFs to promote the survival, phenotype and functionality of neurons, their therapeutic potential to treat neurodegenerative diseases of the brain has been under vigorous research. This literature review has covered a portion of growth factors affecting the DAergic neurons, and described only the outcomes from the 6-OHDA rodent models. If results from other toxin models and the information regarding the development and maintenance of DAergic neurons of the brain were included, the evidence of the potential of various growth factors would be perhaps even more compelling. Still, although many NTFs have been very effective in preclinical models, translation to clinical level remains challenging. This may be due to several factors, and one must always keep in mind the limitations of preclinical models. None of the PD models can fully model all the aspects of the disease, and quite often the experimental animals are a) of reasonably young age, b) typically either only male or female and c) from few litters. Also, when assessing the therapeutic potential of a molecule, it is important to assess behavioural read-outs.

It is a matter of endless discussion, which tests are best suited to give reliable information about the functional benefits. Drug-induced rotation is one of the most common ways to assess the DAergic imbalance between hemispheres in unilaterally 6-OHDA –lesioned rodents. However, some may argue that this experiment does not represent a natural state of the animal, and is a rather robust measure. Therefore, drug-free asymmetry tests seem to be gaining popularity as they measure the spontaneous behaviour as well as finer motor functions of the animal. Also, NTFs often induce behavioural recovery, typically with increased survival of nigral TH-positive neurons but without significant elevations in striatal DA content or TH-immunoreactivity when compared to vehicle –treated animals (see above). These biochemical measures, however, are not descriptive of the functionality of DAergic neurons –such as quanta of DA released, firing pattern, rate of DA reuptake-, although calculation of metabolite/DA –ratios may reveal changes in DA turnover. Also, it is possible that even a small increase in striatal DA is enough to alleviate behavioural deficits that may manifest only after a certain threshold level of DA-depletion has been reached. Therefore, small differences in DA levels that are not statistically significant may still be of functional importance. Finally, it is also important not to limit the screening of neurotrophic effects to one preclinical model. GDNF, for example, is well known to have potent neurotrophic effects in toxin-based models of PD, but is ineffective in rat models based on viral expression of WT or A30P-aSyn (Lo Bianco et al., 2004, Decressac et al., 2011).

Until today, only GDNF and NRTN have been clinically tested in PD patients. Of the four clinical trials with GDNF, there was a positive outcome in two (Gill et al., 2003, Patel et al., 2005, Slevin et al., 2005, Patel et al., 2010), whereas in two studies no improvements were seen (Nutt et al., 2003, Lang et al., 2006). These controversial results have been suggested to be due to, in addition to the open nature of the studies, poor diffusion of GDNF in the brain, differences in the doses, administration site (ICV in Nutt et al. 2003, intraputaminal in the other studies), injection equipment (multiport catheter in Slevin et al. 2005 vs. uniport catheter in others) and delivery mode (pulsatile or convection-enhanced in Slevin et al. 2005 vs. simple infusion in others) (Salvatore et al., 2006, Aron and Klein, 2011). Also, safety issues

arose during the trials. Some patients developed antibodies against GDNF, and cerebellar lesions were observed in a study on monkeys (Lang et al., 2006, Hovland et al., 2007). Because of these reasons and the unsettled clinical efficacy, human trials with GDNF have been discontinued. Of note, there is no compelling evidence so far linking PD and altered GDNF-signalling. Genetic variations in the gene coding for GDNF or RET are not associated with PD (Wartiovaara et al., 1998, Lucking et al., 2010). The expression of nigral GDNF protein in PD patients is either reduced or not changed when compared to control patients (Siegel and Chauhan, 2000, Chauhan et al., 2001, Mogi et al., 2001), but a modest increase in GDNF mRNA in the putamen of PD patients has been reported, which may reflect a compensatory reaction to the ongoing DAergic degeneration (Backman et al., 2006).

NRTN has been delivered intraputaminally to PD patients via an AAV –vector, CERE-120. It was reported that the treatment is well tolerated by the patients and there seemed to be a slight improvement in their symptoms (Marks et al., 2008). The improvements remained modest also in a later phase II trial, but this has still encouraged further studies in which both the putamen and the SN will be targeted (Marks et al., 2010).

Collectively, these clinical trials represent well some of the challenges related to potential NTF therapy in PD. What has been learned is that the method of delivery, as well as the site of administration both need to be carefully planned. Challenges arise partially from the characteristics of the disease itself, as it is a progressive neurodegenerative illness and neuronal death is already rather advanced at the moment of diagnosis. Also, the DAergic neuronal death is still rather poorly understood, and it is not completely clear whether the degeneration affects first the axons or the cell bodies (Cheng et al., 2010). Thus, while there is a great need for restorative therapy, it is equally important to aim for earlier diagnosis and gain further understanding of the mechanisms of neuronal death in PD.

3. Aims of the study

GDNF still remains as one of the most potent NTFs for DAergic neurons, and therefore more effort is needed to study its characteristics and to evaluate its clinical potential further. The discovery of new NTFs for DA neurons could lead to new ideas in developing therapeutic interventions. Furthermore, genetically modified mice with changes in the signalling of a NTF or proper function of DA may provide a powerful tool to dissect the mechanisms of how the protein in question acts on the neurons. PD is a complex disorder with various underlying factors and their interactions. Mice carrying mutated genes associated with PD may open new ways of understanding the pathogenesis and treatment of the disease.

In this context, the specific aims of the study were:

1) To compare the efficacies of GDNF, a N-terminally truncated non-heparin-binding variant (Δ38N-GDNF) and a combination of GDNF and HB-GAM in the unilateral 6-OHDA rat model of PD, and to study if the diffusion pattern of GDNF is changed with the amino acid sequence truncation (Study I).

2) To examine the neuroprotective and neurorestorative potential of VEGF-C in the unilateral 6-OHDA rat model of PD (Study II, neurorestoration unpublished)

3) To characterize the response of two genetically modified mouse strains to intrastriatally administered 6-OHDA. Both genetic modifications are strongly bound to DAergic function: a) MEN2B knock-in mice with constitutively active RET receptor tyrosine kinase, which mediates the effects of GDNF (Study III) and b) A30P-mutated aSyn -expressing transgenic mice in which aSyn aggregates may cause DAergic damage (Study IV)

4. Experimental procedures

4.1 Animals

Wistar male rats weighing ~250 g at the start of each experiment were supplied by Harlan, The Netherlands or the Laboratory Animal Centre of University of Helsinki, Finland. The rats were housed in groups of 2-4, except following any surgical procedure, after which they were housed singly over night before regrouping.

MEN2B (multiple endocrine neoplasia type 2B) knock-in mice and their WT littermates, aged 8-14 weeks, (Smith-Hicks et al., 2000) were bred and supplied by the Laboratory Animal Centre of University of Helsinki, Finland. Transgenic mPrPh(A30P)asyn mice expressing the human A30P-aSyn and WT mice (6-17 months old, Yavich et al., 2004) were bred and supplied by the National Laboratory Animal Centre of Finland, University of Eastern Finland. The mice were housed in groups of 2-8 until surgical procedures, after which they were housed singly until the end of all experiments.

All animals had free access to rodent chow and water, and they were maintained under 12/12 h light/dark cycle at a convenient temperature of +20-22 ºC. The experimental procedures were approved by the Committee for Animal Experiments at the University of Helsinki, Finland, or the National Animal Experiment Board in Finland.

4.2 Drugs and treatments

6-OHDA (Sigma, St Louis, MO, USA) was dissolved in 0.9% NaCl solution containing 0.02% ascorbic acid as an antioxidant, and stereotaxically injected into the brains of mice and rats as described in the original publications. GDNF (Amgen Inc., Thousand Oaks, CA, USA) was diluted with 10 mM citric acid in 0.9% NaCl, Δ38N-GDNF as well as insect cell –derived GDNF and VEGF-C were produced at the University of Helsinki (Joukov et al., 1996) and diluted with phosphate-buffered saline (PBS). Stereotaxic injection of NTFs is also described in original publications I and II. D-amphetamine (2.5 mg/kg intraperitoneally, produced by Faculty of Pharmacy, University of Helsinki, Finland) and tramadol (1 mg/ml/kg subcutaneously, Orion Pharma, Espoo, Finland) were dissolved in 0,9% NaCl. Desipramine (25 mg/10 ml/kg intraperitoneally, Sigma, St Louis, MO, USA) and buprenorphine (0.1 mg/10ml/kg subcutaneously, RB Pharmaceuticals, Slough, UK) were dissolved in sterile water.

4.3 Stereotaxic surgery

The animals were kept under general isoflurane and local lidocaine anaesthesia during the surgical procedures. The rats received tramadol (1 mg/kg) and the mice were administered buprenorphine (0.1 mg/kg) for post-operative pain relief.

4.3.1 Injections of NTFs

In study I, a 23 G guide cannula was inserted 3 mm above the intended injection site in the striatum (A/P 0.0; M/L – 3.5; D/V – 5.0) and fixed on the rat skull with dental cement, 18 hours before the 6-OHDA –infusion. HB-GAM or PBS was injected at 0.5 µl/min via the cannula, using a 30G needle attached to a thin polyethylene tube connected to a glass syringe, which was run by a microinfusion pump. 12 h later GDNF or Δ38N-GDNF was similarly injected through the guide cannula. 6-OHDA was infused 6 h after the administration of GDNF.

In studies I, II, and VEGF-C –neurorestoration experiments, GDNF, Δ38N-GDNF and VEGF-C were injected into the striatum, A/P +1.0; M/L -3.0; D/V -5.0, using a 10 µl Hamilton microsyringe run by a stereotaxic injector at 1 µl/min (Stoelting Co., Wood Dale, IL, USA). The NTFs were injected 6 h before 6-OHDA in the neuroprotection studies, but in the VEGF-C -neurorestoration study the injections were made 4 weeks after 6-OHDA.

In studies I and II, some animals received only NTFs without lesioning procedure to study their diffusion pattern or effects on the intact brain. More detailed information and the dosages can be found in the original papers related to the studies.

4.3.2 6-OHDA –injections

Only in study I, 6-OHDA was infused simultaneously to four coordinates in the rat striatum with a custom-made cannula. Four 29-gauge needles were fixed together with dental cement, and each needle was separately connected via a thin polyethylene tube to a glass syringe run by a microinfusion pump to deliver four 7 µg/3.5 µl deposits of 6-OHDA at 0.5 µl/min. The needles in the block were placed so that the coordinates for the infusions were A/P +1.3/+0.4 /-0.4/-1.3; M/L -2.6/-3.2/-4.2/-4.5; D/V -5.0.

In all four studies 6-OHDA was injected at 1 µl/min using a 10 µl Hamilton microsyringe. In studies I-II on rats, a dose of 16 µg /4 µl was injected into the striatum using the same coordinates as for the NTFs (A/P +1.0; M/L -3.0; D/V -5.0). In addition, the same coordinates and dosage was used also in VEGF-C -neurorestoration study with two types of lesions; the second lesion type was a two-deposit lesion of 2x10 µg in A/P +1.6/-0.4; M/L -2.2/-4.0;D/V -5.0. In studies III-IV on mice 0.33-5 µg / 2 µl of 6-OHDA was administered into the striatum, A/P +0.7; M/L +1.8; D/V -2.7.

4.4 Behavioural methods

4.4.1 Rotational behaviour

A common method to evaluate the function of the nigrostriatal pathway in unilaterally 6-OHDA –lesioned rodents is to measure rotational behaviour, which manifests after administering a DA-releasing or DA-mimicking drug (Ungerstedt and Arbuthnott, 1970). We evaluated the efficacy of NTF treatments and the successfulness of 6-OHDA -lesioning by measuring D-amphetamine –induced rotational behaviour in rats. The rats were fitted with a harness and allowed to habituate for 10-15 min in the rotation bowls (Coulbourn Instruments Inc., Allentown, PA, USA (study I) or Med Associates Inc., St Albans, VT, USA) before an

injection of 2.5 mg/ml/kg D-amphetamine. Full ipsilateral and contralateral rotations were recorded for 120 min in 5 min blocks, and the data were expressed as net ipsilateral rotations (ipsilateral minus contralateral rotations). In study I, rotational behaviour was measured 2, 3, 7 and 8 weeks post lesioning, and in study II 2, 4, 6 and 8 weeks post lesioning. In VEGF-C -neurorestoration experiments, rotational behaviour was first measured 3 weeks post lesioning to divide animals into groups of similar average rotational values. Thereafter, rotational behaviour was measured 6, 8, 10, 12 and 14 weeks post lesioning.

D-amphetamine –induced rotations were also measured in mice to assess the sensitivity of MEN2B and mPrPh(A30P)asyn mice to 6-OHDA on behavioural level. The mice were fitted with plastic collars on the previous day, and on the day of the experiment they were allowed to habituate in the rotation bowls for 10-15 min before an injection of D-amphetamine (2.5 mg/10 ml/kg, i.p.). Full ipsilateral and contralateral rotations were recorded for 90 min, and the data were expressed as net ipsilateral rotations (ipsilateral minus contralateral rotations). The experiment was done at 3 weeks post lesion in MEN2B mice, and at 2 and 4 weeks post lesion in A30P –mice.

4.4.2 Stride length

Measurement of stride length is a simple way to study nigrostriatal dysfunction in mice (Fernagut et al., 2002). This method was employed to study the basal motor differences as well as the effects of the unilateral 6-OHDA-lesion in mPrPh(A30P)asyn mice. Baseline values were measured one week before 6-OHDA, and the effect of lesioning was studied at 3 weeks after 6-OHDA. The average of the longest three steps was used as the representative value for each mouse.

4.4.3 Locomotor activity

24 h spontaneous locomotor activity of aged 13.5-17 month-old mPrPh(A30P)asyn mice was measured after letting the mice to acclimatize to the conditions in the experiment room for a minimum of 3 days. At the start of the lights-on period in the morning, the mice were placed in transparent cage inserts (25 cm × 25 cm × 15 cm, with food pellets and a water burette) in the activity monitor (MedAssociates, St. Albans, GA, USA). The measurement was started immediately to record also the explorative behaviour in a new environment, and the data was collected at 15 min intervals.

D-Amphetamine –induced locomotor activity was measured in 6-month-old mPrPh(A30P)asyn mice before 6-OHDA lesioning and rotational experiments to find out if the genotypes had a basal difference in response to D-amphetamine (1 mg/kg or 2.5 mg/kg i.p.). All mice used in the experiment received both doses with 7 days in between the treatments. The mice were allowed to explore the cages (described above, but without food and water) for 10-15 min before the actual experiment started. Locomotor activity was recorded for 3 h , data collection interval being 5 min.

4.5 Immunohistochemistry and quantification of immunoreactivity

4.5.1 Tissue preparation

In all studies with rats, the animals were terminally anaesthetized with pentobarbital (100 mg/kg), and perfused transcardially with PBS followed by ice-cold 4% paraformaldehyde (PFA, in PB, pH 7.2-7.4). The brains were post-fixed in 4% PFA over night in room temperature, and then switched into 20% sucrose in PB for cryoprotection, and kept at +4°C until they sank. The brains were kept deep-frozen at -84°C until coronal sections (40 µm) were cut on a cryomicrotome. The sections were collected in series of six, and kept free-floating in cryoprotectant buffer containing glycerol and ethylene glycol.

MEN2B mice and mPrPh(A30P)asyn mice were deeply anaesthetized with pentobarbital as above, and quickly perfused transcardially with ice cold PBS. Their brains were removed from the skull and placed on an ice-cooled brain matrix to obtain a 3 mm slice containing both striata which were punched with a 2 mm tool. Alternatively, the brain was cut at the level where the anterior commissure crosses the brain section, and the striata were dissected out using forceps. The samples were immediately frozen on dry ice for later high-performance liquid chromatography (HPLC) -analysis. The remaining block containing substantia nigra was immersion –fixed in 4% PFA over night and thereafter processed for TH-immunohistochemistry as described above. Some mPrPh(A30P)asyn mice were perfused with PFA after PBS to obtain striatal sections for immunohistochemistry as described above for rats.

4.5.2 TH-immunohistochemistry

TH-immunostaining was performed in all studies. The free-floating sections were rinsed to remove cryoprotectant buffer and quenched for 5 min with 3% hydrogen peroxide + 10% methanol. The sections were kept for 1 h in 2% normal serum (normal horse serum for rat tissues, normal goat serum for mouse tissues, Vector laboratories, Burlingame, CA, USA) to reduce non-specific staining. Primary antibody (rat tissue: mouse monoclonal anti-TH MAB318, mouse tissue: rabbit polyclonal anti-TH AB152, both from Millipore/Chemicon, Temecula, CA, USA) was diluted 1:2000 and the sections were incubated over night. Thereafter the sections were incubated with a 1:250 diluted biotinylated secondary antibody (rat tissue: horse anti-mouse BA2001, mouse tissue: goat anti-rabbit BA1000, both from Vector Laboratories, Burlingame, CA, USA), which was combined with a standard avidin-biotin complex (ABC) -procedure (Vectastain standard kit, Vector Laboratories, Burlingame, CA, USA) to enhance the signal. The staining was visualized with 3,3-diaminobenzidine (DAB, Sigma, St. Louis, MO, USA). All incubations were done in room temperature on a sample stirrer. The same protocol was modified for a number of other immunohistochemical procedures described below.

Quantification of TH-positive cells was done using the Stereo Investigator software (MBF BioScience, Williston, VT, USA). Three or four (for rats or mice, respectively) TH-immunostained sections around the medial terminal nucleus of the accessory optic tract were chosen, and the cells were counted according to optical disector rules. SNpc was contoured at 4× magnification, and the cells counted with optical fractionator -application using a 60× oil objective on a microscope (Olympus BX51, Olympus Optical, Tokyo, Japan) that was equipped

with an Optronics digital camera (Goleta, CA, USA). Counting frames of 60×60 μm with optical disector height at 20 μm were placed in a 125×125 μm grid for rat sections and a 80x100 μm grid for mouse sections and cast randomly over the SNpc. The counting was set to yield a coefficient of error (CE Scheffer) under 0.15, based on all three sections of the intact hemisphere. The program estimated the total number of cells in the block of the SNpc defined by these three representative sections, which was used for final data handling.

Densitometric analysis of TH staining in the striatum was performed on pictures of three consecutive striatal sections, obtained with either the Stereo Investigator software and equipment (described above) or a stereomicroscope (Nikon, Japan) fitted with a DS-Fi1 camera head and DS-L2 camera controlling unit (Nikon, Japan). Images were converted to grayscale and inverted, and the measurements done using Image Pro Plus program (vs. 3.0.1., Media Cybernetics, Bethesda, MD, USA). A line was created across the striatum to measure the optical density along the line tool. The effect of background staining was corrected by obtaining correction values from the corpus callosum of each section and subtracting those values from the striatal OD values. The optical density of the lesioned side was expressed as a percentage of the intact side.

4.5.3 aSyn -immunohistochemistry

Detection of aSyn was performed as described in (Myöhänen et al., 2008) with slight modifications. In short, sections were quenched in 10% methanol and 3% H_2O_2 in PBS (pH 7.4) for 10 min, and non-specific binding was blocked with 10% normal donkey serum in PBS (#S30, Millipore, Temencula, CA, USA). The sections were incubated overnight at room temperature with the primary antibody against aSyn (1:500 with 1% serum; AB6162, AbCam, Cambridge, UK). The next day the sections were incubated for 2 h with donkey anti-sheep HRP conjugated secondary antibody (1:500 in 1% NDS in PBS; AB6900, AbCam). Staining was visualized with 3,3'-diaminobenzidine.

The optical density of aSyn –immunoreactivity was measured from striatum, SN and motor cortex as described above for TH-immunoreactivity.

4.5.4 DAT –immunohistochemistry

DAT-immunostaining was performed according to the procedures described for TH-immunohistochemistry with some exceptions. Normal rabbit serum was used for blocking, and a monoclonal rat anti-DAT primary antibody (1:2000, MAB369, Millipore), and biotinylated rabbit anti-rat secondary antibody (1:250, BA4000, Vector laboratories) were combined. The signal was enhanced with the ABC –procedure and visualized with DAB. The optical density of DAT –immunoreactivity in the striata was measured as described above for TH-immunoreactivity.

4.5.5 EBA –immunohistochemistry

Blood vessels with intact BBB in brains injected with VEGF-C were immunostained using a mouse monoclonal antibody raised against rat endothelial barrier antigen (EBA) (anti-SMI-71R, 1:2000, Covance, Emeryville, CA, USA) in combination with a biotinylated horse anti-mouse secondary antibody BA2001 (1:500, Vector Laboratories, Burlingame, CA, USA). ABC procedure was performed before DAB reaction.

Representative pictures of three consecutive striatal sections were taken for analysis using Stereo Investigator software, and the amount of immunoreactive objects was estimated using Image Pro Plus -program. A circular area of preset size was placed above the injection site and the program counted blood vessels of preset intensity and size (larger than 300 U) inside the area.

4.5.6 GDNF-immunohistochemistry

Injected GDNF –protein was visualized from the rat striata using a protocol described by (Kirik et al., 2000b). In short, the sections were quenched in 3% hydrogen peroxide + 10% methanol for 10 min, blocked in 5% normal horse serum for 1 h and incubated in the primary antibody (1:3000, goat polyclonal anti-GDNF, R&D systems, Minneapolis, MN, USA) over night. Biotinylated secondary antibody (1:200, horse anti-goat BA9500, Vector Laboratories, Burlingame, CA, USA) was added in 2% normal horse serum, and the sections were incubated for 2 h before ABC –enhancement and DAB –visualization.

The volume of GDNF-immunoreactivity was estimated using the Cavalieri estimator –probe in Stereo Investigator. The immunostained area was contoured at 4x magnification, and markers were cast over in a 750x750 μm grid. All markers falling inside the contoured area were counted.

4.5.7 GFAP –immunohistochemistry

VEGF-C –induced astrogliosis was studied by staining GFAP-immunoreactive cells from striatal sections around the protein injection site. A polyclonal antibody AB5804 raised in rabbit (1:1500, Millipore, Temecula, CA, USA) and a biotinylated goat anti-rabbit secondary antibody BA1000 (1:500, Vector Laboratories, Burlingame, CA, USA) in 1% normal goat serum were used and signal was enhanced with ABC procedure before DAB reaction.

The number of GFAP-positive cells was estimated using Stereo Investigator as described above using three striatal sections around the injection site, placing approximately 20 counting frames (100×100 μm, optical disector height 20 μm, in a 650×650 μm grid) per striatum. The estimated total number of cells by the program was used as a parameter for calculating the results.

4.5.8 Iba1a –immunohistochemistry

To visualize microglia in striatal sections from rat brains treated with VEGF-C, a polyclonal antibody against the microglial marker, ionized calcium binding adaptor molecule (1Iba1) (1:1000, raised in rabbit, Wako Pure Chemical Industries, Tokyo, Japan) and a biotinylated goat anti-rabbit secondary antibody BA1000 (1:500, Vector Laboratories, Burlingame, CA, USA) were used in combination with ABC procedure before DAB reaction.

Iba1 -positive objects were counted using Stereo Investigator with the same settings as described for GFAP-reactive cells. Resting and activated microglia were roughly separated by their morphology and counted using different markers to make an estimate of the total number of microglia and the number of activated microglia. A microglial cell was considered activated if it appeared very arborous or rod/ amoeba-like (reactive - fully activated).

4.6 Analysis of monoamines from brain tissue

The concentrations of DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), as well as 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were measured from striatal tissue samples obtained from MEN2B and mPrPh(A30P)asyn mice as described in the original publications III and IV. We followed the original method described in detail in (Airavaara et al., 2006), with minor modifications of the mobile phase when needed.

4.7 Western blotting of MAPK/ERK

The ability of VEGF-C to activate MAPK/ERK –pathway in the rat brain was studied with western blot. Striatal tissue samples, obtained 1 or 8 h after an injection of 20 µg VEGF-C into the striatum, were homogenized in buffer containing 0.3 M sucrose, 10 mM Hepes pH 7.4, 1 mM EDTA, PhosSTOP phosphatase inhibitor and Complete protease inhibitor cocktail tablets (both from Roche, Mannheim, Germany) and kept on ice. Protein concentration was measured using a BCA kit (Pierce, Rockford, IL, USA). The samples were electrophoresed on 7.5-10% SDS-PAGE and transferred on nitrocellulose membranes. The membranes were incubated with rabbit polyclonal anti-phospho-ERK1/2 1:1000 in 5% milk (#9101, Cell Signaling Technology, Beverly, MA, USA) and thereafter with goat anti-rabbit secondary antibody 1:3000 in 5% milk (#172-1019, Bio-Rad Laboratories, Hercules, CA, USA). The signal was developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA). The membrane was then stripped, washed and incubated with rabbit polyclonal anti-ERK1 1:500 in 5% milk (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Treatment with secondary antibody and signal development was done as with phospho-ERK1/2. Densitometric analysis was performed in AIDA Image Analyzer software (Raytest Isotopenmessgeräte, Straubenhardt, Germany), or GeneGnome chemiluminescent detector (Syngene, Synoptics LTD, Cambridge, UK) to measure histogram profiles for all bands.

4.8 Statistics

In studies I-II, the data from rotational experiments, TH-cell counts, optical density of TH staining, and distribution of injected GDNF were analyzed with one-way analysis of variance (ANOVA) combined with Tukey's *post hoc* –test, or Tukey's b –test. Two-way ANOVA was used in analysis of rotational behaviour and stride length in mice (study IV), TH-cell counts (Studies I, III, IV), density of TH- and DAT –immunoreactivity (Study IV) and striatal monoamine concentrations (studies III and IV). ANOVA of repeated measures was used for analyzing the data from measuring locomotor activity (study IV). Unpaired Student's t-test was used for comparing EBA-, GFAP-, and Iba1a –stainings between control and treatment (Study II), and rotational behaviour, TH-cell data, striatal DA content, striatal metabolite/DA ratios (Study III), and cumulative locomotor and vertical activity (Study IV) between genotypes.

5. Results

5.1 Effects of truncation of the amino acid sequence or preadministration of HB-GAM on the diffusion pattern and neuroprotective efficacy of GDNF (I)

As shown in Table 10, removal of the first 38 amino acids, containing the sequence responsible for high affinity for heparin-like structures, from the structure of GDNF leads to markedly increased diffusion of the NTF in the rat brain ($P<0.001$ Δ38N-GDNF vs. both full GDNF-proteins). The effect is apparent immediately after administration. Pretreatment with HB-GAM 18 h before GDNF –injection did not alter the diffusion pattern significantly (11.0-12.9 mm^3, result from a pilot study and not included in the table).

Table 10. Diffusion volumes of GDNF –variants after intrastriatal injections

	Diffusion volume, mm^3	
GDNF-variant	10 min	6 h
full GDNF (*E. Coli*)	7.5±1.2	14.1±1.3
full GDNF (baculo)	10.0±1.2	15.1±1.0
Δ38N-GDNF (baculo)	18.2±1.0 ***	25.0±1.4***

Average±SEM, n=5-11. *** $P<0.001$ one-way ANOVA followed by Tukey's *post hoc*

Results from the rotational behaviour experiments (Fig. 5 a) show, that GDNF alone seems to be most effective in reducing D-amphetamine –induced ipsilateral turning in rats with four-site lesions. Δ38N-GDNF could also partially reverse the motoric bias, but not to the same extent as full GDNF. The behavioural measures were supported by the analysis of TH-positive cells of the SNpc showing that Δ38N-GDNF did not protect the cells as effectively as full GDNF (Fig. 5 b). However, when the GDNF –variants were studied employing a considerably smaller one-site 6-OHDA lesion, they were equally effective (see details and full sets of results in original publication I).

Pretreatment with HB-GAM 18 h before GDNF inhibited the functional recovery in a dose-dependent manner (Fig. 5 c), but did not affect the ability of GDNF to protect the DAergic cell bodies in the SNpc (Fig. 5 d). HB-GAM alone did not increase the survival of TH-positive cells, and it only modestly attenuated rotational behaviour in rats.
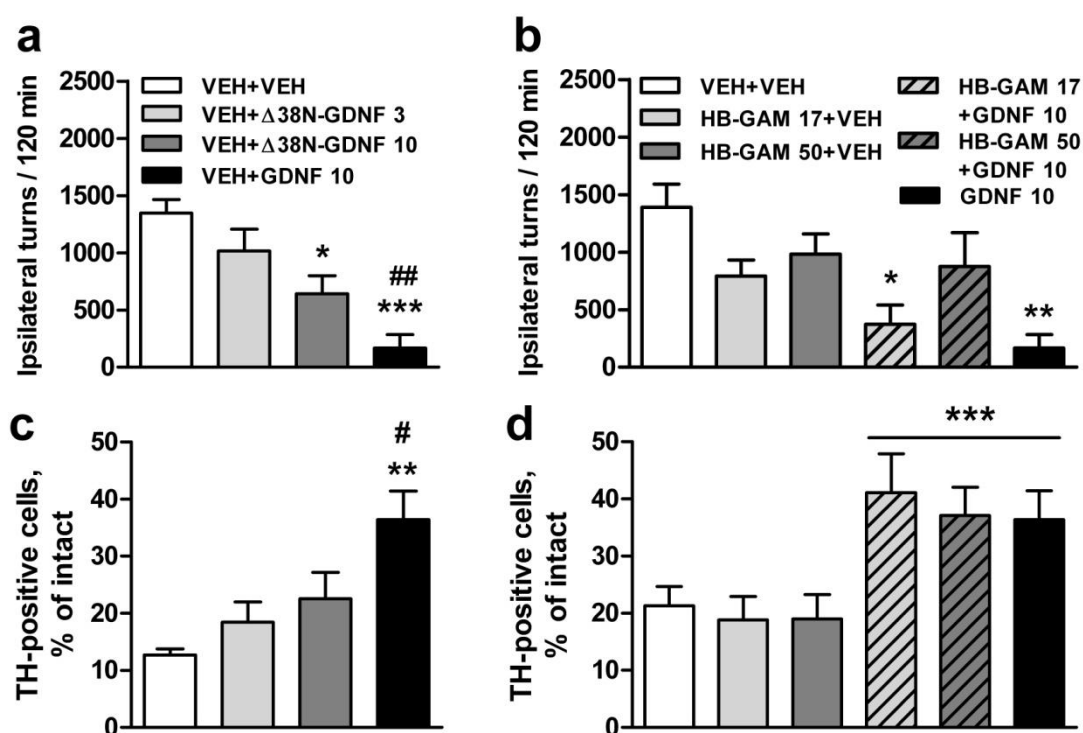
Figure 5. Neuroprotective effects of Δ38N-GDNF and HB-GAM/GDNF –combination treatment in the unilateral 6-OHDA rat model of PD. Rats were injected with 3 or 10 µg of Δ38N-GDNF, 10 µg of GDNF, 17 or 50 µg of HB-GAM, or a combination of HB-GAM and GDNF 6 h before 6-OHDA (4x7 µg). a-b) D-amphetamine -induced ipsilateral rotational behaviour 8 weeks post lesion; c-d) survival of TH-positive cells in the SNpc. *P<0.05, **P<0.01, ***P<0.001 vs. control; #P<0.05, ##P<0.01 vs.3 µg Δ38N-GDNF (one-way ANOVA and Tukey's post hoc test, except in d) two-way ANOVA for the effect of GDNF). average+SEM, n=6-13

5.2 Effects of VEGF-C in the unilateral 6-OHDA rat model (II)

The potential dopaminotrophic effects of VEGF-C were studied both in a neuroprotection protocol, in which VEGF-C was administered 6 h before 6-OHDA into the striatum, and in a neurorestoration protocol, in which VEGF-C was injected 4 weeks after 6-OHDA lesioning.

Fig. 6 summarizes the findings from the neuroprotection experiments (for full sets of data, see original publication II). We found that a 30-µg dose of VEGF-C can reduce D-amphetamine-induced rotational behaviour in rats as effectively as 10 µg of GDNF. However, combining the two NTFs did not result in additional efficacy (Fig. 6 a). When examining the survival of TH-positive cell bodies in the SNpc, it was quite apparent that VEGF-C had only a modest protective effect in comparison to GDNF. In contrast to the behavioural effect, the combination treatment with GDNF and VEGF-C apparently protected the nigral DAergic cells more effectively than GDNF alone (Fig. 6 b), although this effect did not differ statistically significantly from that of GDNF. The analysis of TH-immunoreactivity in the rat striata showed a more robust protective effect of VEGF-C (Fig. 6 c), when compared to its effect on nigral cell bodies, but this was not statistically significant elevation in comparison to control group.
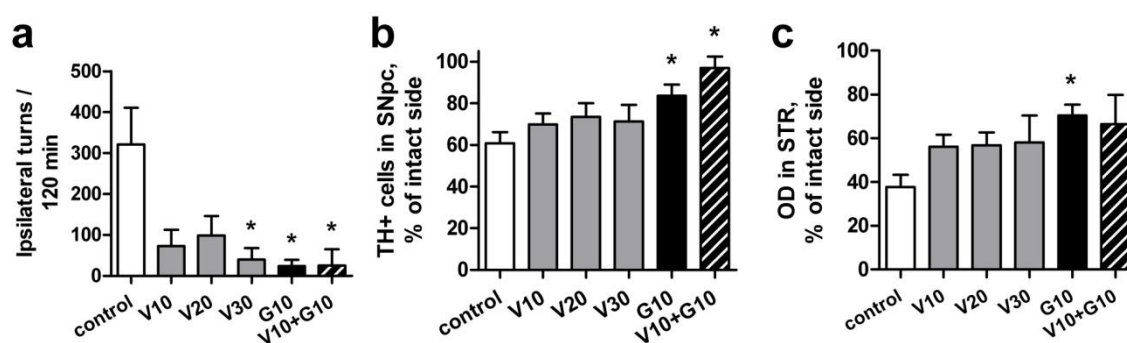
Figure 6. Neuroprotective effects of VEGF-C in the unilateral 6-OHDA rat model of PD. a) D-amphetamine –induced ipsilateral rotational behaviour 4 weeks post lesion in rats treated with 10-30 µg VEGF-C (V), 10 µg GDNF (G) or their combination (V+G, 1o µg each) 6 h before 6-OHDA, b) survival of TH-positive cells in the SNpc, c) optical density of striatal TH-immunoreactivity. *P<0.05 vs. control (one-way ANOVA and Tukey's post hoc test or Tukey's b-test), average+SEM, n=7-17.

After the neuroprotection experiments, the neurorestorative potential of VEGF-C was studied in two unilateral 6-OHDA lesions (Table 11). When a smaller, one site lesion (16 µg 6-OHDA) was employed, an attenuation in rotational behaviour could be observed in all treatment groups (VEGF-C, GDNF and the combination of both), but the rotations were reduced also in the control animals over time. None of the effects of NTFs were statistically significant at any time point or when the AUC curves (change in number of rotations during 3-14 weeks post lesion) were compared due to high variation in each group. No significant differences were observed between the groups in the survival of TH-positive neurons in the SNpc, or TH-immunoreactivity of the striata. However, when comparing the striatal OD values, there is a small positive effect of GDNF.

When a substantially larger lesion (2x10 µg 6-OHDA) was employed, the NTF treatments hardly affected the rotational behaviour of the rats (Table 11). Even GDNF failed to effectively abolish the motoric bias in such a severe lesion, in which only 17 % of nigral DAergic cell bodies could be detected in morphological analysis. Nevertheless, counting of nigral TH-positive cells revealed a clear positive effect of GDNF on the survival of DAergic cell bodies, even though the overall result of the statistical test was not quite significant (*P*=0.054, one-way ANOVA). There were no differences between any groups in striatal immunoreactivities.

Table 11. Results from VEGF-C neurorestoration studies

	control	VEGF-C 10 µg	GDNF 10 µg	VEGF-C 5 µg + GDNF 5 µg	VEGF-C 10 µg + GDNF 10 µg
16 µg 6-OHDA					
AUC; rotations 3-14 weeks post lesion	3,768±1437	1,835±679	1,822±961	n.d.	2,461±672
TH+ cells in SNpc (% of intact)	42.1±11.5	51.9±4.8	47.2±7.5	n.d.	44.2±8.7
TH+ staining in STR (% of intact)	38.4±7.2	39.9±7.0	50.7±2.6	n.d.	41.8±6.3
2x10 µg 6-OHDA					
AUC; rotations 3-14 weeks post lesion	8,005±1,532	6,661±1,518	7,023±1,762	5,883±1,480	6,295±886
TH+ cells in SNpc (% of intact)	17.0±4.4	26.6±6.9	34.2±4.5	33.0±5.7	38.7±3.9
TH+ staining in STR (% of intact)	37.2±7.4	34.2±7.0	30.9±6.9	34.5±7.1	43.8±7.1

AUC= area under the curve. Average±SEM, n=7-8. n.d. = not determined. *P*>0.05 in all comparisons (one-way ANOVA).

5.3 Changes in the intact rat brain after intrastriatal VEGF-C administration (II)

Astroglial and microglial activation as well as BBB disruption was studied by immunohistochemistry 3 days after administration of 20 µg of VEGF-C or vehicle into intact rat striatum. There was a marked increase in GFAP –immunoreactive astroglia and Iba1a-immunoreactive microglia after VEGF-C treatment. The number of activated microglia was increased as well. In addition, VEGF-C caused a reduction in EBA-staining, which indicates a disruption of the BBB. The results are shown in Table 12.

Table 12. Effect of VEGF-C on numbers of astroglia, microglia, and intact blood vessels

	Estimated no. of objects x 10^3	
Biomarker	VEGF-C 20 µg	vehicle
GFAP (astroglia)	50±5**	24±3
Iba1a (microglia), total	208±8**	125±6
-activated	184±9 **	50±6
EBA (BBB)	0.87±0.1 ***	1.4±0.04

Average±SEM, n=5. ** *P*<0.01, *** *P*<0.001 vs. vehicle, unpaired Student's t-test

In search of possible signalling pathways for VEGF-C, the activation of MAPK/ERK –pathway was examined in intact striatal tissue by western blotting 1 and 8 h after an intrastriatal injection of the growth factor. At 1 h post injection, there was an increase of 16±3.7% (average±SEM) in the ERK phosphorylation when compared to the vehicle-injected samples ($P<0.05$, one sample t-test). At 8 h post-injection the trend still existed (26±37% increase), but there was no statistically significant difference between treated and vehicle –samples.

5.4 Effects of 6-OHDA on the nigrostriatal pathway in MEN2B mice (III)

When D-amphetamine –induced ipsilateral rotations were measured in MEN2B mice and their WT littermates 3 weeks after a unilateral 6-OHDA injection, the homozygous M/M knock-in -mice rotated notably more, although the effect was quite not statistically significant (Fig. 7a). Analysis of striatal DA concentrations revealed a slightly greater loss of DA in the WT mice, when the remaining amount of DA was expressed as percentage of the intact samples (Fig. 7 b). Also, M/M mice had greatly elevated basal DA levels, which is in agreement with earlier studies (Mijatovic et al., 2007). Despite the extensive loss of striatal DA in both genotypes, quantification of nigral TH-positive cells revealed only minor degeneration in M/M mice, and approximately 36% neuronal loss in the WT mice (Fig. 7 c).
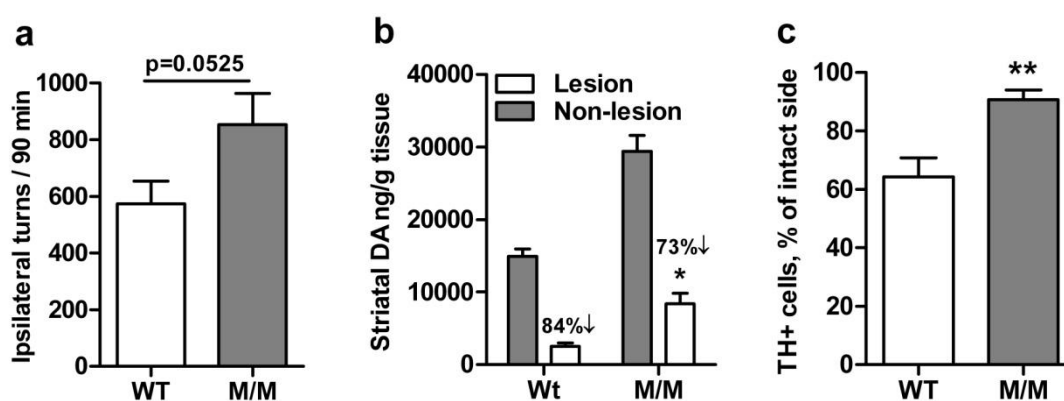

Figure 7. Effects of intrastriatally administered 6-OHDA (5 μg) in MEN2B and WT mice. a) D-amphetamine –induced rotational behaviour 3 weeks post lesion, b) striatal DA concentrations, c) survival of DAergic cell bodies in the SNpc. *P<0.05, **P<0.01 (unpaired Student's t-test). Data expressed as average+SEM, n= 12/group.

5.5 Locomotor activity and rotational behaviour in mPrPh(A3oP)asyn mice (IV)

In mPrPh(A30P)asyn transgenic (+/+) mice, there is an age-dependent development of spontaneous motor impairments. At the age of 6 months there were no significant differences between +/+ mice and their WT littermates in locomotor activity, which was measured for 24 h (data not shown). However, at the age of 14-17 months, we found significant decreases in locomotor activity at certain time points in A30P +/+ mice, even though the cumulative distances did not differ between the genotypes (Table 13). The decreases were especially evident during normal high activity periods, such as the start of the 12-h dark period or the exploratory phase in the beginning of the experiment. During the activity peak periods, the WT mice were actively rearing (vertical movement), which was almost completely absent in +/+ mice.

Since the younger mice did not exhibit any detectable basal motor impairments, they were challenged with D-amphetamine (1 mg/kg and 2.5 mg/kg). This experiment revealed, that the locomotor response to the lower dose was significantly attenuated in +/+ mice. At the higher dose of D-amphetamine, there were no differences in travelled distances between the two genotypes. The higher dose was later used for inducing rotational behaviour in 6-OHDA-lesioned mice, assuming that possible differences in the experiment would not be due to different responses to D-amphetamine.

Table 13. Cumulative locomotor activity counts in mPrPh(A30P)asyn mice

Measurement	Genotype	
	WT/WT	+/+
24 h locomotor activity (aged mice)		
- *distance travelled (m)*	894±93	870±104
- *vertical counts*	2065±200	999±232**
D-AMPH –induced locomotor activity (young mice)		
- *distance travelled (m), 1 mg/kg*	167±23	84±9***
- *distance travelled (m), 2.5 mg/kg*	518±100	632±121

Average ±SEM, n=11-12. ** $P<0.01$, *** $P<0.001$, unpaired Student's t-test

Rotational behaviour was measured in 6-OHDA –treated mPrPh(A30P)asyn mice at two and four weeks post lesion (Fig. 8 a, b, see next section). We observed that, in general, WT/WT –mice required a higher dose of 6-OHDA to exhibit a rotational bias in any direction. The smallest dose of 6-OHDA (0.33 µg) caused the +/+ -mice to rotate contralaterally in response to D-amphetamine, but with higher doses they rotated ipsilaterally, making more rotations in 120 min than their WT littermates. The differences seen between the genotypes were significant at two weeks post lesion (genotype x treatment, $P=0.02$, two-way ANOVA), and there was a similar tendency at four weeks post lesion (genotype x treatment, $P=0.056$, two-way ANOVA).

Between the rotational tests at 3 weeks post lesioning, the stride lengths of mPrPh(A30P)asyn mice were measured. The baseline values, measured four weeks earlier, were not significantly different between the genotypes (6.6±0.54 in WT/WT, 6.5±0.71 in +/+ mice). However, we observed a slight but significant decline in the stride length in +/+ mice after lesioning (6.2±0.95/5.8±0.74/5.9±0.72 for 0.33/1.0/3.0 μg of 6-OHDA, respectively), but not in WT/WT mice (6.8±0.45/6.9±0.8/6.5±1.0 for 0.33/1.0/3.0 μg of 6-OHDA, respectively). There was no treatment effect of 6-OHDA, but a significant genotype effect was revealed by two-way ANOVA ($P<0.001$).

5.6 Biochemical and neuropathological changes in 6-OHDA –treated and aged transgenic mPrP(A3oP)asyn +/+ mice (IV)

Four to seven days after the last test of rotational behaviour, brain samples from 6-OHDA-treated mice were taken for immunohistochemistry and monoamine measurements. We immunostained TH and DAT in striatal, and only TH in midbrain sections of the mice to evaluate, whether the above mentioned behavioural differences could be explained with morphological findings. We saw a clear dose-response of 6-OHDA on the loss of TH-positive neurons in the SNpc (Fig. 8 c-e, treatment effect $P<0.001$, two-way ANOVA), and the loss of TH- and DAT-immunoreactivity in the striatum in both genotypes. However, the extent of these degenerative changes were similar in +/+ and WT/WT –mice, indicating that the sensitivity to 6-OHDA was not increased in +/+ mice. There were no basal differences in DAT-staining or number of TH-cells in SNpc, but we detected a slight decrease in striatal TH-immunoreactivity on the intact side (data not shown).

We studied the 6-OHDA –induced changes in the brain of mPrPh(A30P)asyn mice further by HPLC-analysis of DA and its metabolites, as well as 5-HT in the striatum (Fig. 9). Significant genotype ($P=0.009$, two-way ANOVA) and treatment ($P<0.001$, two-way ANOVA) effects were seen in DA concentrations, but without interaction of these factors (Fig. 9 a). An interesting phenomenon was seen in 0.33 μg 6-OHDA-lesioned +/+ mice, since their striatal DA content was increased with lesioning. DOPAC concentrations were generally higher in +/+ mice (Fig. 9 b, $P=0.007$, two-way ANOVA). There were no significant differences in HVA concentrations due to high variation. 5-HT –concentrations were generally higher in +/+ mice, but the effect of 6-OHDA was similar in both genotypes (Fig 9 d, genotype effect $P<0.001$, treatment effect $P=0.043$, two way ANOVA).
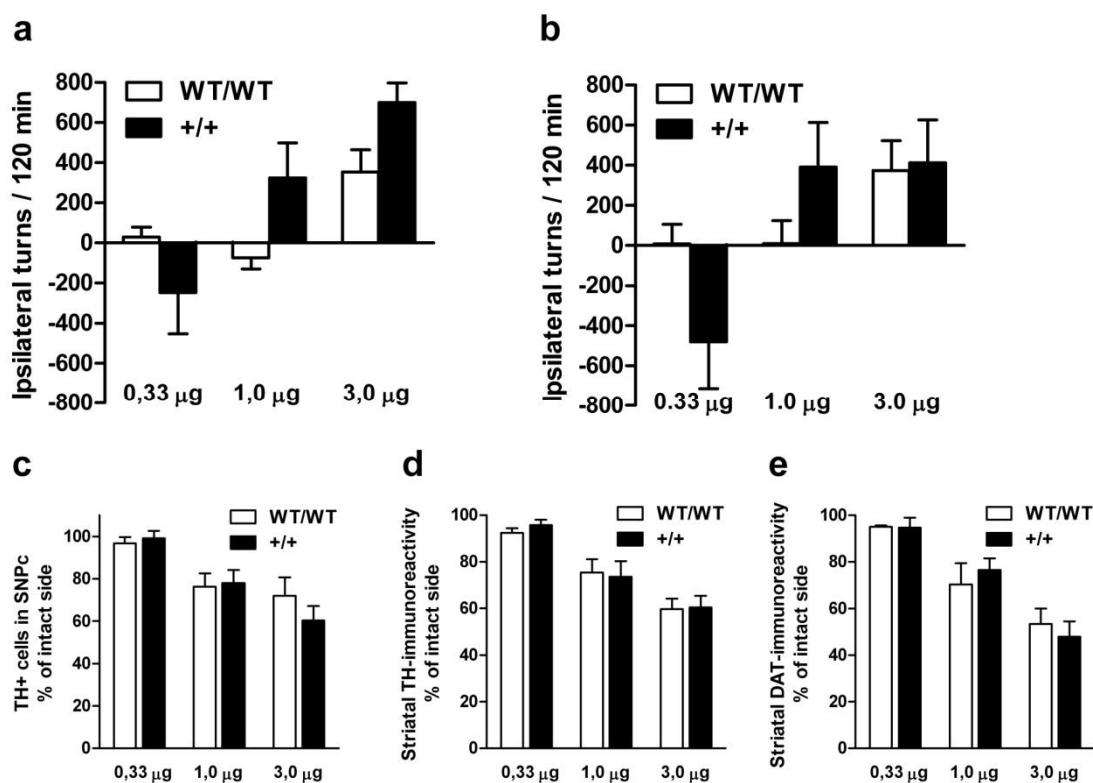
Figure 8. Effects of different doses of 6-OHDA on rotational behaviour and DAergic markers in mPrPh(A30P)asyn mice. a) D-amphetamine –induced (2.5 mg/kg, i.p.) rotational behaviour 2 weeks post lesion; genotype x dose P=0.02 (two-way ANOVA); b) D-amphetamine –induced (2.5 mg/kg, i.p.) rotational behaviour 4 weeks post lesion; genotype x dose P=0.056 (two-way ANOVA); c) Number of TH-positive neurons in the SNpc; d) optical density of TH-immunostaining in the striata; e) optical density of DAT-immunostaining in the striata. 6-OHDA-treatment effect in c, d and e: P<0.001 (two-way ANOVA). Data expressed as average+SEM, n=8-1 in a-c; n=3-5 in d, e.

Metabolite/DA ratios were calculated to detect possible changes in DA turnover (Fig. 9 e). Significant genotype and lesioning effects, but without interaction, were seen in mPrPh(A30P)asyn mice (*P*=0.016 and *P*<0.001 respectively, two-way ANOVA) in DOPAC/DA ratios. In HVA/DA -ratios, only the 6-OHDA-treatment effect was apparent (*P*<0.001, two-way-ANOVA) although in general the concentrations comply with those of DOPAC.

Finally, we investigated the brain accumulation of aSyn in young and aged mPrPh(A30P)asyn mice. We found that in all three brain areas that we analyzed, +/+ mice had significantly increased aSyn immunoreactivity (Fig. 10). This can be explained by the fact that these mice have an inserted A30P –gene, so the total aSyn load is higher. We also saw an age-related increase in aSyn –staining in both genotypes, especially in the STR and M1 cortex.
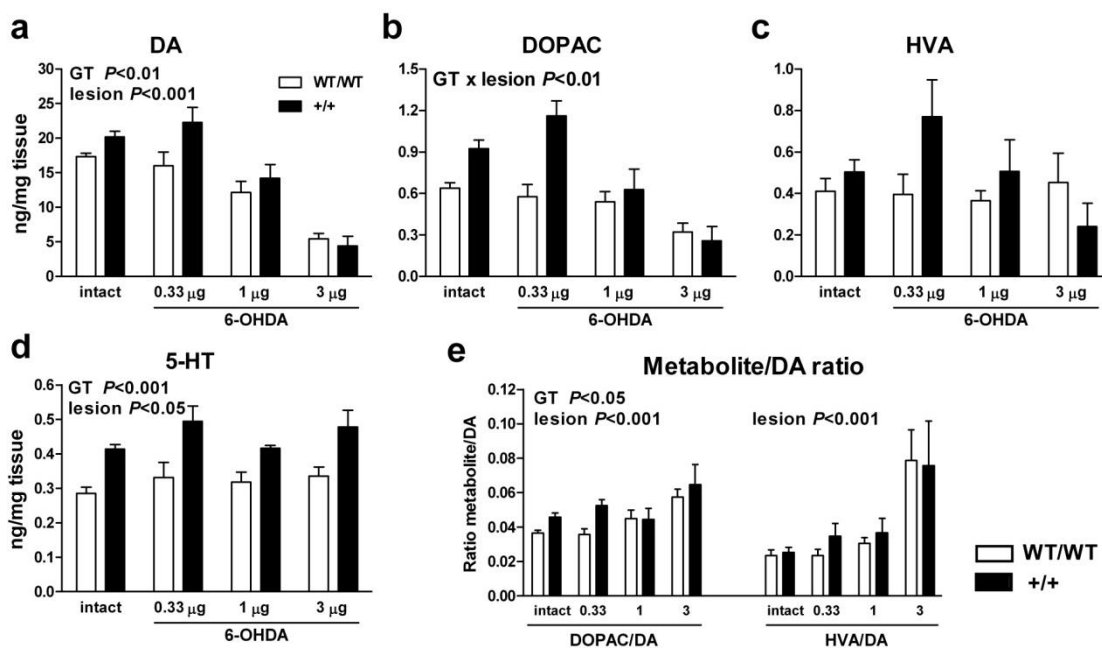
Fig. 9. Striatal concentrations of DA, DOPAC, HVA and 5-HT, and metabolite/DA ratios in mPrPh(A30P)asyn mice after 6-OHDA –lesioning. a-d) Concentrations of each analyte expressed as ng/mg tissue weight. Significant genotype (GT) and lesion effects in a and e, GT x lesion interaction in b (two-way ANOVA). e) metabolite/DA –ratios calculated for indices of DA turnover. Significant GT and lesion effects in DOPAC/DA and lesion effect in HVA/DA (two-way ANOVA). Data expressed as average+SEM, n= 15 in pooled intact samples, n=5 in 6-OHDA –treated samples.
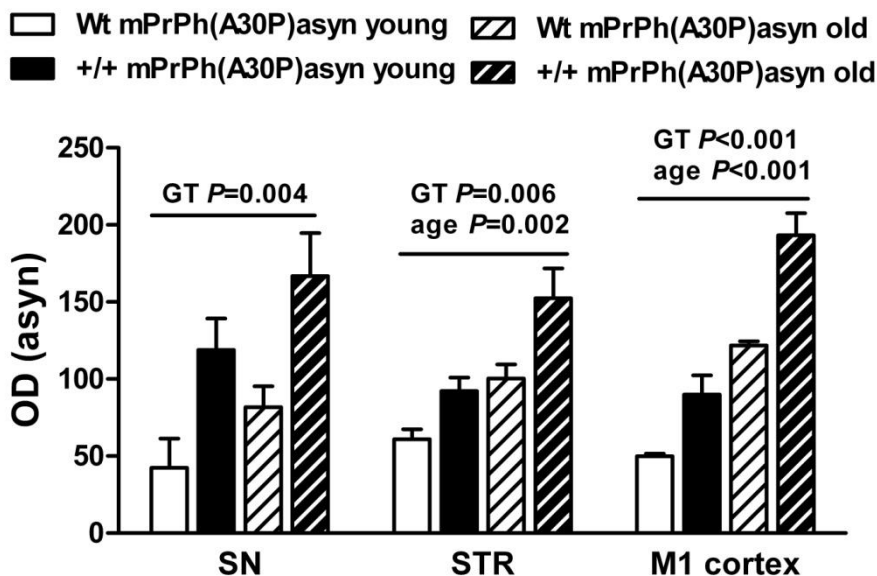

Fig. 10. Optical density of aSyn –immunoreactivity in the substantia nigra (SN), striatum (STR) and motor cortex (M1 cortex) of mPrPh(A30P)asyn mice. In all investigated brain areas, there was a significant genotype effect, and in the STR and M1 cortex also a significant age effect (two-way ANOVA). Data expressed as average+SEM, n=3-4.

Table 14. Summary of findings in mPrPh(A30P)asyn mice

Measure	Change in +/+ mice in comparison to WT/WT mice
Basal locomotor activity, 24 h	0
Basal vertical activity, 24 h	↓↓
D-amphetamine-induced locomotor activity, 1 mg/kg (2.5 mg/kg)	↓↓ (0)
aSyn immunoreactivity of the brain	↑↑
Basal DA and DA turnover	↑
After 6-OHDA –lesioning:	
Stride length	↓
D-amphetamine –induced rotational behaviour	↑
Loss of DAergic markers in the nigro-striatal pathway	0
Loss of striatal DA, DA turnover (except with 0.33 µg 6-OHDA ↑)	0

6. Discussion

6.1 Truncation of GDNF increases the diffusion of the protein *in vivo* (I)

GDNF has a high affinity for heparin-like structures, which is also made use of in the protein purification process (Lin et al., 1993, Lin et al., 1994). This phenomenon may also lie behind the observations that GDNF diffuses poorly in the brain. It has even been suggested that inadequate diffusion of the protein was one of the pitfalls in the clinical trials. Therefore, it was an intriguing idea to test, whether a change in the heparin-binging affinity of GDNF would increase the spreading in the brain. It was shown earlier that a co-infusion of GDNF and heparin increases the biodistribution of GDNF in the rat striatum, most likely due to GDNF binding to heparin instead of heparan sulphates in the brain, but at that time no behavioural responses were measured (Hamilton et al., 2001).

The heparin-binding motifs of GDNF are located within the first 16 amino acids of the sequence, discarding of which greatly reduces the affinity of GDNF to heparin without affecting its ability to bind GFRα1 (Alfano et al., 2007). Therefore, we hypothesized that the N-terminally truncated GDNF-variant, Δ38N-GDNF, would diffuse farther in the brain in comparison to the full protein due to reduced binding to heparan sulphate structures on the cell surfaces and in the extracellular matrix of the brain. Indeed, we found that the truncated variant has a markedly increased diffusion volume when injected into the rat striatum. The effect was evident already immediately after the injection, and persisted at least until 6 h post injection. Surprisingly, both our behavioural and morphological data suggest that despite being biologically active and stable, Δ38N-GDNF is not as potent as full GDNF in the 6-OHDA rat model of PD. Based on these findings we concluded that the poorer *in vivo* effect of Δ38N-GDNF was due to impaired heparin binding, and not because of loss of activity.

Our second approach in trying to increase the distribution of GDNF was to first inject HB-GAM to saturate its heparan sulphate proteoglycan (HSPG) receptor, syndecan-3, and thus reduce the binding of GDNF to this receptor. In a pilot study, we did not detect any signs of enhanced biodistribution, but later saw an impaired behavioural recovery in comparison to animals treated with GDNF alone. Pretreatment with HB-GAM dose-dependently prevented the GDNF-induced reduction of ipsilateral rotations, but did not affect the ability of GDNF to protect nigral DAergic cells. HB-GAM alone, however, did not have a significant effect on the rotational behaviour, and it did not protect the DAergic cell bodies in the SN when compared to vehicle control group. As mentioned before, sustained delivery of HB-GAM via viral vectors has been a more successful approach, leading to protection of TH-positive cells in the SN and TH-positive fibers in the striatum, as well as amelioration of paw use asymmetry depending on the experimental setup (Taravini et al., 2011, Gombash et al., 2012).

Interestingly, Bespalov et al. (2011) have shown that indeed, GDNF binds to syndecan-3 with high affinity. They suggested that the interaction between soluble GDNF and syndecan-3 serves to keep GDNF available for its receptors, whereas the same interaction with immobilized GDNF in the extracellular matrix is involved in neurite outgrowth and migration of embryonic cortical neurons. These effects are markedly reduced when Δ38N-GDNF or

syndecan-3 deficient cells/animals are used, or when heparan sulphate chains of the receptor are enzymatically cleaved by heparinase III. In this context, it is possible that the pretreatment with HB-GAM may interrupt the neuritogenic effects of GDNF as they can both bind syndecan-3, and so attenuate the positive behavioural response. Also, this may explain the weaker effect of Δ38N-GDNF in our experiments as the protein is not capable of binding to syndecan-3. Thus, it would not be kept concentrated near its receptors, which seems to be needed to gain adequate response. As we did not measure the optical density of striatal TH-immunoreactivity in Study I, this putative effect still remains to be confirmed.

6.2 VEGF-C reduces rotational bias in a neuroprotection set-up in the 6-OHDA rat model (II)

VEGF-family proteins have consistently been shown to have effects in the central nervous system. VEGF-A has been extensively studied, and it is known to directly promote the survival of neurons through various mechanisms. Also VEGF-B has been shown to protect a variety of neurons, and it is effective in cellular and animal models of PD (Falk et al., 2011). Neuronal effects of VEGF-C, on the other hand, have been fairly little studied. It is required for the proliferation of neural progenitors as well as oligodendrocyte precursor cells of the optic nerve, and it has been detected in the brains of embryonic, neonatal and adult mice (Le Bras et al., 2006, Kranich et al., 2009). Also, both the VEGF-C protein and its receptor VEGFR-3 were upregulated in the ischaemic rat hippocampus (Shin et al., 2008). After our initial finding demonstrating that *Vegfc* is one of the genes upregulated in N2A –cells after GDNF treatment, we hypothesized that VEGF-C has neurotrophic actions against neurodegeneration.

Before conducting *in vivo* experiments, we examined the ability of VEGF-C to protect DAergic cells in a primary embryonic midbrain culture. We found a modest increase in the survival of TH-positive neurons in comparison to the strong effect of GDNF, which was used as a positive control (see original publication II for details and figures). In the 6-OHDA rat model of PD however, we observed a robust reduction in the rotational behaviour of the rats that were treated with VEGF-C 6 h before lesioning. The effect was even comparable to that of GDNF, albeit with a higher dose. Surprisingly, TH-positive cells of SNpc were not significantly protected against 6-OHDA by VEGF-C, but the effect of GDNF was clear. Striatal TH-immunoreactivity was slightly better preserved with VEGF-C than were nigral DAergic cells, but not significantly more than in the control group. Since it is thought that the rotational behaviour induced by amphetamine is a measure of imbalance in striatal DA release between the two brain hemispheres, it must be taken into account that measuring the number of TH-positive cells or striatal DAergic fiber density will not reveal any changes in storage, release, or metabolism of DA in the surviving neurons, which can contribute to behavioural recovery. On the other hand, although the effect of VEGF-C on the survival of TH-neurons was modest and statistically non-significant (on average +11 %-points for TH-positive neurons and +19 %-points for striatal optical density in comparison to the control group), it may still have a role in the robust reduction of the rotational behaviour. The control group had on average a 40 % reduction in TH-positive cells and a 60 % reduction in striatal TH-immunoreactivity. These values seem to lie close to the border of whether consistent turning behaviour can be induced

by amphetamine (Schwarting and Huston, 1996a). Therefore, even a small 10-20% increase in the preservation of DAergic function may notably reduce the turning behaviour of a rat.

GDNF, as a powerful NTF for DAergic neurons, does not only promote neuronal survival and axonal branching but also enhances the functionality of these neurons. Cass et al. (1999) reported that already after 24 h after intrastriatal administration of GDNF, there was an increase in methamphetamine-stimulated DA release both in the striatum and SN. Even though the basal release and tissue concentration of DA were not augmented by GDNF, increased concentrations of its metabolites indicate an increase in DA turnover. Therefore, if the mechanisms behind the positive behavioural response to VEGF-C were to be explored further, measurements of eg. TH activity or DA release might clarify our findings.

We found that the MAPK/ERK –pathway was activated by VEGF-C in the rat striatum. This intracellular signalling pathway is also activated by numerous other neurotrophic factors. ERK phosphorylation was clear at 1 h after VEGF-C injection, but at 8 h post-injection the variation between samples had increased and the level of activation was not statistically significantly different from control levels. It is known that ERK activity can lead to distinct responses even in the same cell type depending on e.g. duration and magnitude of phosphorylation (Ebisuya et al., 2005). We postulate that if VEGF-C induces only a transient activation of ERK, 8 h post-injection may represent a kind of threshold time point, when the activation starts to disappear, and therefore can be seen only in some samples.

6.3 Neither VEGF-C nor GDNF are neurorestorative in a severe unilateral 6-OHDA rat model (II)

Since we observed a decrease in D-amphetamine –induced rotational behaviour in 6-OHDA –lesioned rats that were injected intrastriatally with VEGF-C before lesioning, we decided to assess if it has any neurorestorative activity after 6-OHDA -administration. When 6-OHDA and VEGF-C or GDNF were given as one deposit with one-month interval, there was a trend towards reduced rotational behaviour during the 10 weeks of follow-up in both experiments in NTF-treated groups. However, the effects were not significantly different from the vehicle group. In our hands, a one-site 6-OHDA –lesion seems to produce highly variable rotational behaviour and may be considered even unstable, since many rats show spontaneous functional recovery. This phenomenon may interfere with the evaluation of neuroprotective or neurorestorative efficacy of new NTFs. Therefore another model, created with two deposits of 6-OHDA, was employed. This approach, however, produced such a severe lesion that even GDNF was without significant effects. In this context, since GDNF is one of the most potent NTFs for DAergic cells known to date, it is not surprising that we could not detect any effect of VEGF-C in our neurorestoration experiments.

Striatal reinnervetion has been observed after a single GDNF –injection (Aoi et al., 2000a, Lindholm et al., 2007, Voutilainen et al., 2009) and after sequential GDNF –administration (Rosenblad et al., 1998) but all of these experiments were based on the smaller, single-site

striatal 6-OHDA lesion. On the other hand, Rosenblad et al. (2000) did not find any consistent striatal improvements in rats with extensive striatal lesions when the animals received a long-term GDNF –treatment first in the SN followed by sequential administration into the striatum. However, an increased survival of TH-positive cells in the SN and axonal sprouting in the globus pallidus were seen, proving that GDNF had a positive effect on the DAergic cells. We postulate that the lack of efficacy of GDNF in the more extensive lesions, such as ours, may be due to insufficient retrograde transport of GDNF as the axonal terminals are almost abolished, and therefore also the axonal sprouting is minimal. This would interrupt the retrograde transport of GDNF, which has been suggested to be important for the survival-promoting effects of GDNF (Tomac et al., 1995). However, there are reports describing also anterograde transport, evidently via GABAergic neurons, when GDNF is expressed in the striatum using viral vectors (Kordower et al., 2000, Georgievska et al., 2004). If GDNF is then released in the SN after anterograde transport, it may contribute to the survival of DAergic cells, and play a role also in our findings of increased TH-cell survival while a robust striatal and behavioural recovery was lacking. It is also possible, that VEGF-C does not possess any neurorestorative activity, but its positive effects are restricted to neuroprotection.

6.4 VEGF-C induces gliosis and disrupts the BBB (II)

We detected a prominent activation of microglia and expression of GFAP-positive astroglia, as well as a reduction in immunoreactivity of intact blood vessels (endothelial barrier antigen staining) three days after VEGF-C was injected into intact rat brain. These results are similar to those reported for VEGF-A. Although VEGF-A clearly has supportive effects on various neurons, and outcomes in preclinical animal models of neurodegenerative disorders have been positive, the safety of a vascular growth factor may be an issue when considering its therapeutic use. VEGF-A-induced neovascularisation may be a desired and positive effect in certain disorders, but detrimental when uncontrolled and accompanied by vascular leakage that may follow after higher doses. VEGF-B, however, seems to be devoid of vascular and glia-inducing effects in the brain, which is likely due to its high receptor selectivity (Poesen et al., 2008).

These signs on one hand suggest that VEGF-C may not be safe as a therapeutic molecule, but on the other hand remind that little is known about the borderline between beneficial and harmful astro- and microgliosis. As reviewed by Hanisch and Kettenmann (2007), activation of microglia can produce protective effects in some neuropathological states. Even though current evidence proposes that microglial activation is detrimental in models of PD, this may be partly due to experimental conditions that often provoke robust defence mechanisms and lead to a highly reactive microglial state (for example when using lipopolysaccharide). Astroglial cells, however, are known to secrete survival-promoting agents such as NTFs. Since we studied the occurrence of glial activation only at one time point, chosen to reveal the effect at maximum, it is not known how long the phenomenon exists after VEGF-C administration. This could be crucial information, since sustained reactive microgliosis may be the more harmful phenomenon rather than astrogliosis. VEGF-A, for example, causes a transient activation of microglia when injected to intact rat SN, the effect peaking at 1 day post-injection

and reaching control values in a week (Rite et al., 2007). In the same study, astrogliosis peaked at day 4 post-injection and but was still evident at 3 weeks post-injection.

6.5 Nigral DAergic neurons of MEN2B knock-in mice are protected from 6-OHDA –toxicity (III)

MEN2B knock-in mice carry the Met918Thr –missense mutation in the RET receptor tyrosine kinase. The mutation renders the receptor constitutively active, and causes a cancer syndrome (multiple endocrine neoplasiatype 2). RET is also one of the main signaling receptors for GDNF, and it is expressed by nigrostriatal DAergic neurons (Nosrat et al., 1997, Trupp et al., 1997). Mijatovic et al. (2007) have studied changes in the brain DAergic system of these mice and have observed robustly elevated concentrations of DA and its metabolites in the striatum, cortex and hypothalamus, increased levels of TH (striatum and SN) and DAT (striatum), and even slightly increased numbers of TH-positive cells in the SNpc and DAT-positive varicosities in the striatum. The peculiarities in the DAergic transmission of these mice were further confirmed by findings of augmented DA synthesis, storage, and stimulated release (Mijatovic et al., 2008). The next step after these findings was to study the effects of known DAergic neurotoxins, 6-OHDA and MPTP, in these mice and to assess how constitutive RET signalling affects the neurodegenerative process induced by the toxins. The elevated levels of DA and increased expression of DAT in MEN2B M/M- mice could sensitize them to the effects of the toxins, since excess DA is harmful to neurons, and both 6-OHDA and the toxic metabolite of MPTP (MPP+) enter the neurons via DAT (Blum et al., 2001, Chen et al., 2008).

We induced rotational behaviour in 6-OHDA –lesioned MEN2B mice to monitor the success of lesioning procedure. The result from the rotational experiment cannot reliably be used to assess the extent of the lesion in these mice because of the special features in their DAergic transmission. In comparison to WT littermates, M/M mice rotated more despite a lower percentual drop in striatal DA content. The behavioural effect is most likely due to higher release of DA in the intact hemisphere in M/M mice. Additionally, we found a notable difference in the survival of nigral TH-positive cells between the genotypes. While in the WT mice approximately 36 % of cells had degenerated (or lost their TH-phenotype) at 3 weeks post lesion, hardly any changes could be observed in M/M mice. The nigral DAergic cells were almost completely preserved despite a substantial loss of striatal DA, which interestingly was not compensated by elevated DA turnover in M/M mice in contrast to WT mice. Therefore, we suggested that constitutive RET activity is an important mediator of neuroprotection by GDNF in the DAergic cell bodies, whereas other mechanisms independent of RET are needed to preserve the axons. It must be noted though, that we studied rather acute neurodegeneration during the course of 3 weeks and therefore cannot completely rule out the possibility that constitutive RET activity might lead to enhanced axonal regeneration with time.

In the same publication, we report results from an experiment in which MEN2B mice, their heterozygous and WT littermates were given MPTP twice a day with a 3-hour interval, for two

consecutive days. This intoxication regime resulted in striatal depletion of DA, which was approximately 50 % in all genotypes. Striatal TH-immunoreactivity was likewise reduced by about 25 % in all groups, but the DAergic cells in the SN were left intact. We speculated that our choice of MPTP –intoxication, milder than usual, may have been inefficient in inducing proper neurodegeneration. It has been also shown that mice can exhibit spontaneous recovery from MPTP –treatment, manifested as a gradual increase in striatal TH-immunoreactivity and recovery of striatal DA, albeit that these effects may stay incomplete (Mitsumoto et al., 1998). Therefore, it is difficult to make conclusions about the MPTP –experiment in MEN2B mice.

6.6 D-Amphetamine-induced locomotor activity is attenuated in mPrPh(A30P)asyn +/+ mice (IV)

mPrPh(A30P)asyn transgenic +/+ -mice express the mutated human A30P-aSyn under the control of the pan-neuronal mouse prion protein promoter. The mice have been studied to some extent earlier (Yavich et al., 2004, Yavich et al., 2005, Oksman et al., 2009). According to these reports, +/+ -mice exhibit several motor impairments, including reduced locomotion and rearing, weaker performance on the rotarod, as well as attenuated locomotor response to L-DOPA. They also have a higher brain aSyn load as assessed by western blotting, which we confirmed by immunohistochemistry. Nevertheless, DAergic neuropathology, such as loss of neurons or striatal DA, has not been observed. Instead, the readily releasable pool of DA is exhausted faster by intensive electric stimulation in these mice (Yavich et al., 2004, Yavich et al., 2005). This finding implies that there is a lower capacity or inadequate recruitment of DA storage pool, which supposedly offers replenishment to the readily releasable DA pool under heavy stimulation.

D-amphetamine-induced locomotor activity was measured in 6-month-old mPrPh(A30P)asyn mice, since there were no detectable differences in their basal 24-h activity. Also, we intended to use D-amphetamine later in the experiments to induce rotational behaviour in 6-OHDA-lesioned mice, and decided to evaluate whether those results could possibly be influenced by basal differences in response to the drug. We observed that +/+ mice have a significantly reduced response to 1 mg/kg of D-amphetamine when compared to their wild type littermates. Amphetamines can affect the release of DA through various mechanisms, one of the main routes being the inhibition and reversal of DAT function to increase extracellular DA concentrations (Sulzer et al., 2005). However, since mPrPh(A30P)asyn +/+ mice have normal levels of DAT in the striatum and there are no significant changes in the rate of DA reuptake (Yavich et al., 2005), defects related to DAT can most likely be ruled out as an underlying cause for our observation. We suggest that the described deficits in DA dynamics play a part in our observation of decreased locomotor response to a small dose of D-amphetamine. Interestingly, when the dose was increased to 2.5 mg/kg, the distance travelled did not differ significantly between the genotypes. We cannot rule out that the earlier low dose of the drug did not play any role in how the mice responded to the higher dose. Vanderschuren et al. (1999) have reported that a single injection of amphetamine (5 mg/kg) could sensitize rats to the stimulating effects of the second injection, which followed 3, 7 or 21 days later. The sensitization at 3 days after the pretreatment was modest compared to the later time points,

but still points out the problem of interpreting behavioural responses after repeated injections of a psychostimulant drug. It is also possible that 2.5 mg/kg was simply too high a dose to detect similar differences as with 1 mg/kg in locomotor activity in our mice.

6.7 The effects of 6-OHDA in mPrPh(A30P)asyn mice (IV)

Considering the complex etiology of PD, it has been hypothesized that more than one "triggering factor" may be needed to start the neuropathological process. There is an increasing amount of evidence of susceptibility genes that do not alone explain the appearance of PD (Wirdefeldt et al., 2011). For example, polymorphisms in the *SNCA* gene encoding for aSyn are related to sporadic PD in addition to the point mutations and multiplications of the gene that are linked to familial PD (Bekris et al., 2010). In this context, and since the mPrPh(A30P)asyn mice have a rather mild motor phenotype and are without PD-like neuropathology, we decided to challenge the mice with 6-OHDA to see if the expression of human mutated aSyn would predispose them to the effects of the toxin.

Stride lengths were not significantly affected by 6-OHDA in either genotype, but it was generally shorter in 6-OHDA –treated mPrPh(A30P)asyn +/+ mice. However, the reduction in stride length does not necessarily correlate with the magnitude of nigral cell loss, which may explain the lack of 6-OHDA dose effect (Iancu et al., 2005). More robust changes were seen in D-amphetamine –induced rotational behaviour, and the general conclusion from these experiments is that the WT/WT mice required a higher dose of 6-OHDA than +/+ mice to exhibit a robust ipsilateral rotational bias. This, however, was not a sign of increased sensitivity to 6-OHDA, since striatal DA was similarly depleted in both genotypes at higher doses of 6-OHDA, and there were no genotype effects in the reductions of DAergic immunohistochemical markers. At the lowest dose of 6-OHDA (0.33 μg), the +/+ mice paradoxically rotated mainly contralaterally, and also had an elevated amount of DA in the toxin-treated striatum. We suggest that the differences between the genotypes in rotational behaviour are due to the functional deficits in DA release (Yavich et al., 2004, Yavich et al., 2005). These defects may have been enhanced with 6-OHDA -treatment in +/+ mice. What comes to the puzzling effect of the lowest dose of 6-OHDA, it may well be that the dose was so low, that it corresponds to a mere mechanical lesion caused by the injection needle. Such a mechanical damage in the mouse striatum can result in increased TH-activity, increased DA, DOPAC and HVA, as well as proliferation of presynaptic DA uptake sites (Howells et al., 1996). There was no difference in striatal DAT –immunoreactivity between the mPrPh(A30P)asyn genotypes, but DA and its metabolites were clearly increased in +/+ mice. However, our experiments do not offer an explanation to why this phenomenon does not happen in both genotypes.

In contrast to the earlier report (Yavich et al. 2005), basal DA and DOPAC –levels were slightly elevated in the mPrPh(A30P)asyn +/+ mice in our studies. Also, DOPAC/DA –ratio was significantly raised in the intact and 0.33 μg of 6-OHDA –treated striata in +/+ mice. This rise in metabolite/DA –ratio is an indicator of increased DA turnover, which is a known

compensatory mechanism to make up for reduced DAergic transmission eg. in 6-OHDA – lesions (Melamed et al., 1980). Therefore, we speculate that the defects in DA release in mPrPh(A30P)asyn +/+ lead to increased DA and DOPAC tissue concentrations and are reflected in presynaptic DA turnover, since the HVA/DA –ratio was clearly elevated only by the highest dose of 6-OHDA and not affected by genotype.

Also striatal 5-HT concentrations in mPrPh(A30P)asyn +/+ mice were higher than in WT/WT mice. Since 5-HT has complex modulatory effects on striatal DAergic function and output, this finding might require some further investigations. It has been reported that WT aSyn can attenuate the cell-surface availability of the serotonin transporter (Wersinger et al., 2006). Although the interaction does not seem to be mediated by the lipid binding domain of aSyn (residues 1-57, affected by the A30P -mutation), it might be of interest to study the trafficking and function of serotonin transporter in mPrPh(A30P)asyn mice, since they also express the WT aSyn.

6.8 Relevance of the current studies to clinical research

As mentioned before, there are several NTFs that have been effective in preclinical models of PD, but only two have made it to clinical trials with controversial outcomes. One of the suggested reasons for why GDNF-trials failed, is the poor diffusion of the protein in the brain parenchyma. Studies in rhesus monkeys have shown that the diffusion pattern of infused GDNF is very unpredictable and can vary over four-fold between individuals (Salvatore et al., 2006). In another study, there was a positive correlation between the volume of GDNF distribution, motor improvement and preservation of striatal TH-innervation in MPTP-intoxicated monkeys (Gash et al., 2005). These findings may be of pivotal importance when planning new clinical trials with NTFs. Our studies demonstrated that one possible approach to improve the diffusion properties of a protein is structural modification, in this case the removal of heparin-binding domain of GDNF. Although this compromised the neurotrophic potency of the protein, the molecule was not completely ineffective *in vivo*. It would be interesting to know if the efficacy of the truncated protein could be improved by longer term administration. Still, it should be kept in mind that improving the diffusion may bring about other challenges, for example how to control the diffusion. If it is only a certain neuronal population that needs to be targeted, how can it be ensured that no severe adverse effects are caused by uncontrolled diffusion to other brain areas? Also, the safety of long-term delivery of NTFs needs to be carefully assessed, since NTFs are involved in neuronal cell growth, migration, differentiation and survival, which could possibly promote the appearance of malignancies. MEN2B syndrome, for example, is characterized by a high frequency of medullary thyroid carcinomas and pheocromocytomas (Moline and Eng, 2011). The syndrome is caused by mutations in the gene coding for RET, rendering it constitutively active and perhaps leading to loss of substrate specificity. As mentioned, RET is one of the main signalling receptors for GDNF. Although brain malignancies are not commonly found in MEN2B –patients, the syndrome is still a warning example of what may follow of sustained activation of a growth factor receptor.

It is also intriguing that many growth factors serving their main functions outside the nervous system can have neurotrophic properties in mature neurons. These findings offer us new insights into the complex regulation and maintenance of neurons under both physiological and pathological states. It is possible that additional benefit can be achieved by combining NTFs, perhaps even specifically for different neurodegenerative disorders. For example, we found in our studies that nigral TH-positive cells were slightly more efficiently protected in the 6-OHDA-lesioned rats by combining VEGF-C and GDNF, than with either of the proteins alone. The benefit of combination therapy could be mediated by either activation of separate survival-promoting intracellular signalling pathways by the trophic factors, or simply through actions on different cells. However, great caution is required when considering growth factors that primarily affect other tissues for treating brain disorders. VEGFs undoubtedly have positive effects on neurons and could be potential targets in drug development, but they may have severe adverse effects such as vascular leakage that can lead to brain oedema. We also demonstrated that VEGF-C activates microglia, which may cause neuroinflammation, and it may also open the BBB. Additionally, although it was reported only once by Xue et al. (2010) that striatal expression of EPO in the rat brain via AAV-vector leads to systemic effects in rats (ie. increase in red blood cells), the chance for unexpected systemic adverse effects should be kept in mind.

Emergence of genes associated with PD, as well as identification of NTFs for DAergic neurons have allowed the development of genetically modified mouse strains. Although these mouse strains rarely and inconsistently exhibit both behavioural and neuropathological hallmarks of PD, their usefulness may rather lie in clarifying the mechanisms of neurodegeneration. The growing list of PD-related genes, as well as all the putative causes of sporadic PD suggest that what we clinically know as PD is the manifestation of various underlying factors. It is possible, that the sequences of events that finally lead to neurodegeneration differ depending on the background of the patient. Therefore, better understanding of how neurons degenerate may help to design targeted and customized therapy in the future.

7. Conclusions

The aims of the present studies were to analyse the effects of novel or modified known NTFs and to investigate the effects of selected genetic backgrounds on induced DAergic degeneration by utilizing the unilateral 6-OHDA –model of PD in rodents. The principal findings were:

1. The removal of the first 38 amino acids from the structure of GDNF results in increased diffusion of the protein in the rat striatum, but its effects on functional recovery and preservation of the nigrostriatal DAergic pathway are attenuated in the 6-OHDA –lesioned rats when compared to the full-sequence GDNF.

2. VEGF-C has neuroprotective effects in the 6-OHDA –treated rats, but its neurorestorative activity remained unconfirmed. VEGF-C effectively abolishes the rotational bias although its effect on the survival of DAergic neurons was rather modest. It was also seen that VEGF-C can induce astro- and microgliosis and disruption of the BBB when injected intrastriatally.

3. Constitutive RET signalling provides a robust protection of nigral DAergic neurons in MEN2B M/M mice against 6-OHDA in spite of substantial depletion of striatal DA. This suggests that other mechanisms than RET –signalling are involved in axonal protection by GDNF.

4. Transgenic mice expressing the human A30P-aSyn are not sensitized to the neurotoxic effects of 6-OHDA although they exhibit a particular rotational bias and changes in striatal DA after the toxin treatment. These mice have an attenuated locomotor response to D-amphetamine and they develop notable motor defects with age.

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Helsinki, October 2012

Marjo

> *"I almost wish I hadn't gone down that rabbit-hole*
> *— and yet — and yet — it's rather curious,*
> *you know, this sort of life!"*
>
> *Lewis Carroll: Alice's adventures in Wonderland (1865)*

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