CDNF AND MANF IN AN EXPERIMENTAL MODEL
OF PARKINSON’S DISEASE IN RATS

Merja H. Voutilainen

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy, University of Helsinki, for public examination at Viikki Biocentre, auditorium 1041, University of Helsinki, on December 18th 2010 at 12 noon.
Supervisors:  
Professor Raimo K. Tuominen, MD, PhD  
Division of Pharmacology and Toxicology  
Faculty of Pharmacy  
University of Helsinki  
Finland

Professor Mart Saarma, PhD  
Institute of Biotechnology  
Viikki Biocentre  
University of Helsinki  
Finland

Professor Pekka T. Männistö, MD, PhD  
Division of Pharmacology and Toxicology  
Faculty of Pharmacy  
University of Helsinki  
Finland

Reviewers:  
Docent Matti Airaksinen, MD, PhD  
Neuroscience center  
Viikki Biocentre  
University of Helsinki  
Finland

Professor Deniz Kirik, MD, PhD  
Section of Neuroscience  
Department of Experimental Medical Science  
Lund University  
Sweden

Opponent:  
Professor Lars Olson, MD, PhD  
Department of Neuroscience  
Karolinska Institutet  
Stockholm, Sweden

©Merja Voutilainen 2010  
ISSN 1795-7079  
Yliopistopaino, University Press  
Helsinki, Finland 2010
To Elias
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>VI</td>
</tr>
<tr>
<td>ABSTRAKTI</td>
<td>VII</td>
</tr>
<tr>
<td>ORIGINAL PUBLICATIONS</td>
<td>VIII</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>IX</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2. REVIEW OF THE LITERATURE</td>
<td>2</td>
</tr>
<tr>
<td>2.1 Parkinson’s disease</td>
<td>2</td>
</tr>
<tr>
<td>2.1.1 Etiology and pathology of Parkinson’s disease</td>
<td>2</td>
</tr>
<tr>
<td>2.2 The dopaminergic system of the brain</td>
<td>4</td>
</tr>
<tr>
<td>2.2.1 Synthesis and metabolism of dopamine</td>
<td>7</td>
</tr>
<tr>
<td>2.2.2 Neural circuits of the basal ganglia</td>
<td>7</td>
</tr>
<tr>
<td>2.3 Current drug therapy for Parkinson’s disease</td>
<td>8</td>
</tr>
<tr>
<td>2.3.1 Dopaminergic drugs</td>
<td>8</td>
</tr>
<tr>
<td>2.3.2 Pharmacotherapy beyond the dopamine system</td>
<td>10</td>
</tr>
<tr>
<td>2.4 Experimental animal models for studying Parkinson’s disease pathology and therapy</td>
<td>12</td>
</tr>
<tr>
<td>2.4.1 Criteria for a good animal model of human disease</td>
<td>12</td>
</tr>
<tr>
<td>2.4.2 6-hydroxydopamine (6-OHDA)</td>
<td>13</td>
</tr>
<tr>
<td>2.4.3 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)</td>
<td>17</td>
</tr>
<tr>
<td>2.4.4 Genetic parkinsonian models</td>
<td>18</td>
</tr>
<tr>
<td>2.5 Neurotrophic factors and midbrain dopaminergic neurons</td>
<td>19</td>
</tr>
<tr>
<td>2.6 The GDNF family of neurotrophic factors</td>
<td>20</td>
</tr>
<tr>
<td>2.6.1 Expression of GDNF in the brain</td>
<td>22</td>
</tr>
<tr>
<td>2.6.2 Physiological role of GDNF in the midbrain dopaminergic system</td>
<td>23</td>
</tr>
<tr>
<td>2.6.3 The effect of exogenous GDNF on intact dopaminergic system</td>
<td>23</td>
</tr>
<tr>
<td>2.6.4 The effects of exogenous GDNF on the lesioned dopaminergic system</td>
<td>24</td>
</tr>
<tr>
<td>2.6.5 GDNF administration in Parkinsonian patients</td>
<td>26</td>
</tr>
<tr>
<td>2.7 The CDNF/MANF family of neurotrophic factors</td>
<td>28</td>
</tr>
<tr>
<td>2.7.1 Mesencephalic astrocyte-derived neurotrophic factor (MANF)</td>
<td>28</td>
</tr>
<tr>
<td>2.7.2 Cerebral dopamine neurotrophic factor (CDNF)</td>
<td>29</td>
</tr>
<tr>
<td>2.7.3 Expression of CDNF and MANF</td>
<td>31</td>
</tr>
<tr>
<td>2.7.4 Structure of CDNF and MANF</td>
<td>32</td>
</tr>
<tr>
<td>3. AIMS OF THE STUDY</td>
<td>33</td>
</tr>
<tr>
<td>4. MATERIALS AND METHODS</td>
<td>34</td>
</tr>
<tr>
<td>4.1 DNA and RNA methods</td>
<td>34</td>
</tr>
<tr>
<td>4.1.1 RNA isolation and reverse transcription</td>
<td>34</td>
</tr>
<tr>
<td>4.1.2 PCR, cloning and DNA sequencing</td>
<td>34</td>
</tr>
<tr>
<td>4.1.3 In situ hybridization</td>
<td>34</td>
</tr>
<tr>
<td>4.2 Cell culture methods</td>
<td>34</td>
</tr>
<tr>
<td>4.2.1 Primary neuronal cultures</td>
<td>34</td>
</tr>
<tr>
<td>4.2.2 Transfection</td>
<td>34</td>
</tr>
<tr>
<td>4.3 Animals</td>
<td>34</td>
</tr>
<tr>
<td>4.4 Drugs and treatments</td>
<td>35</td>
</tr>
<tr>
<td>4.4.1 Production and purification of rhCDNF and MANF</td>
<td>35</td>
</tr>
<tr>
<td>4.4.2 Protein stability (III)</td>
<td>35</td>
</tr>
</tbody>
</table>
4.4.3 Preparation of $^{125}$I-labeled neurotrophic factors (II,III).......................... 35
4.5 Stereotaxic surgery....................................................................................................... 35
4.5.1 Stereotaxic operation............................................................................................... 35
4.6 Rotational behavior....................................................................................................... 37
4.7 Immunohistochemistry................................................................................................. 38
  4.7.1 Tissue preparation .................................................................................................. 38
  4.7.2 Immunohistochemistry ......................................................................................... 38
  4.7.3 Quantification of immunoreactivity ....................................................................... 38
4.8 Neurotrophic factor transportation studies (II,III).......................................................... 39
  4.8.1 Quantification of $^{125}$I-neurotrophic factors in CNS ............................................ 39
  4.8.2 Emulsion autoradiography .................................................................................... 39
  4.8.2 Autoradiographic analysis of the distribution of $^{125}$I-neurotrophic factors 39
4.9 Statistical analysis........................................................................................................ 39

5. RESULTS ......................................................................................................................... 40
  5.1 CDNF - an evolutionarily conserved protein ............................................................... 40
  5.2 CDNF expression in neuronal and non-neuronal tissue ............................................... 40
  5.3 Testing of survival promoting activity of CDNF on primary neuronal cultures (I)........ 41
  5.4 Neuroprotective and restorative effects of CDNF (I)..................................................... 41
  5.5 Neuroprotective and restorative effects of MANF (II) ............................................... 42
  5.6 Stability of the neurotrophic factors in buffer solution ............................................. 43
  5.7 Chronic infusion of CDNF reduce amphetamine-induced ipsilateral rotations and protects TH-positive cell bodies in the SNpc and their fibres from degeneration (III).... 43
  5.8 Lack of neurorestorative effect of chronic infusion of MANF in 6-OHDA model of PD (III) ................................................................. 44
  5.9 Effect of chronic infusion of CDNF and MANF on TH-immunoreactive neurons in SNpc and fibers in striatum in non-lesioned animals (III).................... 45
  5.10 Distribution of intrastratial CDNF and MANF (II, III).............................................. 45
  5.11 Transportation experiments (II, III)........................................................................... 45

6. DISCUSSION ...................................................................................................................... 47
  6.1 CDNF: a promising trophic factor in 6-OHDA PD model...................................... 47
  6.2 MANF: neuroprotective and neurorestorative properties in the 6-OHDA PD model.................................................. 48
  6.3 Continuous infusion of CDNF and MANF ................................................................. 49
  6.4 Methodological consideration .................................................................................... 49
  6.5 Distribution properties of CDNF and MANF ............................................................. 50
  6.6 Neuroprotective mechanisms of neurotrophic factors in PD models ..................... 51
    6.6.1 Endoplasmic reticulum-stress ........................................................................... 51
    6.6.2 Oxidative stress .................................................................................................. 52
  6.7 Two putative activities of CDNF and MANF ............................................................. 52
  6.8 CDNF and MANF as potential therapeutic proteins ................................................. 53

7. CONCLUSIONS .............................................................................................................. 56

8. ACKNOWLEDGEMENTS ............................................................................................. 57

9. REFERENCES ................................................................................................................... 59
ABSTRACT

Parkinson’s disease (PD) is a neurodegenerative disorder associated with a progressive loss of dopaminergic neurons of the substantia nigra (SN). Current therapies of PD do not stop the progression of the disease and the efficacy of these treatments wanes over time. Neurotrophic factors are naturally occurring proteins promoting the survival and differentiation of neurons and the maintenance of neuronal contacts. Neurotrophic factors are attractive candidates for neuroprotective or even neurorestorative treatment of PD. Thus, searching for and characterizing trophic factors are highly important approaches to degenerative diseases.

CDNF (cerebral dopamine neurotrophic factor) and MANF (mesencephalic astrocyte-derived neurotrophic factor) are secreted proteins that constitute a novel, evolutionarily conserved neurotrophic factor family expressed in vertebrates and invertebrates. The present study investigated the neuroprotective and restorative effects of human CDNF and MANF in rats with unilateral partial lesion of dopamine neurons by 6-hydroxydopamine (6-OHDA) using both behavioral (amphetamine-induced rotation) and immunohistochemical analyses. We also investigated the distribution and transportation profiles of intrastriatally injected CDNF and MANF in rats.

Intrastrial CDNF and MANF protected nigrostriatal dopaminergic neurons when administered six hours before or four weeks after the neurotoxin 6-OHDA. More importantly, the function of the lesioned nigrostriatal dopaminergic system was partially restored even when the neurotrophic factors were administered four weeks after 6-OHDA. A 14-day continuous infusion of CDNF but not of MANF restored the function of the midbrain neural circuits controlling movement when initiated two weeks after unilateral injection of 6-OHDA. Continuous infusion of CDNF also protected dopaminergic TH-positive cell bodies from toxin-induced degeneration in the substantia nigra pars compacta (SNpc) and fibers in the striatum. When injected into the striatum, CDNF and GDNF had similar transportation profiles from the striatum to the SNpc; thus CDNF may act via the same nerve tracts as GDNF. Intrastrial MANF was transported to cortical areas which may reflect a mechanism of neurorestorative action that is different from that of CDNF and GDNF. CDNF and MANF were also shown to distribute more readily than GDNF.

In conclusion, CDNF and MANF are potential therapeutic proteins for the treatment of PD.
ABSTRAKTI


Hermokasvutekijät CDNF (cerebral dopamine neurotrophic factor, dopamiiniisoluen hermosokasvutekijä) ja MANF (mesenkefaalinen astrocytyliperäinen hermosokasvutekijä) ovat läheistä sukea keskenään ja kuuluvat evoluuttiossa varhainen syntyneeseen CDNF/ MANF-proteiiniperheeseen. Kyseessä on ensimmäinen hermosokasvutekijäperhe, jolla on sukulaiskasvutekijä myös sellarangattomissa.

Tässä työssä tutkimme CDNF:n ja MANF:n tehokkuutta kokeellisessa Parkinsonin taudin mallissa rotilla. Rotille aiheutetettiin Parkinsonin taudille ominainen hermovaario ruiskuttamalla 6-hydroksidopamiini (6-OHDA)-toksiniä aivojuovioon (striatum) toiseen aivosulokseen, jolloin dopamiinihermot tuhoutuvat hitaasti muutamien viikojen ja kuukausien aikana. Samalla kehitteytyi toispulinen liikkehäiriö, joka ilmenee, kun rotille annetaan dopamiiniaktiivisuutta lisäävää lääketä.


Tutkimuksen perusteella CDNF:stä ja mahdollisesti myös MANF:sta saattaa olla mahdollista kehittää uudentyyppinen Parkinsonin taudin hoito, joka suojaa dopamiinihermosoluja ja estää niiden rappautumisen.
This dissertation is based on the following publications, herein referred by their Roman numerals (I-III):


The original publications are reprinted with permission of the copyright holders.

Cover Image:
Schematic illustration of amino acid sequences of CDNF and MANF. Pre corresponds to the signal sequence, and the numbers show the length of the polypeptides in amino acids. Conserved cysteine residues are shown by yellow vertical bars. Below the illustration of amino acid sequences of CDNF and MANF is photomicrograph showing the neuroprotective effect of CDNF and GDNF in a rat model of Parkinson's disease (PD). In a rat model of PD the dopaminergic neurons (revealed with brown colour) die by 6-OHDA treatment (see reduction of brown colour in the 6-OHDA-treated left hemisphere). The rats were given vehicle, GDNF (10 pg) or CDNF (10 pg) unilaterally into the striatum 6 h before injection of 6-OHDA (8 pg) in to the same location. GDNF (B) and CDNF (C) protect dopamine neurons and fibres in this model. Modified from Lindholm et al., 2007 and Lindholm and Saarma, 2010. This material is reproduced with permission of Nature Publishing group (Lindholm et al 2007) and John Wiley & Sons Inc. (Lindholm and Saarma, 2010).
LIST OF ABBREVIATIONS

6-OHDA 6-hydroxydopamine
ANOVA Analysis of variance
ARTN Artemin
BDNF Brain-derived neurotrophic factor
CDNF Cerebral dopamine neurotrophic factor
CNS Central nervous system
COMT Catechol-O-methyltransferase
Cpu Caudate putamen
DAT Dopamine transporter
L-DOPA, DOPA 3,4-dihydroxyphenylalanine, L-dopa, as a drug substance Levodopa
DOPAC 3,4-dihydroxyphenyl acetic acid
DRG Dorsal root ganglion
ER Endoplasmic reticulum
GABA \(\gamma\)-aminobutyric acid
GDNF Glial cell line-derived neurotrophic factor
GFL GDNF family ligands
GFR\(\alpha\) GDNF family receptor alpha
GPe Globus pallidus external segment
GPI Globus pallidus internal segment
HVA Homovanillic acid
IHC Immunohistochemistry
i.p. Intraperitoneally
kDa KiloDalton
LC Locus coeruleus
MANF Mesencephalic astrocyte-derived neurotrophic factor
MAO Monoamine oxidase
MFB Medial forebrain bundle
MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NRTN Neurturin
NTF Neurotrophic factor
PD Parkinson’s disease
pl Isometric point
PSPN Persephin
ROS Reactive oxygen species
SCG Superior cervical ganglion
SEM Standard error of mean
SN Substantia nigra
SNpc Substantia nigra pars compacta
SNpr Substantia nigra pars reticulate
Str Striatum
TH Tyrosine hydroxylase
UPR Unfolded protein response
VTA Ventral tegmental area
1. INTRODUCTION

Parkinson’s disease is an age-related, progressive neurodegenerative disorder in which patients experience a debilitating loss of movement functionality that includes muscle rigidity, bradykinesia (slowness of movement) and resting tremors. These motor symptoms of PD result from the degeneration of neurons of the substantia nigra (SN) in the midbrain, which leads to a loss of dopamine (DA) in the striatum and disrupts the neural circuitry that controls movements (Thomas and Beal, 2007). The current therapy for PD is symptomatic and cannot halt or retard the neurodegeneration in patients suffering from the disease.

Neurotrophic factors (NTFs) are proteins important for the survival and function of specific neuronal populations. The first neurotrophic factor, nerve growth factor (NGF), was discovered in the early 1950s (Cohen et al., 1954). Because of the therapeutic potential of trophic factors for neurological disorders, research in this area has been growing rapidly, and a large number of trophic factors and their receptors have been identified (Hefti 1997, Skaper and Walsh 1998, Lanni et al., 2010). GDNF (glial cell line-derived neurotrophic factor) was originally characterized in 1993 as a growth factor that promotes the survival of ventral midbrain dopamine neurons in vitro (Lin et al., 1993). GDNF protects dopamine neurons and shows therapeutic potential in rodent (Hoffer et al., 1994, Tomac et al., 1995b, Rosenblad et al., 1998) and primate (Gash et al., 1996, Zhang et al., 1997) PD models. These positive results led to the initiation of clinical trials of GDNF in PD patients. Results from GDNF open-label trials have shown benefits of continuous infusion of GDNF into the putamen in PD patients (Gill et al., 2003, Slevin et al., 2005).

Nevertheless, these promising results with GDNF were not confirmed in later double-blind studies (Nutt et al., 2003, Lang et al., 2006). Thus, it is important to search for and study novel NTFs that could offer new therapeutic approaches for the treatment of neurodegenerative diseases.

CDNF and MANF are secreted proteins that constitute a novel, evolutionarily conserved neurotrophic factor family expressed in vertebrates and invertebrates (Lindholm et al., 2007, Petrova et al., 2003, Palgi et al., 2009, Lindholm and Saarma, 2010). The purpose of the present studies was to characterize the neuroprotective and restorative effects of CDNF and MANF in a rat 6-hydroxydopamine (6-OHDA) PD model. Moreover, the distribution and transportation profiles of intrastriatally injected CDNF and MANF in rats were explored.
2. REVIEW OF THE LITERATURE

2.1 Parkinson’s disease

Parkinson’s disease was first described in an essay entitled “An Essay of the shaking Palsy” by James Parkinson in 1817 (Parkinson, 2002). PD is a progressive neurodegenerative movement disorder affecting primarily the nigrostriatal system. It is an age-related disease with a prevalence of 1-3 % in the population over 50 years of age, and it is characterized by relatively selective nigrostriatal dopamine depletion. The clinical features of PD include motor impairments involving resting tremor, bradykinesia, postural instability and rigidity along with non-motoric symptoms such as autonomic, cognitive and psychiatric problems (Lees et al., 2009).

2.1.1 Etiology and pathology of Parkinson’s disease

The specific etiology of PD is not known, but both environmental and genetic factors are thought to play a role.

Environmental factors There are a number of factors that may increase the risk of developing PD (Tanner and Langston, 1990), including exposure to pesticides, herbicides or industrial chemicals. A hint for an environmental factor in PD relates to the toxin 1,2,3,6-methyl-phenyl-tetrahydropyridine (MPTP) (Langston et al., 1983), which was discovered accidentally. As a by-product of a pethidine analogue (1-methyl-4-phenyl-propion-oxyppiperidine; MPPP) used as ”synthetic heroin,” MPTP produced a parkinsonian syndrome in drug addicts (Langston et al., 1983, Davis et al., 1979). Paraquat is structurally similar to 1-methyl-4-phenylpyridium (MPP+), the active metabolite of MPTP, and has been used as an herbicide. Like MPP+, rotenone is also a mitochondrial toxin present in the environment, and it is used as an insecticide. Human epidemiological studies have suggested a link between residence in a rural environment (and associated exposure to herbicides and pesticides) and elevated risk of PD (Tanner 1992). However, there are no compelling data to implicate any specific toxin as a cause of sporadic PD, and chronic environmental exposure to MPP+ or rotenone is unlikely to cause PD (Dauer and Przedborski, 2003).

Genetic factors The majority of PD cases are sporadic; however, the discovery of genes linked to relatively rare familial forms of the disease and studies from experimental animal models have provided some insights into molecular mechanisms in PD pathogenesis and identified probable targets for therapeutic intervention. Currently, approximately 10 genetic loci have been identified as being associated with parkinsonism, and mutations of the α-synuclein, LRRK2 (encoding leucine-rich repeat kinase 2), GBA (glucocerebrosidase), tau, parkin, PINK and DJ-1 genes can be counted as genetic causes of PD (Lee and Liu, 2008, Hardy 2010, Obeso et al., 2010). α-Synuclein, a natively unfolded protein, is highly expressed throughout the mammalian brain and is enriched in presynaptic nerve terminals where it has a role in vesicle transport and dopamine neurotransmission (Murphy et al., 2000, Abieliovich et al., 2000). At the cellular level, the characteristic pathology of PD is the accumulation of intracytoplasmic protein aggregates called Levy Bodies that contain misfolded protein α-synuclein and other proteins such as neurofilaments and ubiquitin. However, the mechanism by which α-synuclein aggregates in sporadic forms of PD is poorly understood (Moore et al., 2005). Parkin was originally considered
to be a ubiquitin E3-protein ligase involved in proteasomal degradation of proteins (Kitada et al., 1998) that functions as a multipurpose neuroprotective protein crucial for dopamine neuron survival after a variety of toxic insults (Feany and Pallanck, 2003). It now appears that both Parkin and PINK1 (a mitochondrial kinase) are genetically in the same pathway which is involved in the elimination of damaged mitochondria (Park et al. 2006) and they are also functionally linked since their expression induces mitochondrial fission (Lutz et al., 2009). DJ-1 is also likely to be involved in mitochondrial physiology (Cookson et al. 2010), because it has been shown to translocate to the mitochondria and exert a protective effect on the application of oxidative stress. In addition, DJ-1 has been proposed to have antioxidant activity (Thomas and Beal, 2007), but it also functions as a redox-dependent chaperone that inhibits α-synuclein aggregation and subsequent death (Shendelman et al., 2004). LRRK2 is a protein kinase that modulates synaptic vesicle recycling and neurite outgrowth and has a role in mitochondrial functions (Li and Beal, 2005). LRRK2 is almost certainly involved in signaling cascades (Dauer and Ho, 2010), possibly relating to cytoskeletal dynamics (Meixner et al. 2010). Mutations in LRRK2 represent the highest risk of familial and, apparently, sporadic PD (Zimprich et al. 2004, Floris et al., 2009).

Neuropathology: The neuropathological hallmarks of PD are the loss of neuromelanin-containing dopaminergic neurons in the substantia nigra pars compacta (SNpc) resulting in a depletion of dopamine along the nigrostriatal pathway and the presence of intraneuronal cytoplasmic inclusions (Bernheimer et al., 1973, Marsden 1983, Forno 1996). These inclusion bodies, termed Lewy bodies, are spherical eosinophilic cytoplasmic protein aggregates composed of numerous proteins including α-synuclein, parkin, ubiquitin, synphilin and neurofilaments (Forno 1996, Spillantini et al., 1998).

Animal studies have revealed that the degeneration of dopaminergic neurons starts from the nerve endings and is followed by axon and cell body degeneration (Björklund et al., 1997). How the degeneration proceeds in humans is less clear. At the onset of first motor symptoms, approximately 60% of the SNpc neurons are lost and there is an approximate 80% decrease in dopamine content in the putamen (Bernheimer et al., 1973, Agid 1991). The loss of terminals in the striatum is more significant than the loss of dopaminergic cells in the SNpc (Bernheimer et al., 1973). Dopaminergic neurons of the ventral tegmental area are much less affected in PD and, as a result, there is significantly less depletion of dopamine in the caudate nucleus (Price et al., 1978, Uhl et al., 1985). A loss of tyrosine hydroxylase (TH) activity accompanies the loss of dopamine (Birkmeyer and Riederer 1989).

As discussed above, dopamine neuron loss in PD starts several years before the emergence of neurological symptoms. During this period, the loss of dopaminergic neurons is counterbalanced by endogenous compensatory mechanisms (Bernheimer et al., 1973, Zigmond et al., 1990). There are both dopaminergic compensatory mechanisms, which enhance the effects of or exposure to existing dopamine, and non-dopaminergic mechanisms, which reduce the activity of the indirect striatal output pathway (Brotchie and Fitzter-Attas, 2009).

Oxidative stress and mitochondrial dysfunction have been strongly implicated in PD pathogenesis (Betarbet et al., 2002). Accumulating evidence indicate that both excitotoxicity and the generation of
reactive oxygen species (ROS) are important mediators of neuronal death in PD (Jenner 2003, Chen et al., 2004). In addition, recent studies point to endoplasmic reticulum (ER) stress as a potential mediator of PD pathogenesis (Hoozemans et al., 2007, Wang et al., 2007, Wang and Takahashi, 2007). Activation of the unfolded protein response (UPR) together with increased immunoreactivity for α-synuclein has been reported in dopaminergic neurons of the SN in PD cases. Furthermore, certain toxins, (such as 6-OHDA) that partly mimic the pathology of PD both in cell culture and in vivo have also been reported to trigger ER stress (Holtz and O’Malley, 2003, Ryu et al., 2002).

**Immunological factors** Epidemiological studies, analysis of post-mortem PD brains and animal PD models have all provided evidence for a role of brain inflammation in PD pathogenesis (McGeer et al., 2003). Activated microglia have been reported in the SN of post-mortem PD patients (McGeer et al., 1988). Microglial activation has also been detected in post-mortem putamen, hippocampus, cingulate cortex and temporal cortex tissue samples from PD patients (Imamura et al., 2003). A lack of correlation between microglial activation and disease severity suggests that early and persistent activation of microglia in PD may slowly drive the disease process (Gerhard et al., 2006).

However, the neuropathology of PD is not restricted to the nigrostriatal dopaminergic pathway. Other neuronal populations are affected as well, including the locus coeruleus (LC) (noradrenergic); the raphe nucleus (serotonergic); the nucleus basalis of Meynert and the dorsal motor nucleus of vagus (cholinergic); the cingulate cortex; the entorhinal cortex; the olfactory bulb; the sympathetic ganglia and parasympathetic neurons in the gut, (reviewed by Hornykiewicz and Kish, 1987). The degeneration of some of these non-dopaminergic areas correlates with secondary symptoms of PD such as dementia (Lee and Liu, 2008). Additionally, upregulation of corticostriatal glutamatergic synaptic transmission is evident in PD. Changes involving kinase and phosphatase signaling pathways within striatal dopaminergic and medium-spiny neurons seem to increase the synaptic efficacy of glutamatergic receptors in these neurons. Glutamate-mediated striatal sensitization subsequently modifies basal ganglia output in ways that favor the appearance of parkinsonian motor complications (Oh and Chase, 2002).

In summary, PD is a complex disease that is not caused by any one genetic or environmental factor. While some forms of PD are genetic, most cases are idiopathic, and the underlying environmental causes remain to be discovered (Hardy 2010, Obeso et al., 2010).

### 2.2 The dopaminergic system of the brain

Dopamine was initially considered just to be a precursor in the synthesis of noradrenaline and adrenaline and not an independent neurotransmitter in the brain. In the late 1950s, Arvid Carlsson and co-workers found large amounts of dopamine (DA) in basal ganglia and implicated it in the parkinsonian-like symptoms induced by reserpine (Carlsson et al., 1957, Carlsson et al., 1958, Carlsson 1959). Dopamine is involved in many central nervous system (CNS) functions, including motor control, emotions, cognition and endocrine regulation.

Dopaminergic neurons form three major ascending pathways in the brain, the
nigrostriatal, mesolimbic/mesocortical and
tuberohypophyseal pathways (Fig. 1). Cell
bodies of these pathways are located in
the ventral midbrain, in the SNpc (A9) and
in the ventral tegmental area (VTA, A10)
(Dahlstroem et al., 1964, Ungerstedt 1971a).
Cells in the SNpc project mainly to the
dorsal part of the striatum in the caudate/
putamen (CPu), forming the nigrostriatal
pathway. This pathway accounts for about
75% of the dopamine in the brain, and in
fact, over 80% of dopamine in the brain
is in the STR. Furthermore, a minor part
of the nigrostriatal pathway consists of
axons from cell bodies in the A8 area
(retorubral field) projecting to the ventral
putamen. Axons of the nigrostriatal
pathway run alongside fibers containing
other transmitters including noradrenaline
and 5-hydroxytryptamine (5-HT, serotonin)
through the medial forebrain bundle (MFB)
to the striatum. The nigrostriatal pathway
is involved in the control of movement,
posture, the acquisition of motor programs
and habit formation. In rats, the total
number of TH-positive cells in all three cell
groups is 40 000-45 000, with about half
of the cells located in the SN (German and
Manaye, 1993).

**Figure 1.** Schematic representation of the ascending dopaminergic pathways in the rat brain. Topographical location and terminal fields of the main central dopaminergic neurotransmission systems. A) Dopaminergic cell groups constituting the so-called meso-striatal pathway. These
dopaminergic neurons located in the substantia nigra pars compacta (SNpc, A9) and the ventral
tegmental area (VTA. A10 cell group) innervate the dorsal striatum (STR) and the nucleus
accumbens (NAc, ventral striatum), respectively. B) Meso-limbo-cortical dopaminergic pathways
are constituted by A9 and A10 dopamine neurons projecting to other brain regions such as
the prefrontal cortex (PF Cx), the septum, the olfactory tubercles (Olf Bulb) and the amygdala
It is often oversimplified that the nigral A9 neurons project to the striatum along the nigrostriatal pathway and the A10 neurons of the VTA project to limbic and cortical areas along mesolimbic and mesocortical pathways. In fact, the SN contains not only neurons projecting to the striatum, but also neurons that innervate cortical and limbic areas; in addition, the dopamine neurons of the VTA project to the ventral striatum and the ventro-medial part of the head of the caudate-putamen in rodents (Björklund and Dunnett, 2007).

The cells of the ventral tegmental area project to limbic structures including the nucleus accumbens, the amygdala, the hippocampus, the septum and the olfactory tubercle, forming the mesolimbic dopamine pathway (Ungerstedt 1971a). In addition, ventral tegmental cells project to the cortical areas including the medial prefrontal, entorhinal and cingulate cortices, forming the mesocortical dopamine pathway. The mesolimbic pathway is involved in the control of motor behavior as well as emotions and motivation, while the mesocortical pathway regulates higher cognitive functions such as reward and learning (Wise and Rompre, 1989, Björklund and Dunnett, 2007).

In addition to the long ascending pathways mentioned above, there are

\[ \text{Figure 2.} \] Dopamine synthesis and its metabolism at dopaminergic nerve terminals, postsynaptic neuron and glial cell. DA (Dopamine), TH (tyrosine hydroxylase), DOPA (3,4-dihydroxyphenylalanine), AAADC (aromatic amino acid decarboxylase), VMAT2 (vesicular monoamine transporter 2), DAT (dopamine transporter), MAO-A and MAO-B (monoamine oxidase type A, B), DOPAC (3,4-dihydroxyphenylacetic acid), COMT (catechol-O-methyltransferase), HVA (homovanillic acid), 3-MT (3-methoxytyramine). Figure modified from Youdim et al. (2006).
intermediate-length and ultra-short-length dopaminergic pathways. The ultra-short systems include interplexiform amacrine-like neurons, which originate from the A17 area in the retina, and the periglomerular dopamine cells originating in the olfactory bulb (Cooper et al., 2003). Intermediate-length systems include the tuberoinfundibular system, which originates from the arcuate and periventricular nuclei and affects the pituitary gland. This thesis focuses on the long ascending nigrostriatal dopaminergic pathway described above.

### 2.2.1 Synthesis and metabolism of dopamine

Dopamine synthesis starts with conversion of the amino acid tyrosine (Hornykiewicz 1966) to L-dihydroxyphenylalanine (L-DOPA) by the rate limiting enzyme TH (Carlsson and Lindqvist, 1978) (Figure 2.2). L-DOPA is then subsequently converted to dopamine by aromatic L-amino acid decarboxylase. Newly synthesized dopamine is transported by vesicular monoamine transporter 2 (VMAT2) and stored into vesicles within dopamine neurons.

Because TH is a rate-limiting enzyme in dopamine synthesis, dopamine levels are very sensitive to changes in TH activity. Endogenous mechanisms that regulate the rate of dopamine synthesis in dopamine neurons modulate TH activity in several different ways (Cooper et al., 2003). First, TH activity is inhibited by the ability of dopamine and other catecholamines to compete with the tetrahydrobiopterin (BH$_4$) cofactor for a binding site on the enzyme. End-product inhibition is maximal when neuronal activity and transmitter release are low and catecholamine concentration in storage vesicles is high. Second, the availability of BH$_4$ also has a role in regulating TH activity. Additionally, presynaptic dopamine receptors can modulate the rate of tyrosine hydroxylation. Feedback inhibition of dopamine synthesis occurs when presynaptic receptors are activated by dopamine released from the nerve terminal. Long-term regulation of TH takes place, for example, via increased gene transcription (Kumer and Vrana, 1996).

As an action potential reaches the dopaminergic nerve terminal, dopamine is released in a calcium-dependent manner from storage vesicles into the synaptic cleft. Dopamine metabolism takes place in the cytoplasm of the nerve terminal and inside glial cells. Extracellular dopamine is inactivated mainly via non-enzymatic mechanisms. Most of the released dopamine is taken up into the dopaminergic neurons by the membrane dopamine transporter (DAT), which is probably the primary and most efficient mechanism of inactivation in the meso-striatal pathways.

Inside the cell, dopamine is either taken by VMAT and re-stored in vesicles for later use or metabolized by monoamine oxidase (MAO) to dihydroxyphenylacetic acid (DOPAC), which diffuses out of the neurons (Fig. 2). Released dopamine is converted into 3-methoxytyramine (3-MT) through catechol-O-methyltransferase (COMT). 3-MT and DOPAC are further converted through MAO and COMT, respectively, into homovanillic acid (HVA), which is often viewed as the final metabolite of dopamine. The primary metabolites of dopamine in the CNS are DOPAC, HVA and a small amount of 3-MT.

### 2.2.2 Neural circuits of the basal ganglia

The basal ganglia are a group of subcortical nuclei that are involved in a variety of processes including motor, associative and cognitive functions (Bolam et al., 2000). The subset of these nuclei relevant
to motor function are the caudate and putamen (striatum in rodents), the external (GPe) and the internal segments of globus pallidus (GPi; also called entopeduncular nucleus (EP) in rodents). The substantia nigra pars reticulata (SNpr) in the base of the midbrain and the subthalamic nucleus (STN) in the ventral thalamus also belong to the basal ganglia. The motor circuit (Fig. 3) has received particular attention because of its relevance to movement disorders.

The predominant neurotransmitter in the basal ganglia is \(\gamma\)-aminobutyric acid (GABA), and the primary site of functional interactions in the basal ganglia is the striatum, which receives inputs from the cortex and midbrain. More than 90% of all cell bodies in the striatum are GABAergic medium spiny neurons, and they are classified into two subclasses according to the different neuropeptides and dopamine receptors that they express (Surmeier et al., 2007): medium spiny neurons that express D1 dopamine receptors and contain substance P and neurons that express D2 receptors and contain encephalin (Surmeier et al., 2007, Gerfen 2006). The remaining 5-10% of neurons in the striatum are either GABAergic or cholinergic interneurons (Tepper and Bolam, 2004). The GABAergic medium spiny neurons of the striatum receive dense glutamatergic inputs from the cerebral cortex and thalamus and dopaminergic inputs from the midbrain (SNpc and VTA; Fig. 3) (Bolam et al., 2000). The major output from the striatum is the medium spiny GABAergic neurons projecting to midbrain nuclei (Wichmann and DeLong, 1996).

The cortical information is processed within the striatum and passed via direct and indirect pathways to the output nuclei of the basal ganglia, the GPi and the SNpr (Albin et al., 1989). In the direct pathway, corticostriatal information is transmitted directly from the striatum to the output nuclei (Shink et al., 1996). The indirect pathway, in turn, involves an inhibitory projection from the GP to the STN and to the output nuclei and an excitatory projection from the STN to the SNpr/EP. The information is transmitted back to the cerebral cortex via the thalamus or conveyed to various brainstem structures. In this model, SN dopamine neurons differentially influence the basal ganglia system by activating D1-containing and inhibiting D2-containing striatal neurons of the direct and indirect pathway, respectively, thereby facilitating movement (Gerfen 2000).

In parkinsonism, loss of dopamine leads to a shift of the balance to the indirect pathway, with increased activity of GABAergic GPi/SNr neurons and greater inhibition of glutamatergic thalamocortical cells. This results in decreased facilitation of cortical motor areas and subsequent development of akinesia and bradykinesia (Wichmann and DeLong, 1996, Albin et al., 1989).

### 2.3 Current drug therapy for Parkinson’s disease

#### 2.3.1 Dopaminergic drugs

Since its introduction in the late 1960s, treatment for PD has been primarily based on dopamine replacement by means of the dopamine precursor levodopa (Birkmayer and Hornykiewicz, 1961). Levodopa is mainly metabolized by two enzymatic pathways: it is converted either to dopamine by dopa decarboxylase (DDC) (also known as aromatic amino acid decarboxylase (AAADC)) (Fig. 2) (Cotzias et al., 1967) or to 3-O-methyl-dopa by catechol-O-methyltransferase (COMT)
(Axelrod and Tomchick, 1958, Männistö et al., 1992). Levodopa has both central and peripheral effects, of which only central are desirable. Conversion to dopamine in the periphery, which would otherwise account for about 95% of the levodopa dose, is largely prevented by using a DDC inhibitor such as carbidopa or benserazide (Papavasiliou et al., 1972, Hagan et al., 1997), which enables more dopamine to enter the brain. DDC inhibitors also prevent adverse effects such as nausea and vomiting due to activation of dopamine receptors in the area postrema of the medulla that are not protected by the blood brain barrier (Olanow 2004). Chronic levodopa treatment is accompanied by the development of severe motor side-effects after a period of maximal benefit, which usually lasts three to five years (Sage and Mark, 1994). There follows a progressive loss of efficacy ("wearing-off") in about 60% of initial responders (Marsden and Parkes, 1977). Wearing-off, rapid "on/off" fluctuations in symptom control and dyskinesias are all severe clinical problems. "Off-time" refers to periods of the day when the medication is not working well, causing worsening

**Figure 3.** Schematic diagram of the principal basal ganglia thalamocortical circuitry under normal conditions (A) and in Parkinson’s disease (B). The inhibitory dopaminergic pathway from the substantia nigra pars compacta (SNpc) to the striatum is impaired due to dopamine deficiency, increasing the activity of GABAergic cells in the striatum, which in turn inhibit GABAergic cells in the substantia nigra pars reticulate (SNpr), thus reducing the restraint on the thalamus and cortex, causing rigidity. Inhibitory connections are illustrated as black arrows and excitatory connections as white arrows. GPe (external segment of the globus pallidus), GPi (internal segment of the globus pallidus), STN (subthalamic nucleus), DA (dopamine), GABA (γ-Aminobutyric acid), Glu (glutamate). Modified from Wichmann and DeLong (1996).
of parkinsonian symptoms. In contrast, the term “on-time” refers to periods of adequate control of PD symptoms. The COMT inhibitors entacapone and tolcapone reduce O-methylation in the gut, increase levodopa bioavailability and prolong the half-life of levodopa (Kaakkola et al., 1994, Nutt et al., 1994, Merello et al., 1994). Entacapone is currently in wide clinical use, while tolcapone can be used in restricted cases only due to its hepatotoxicity (Assal et al., 1998).

Levodopa is unquestionably still the most potent dopaminergic drug, but also other dopaminergic drugs (e.g. dopamine agonists and MAO-B inhibitors) can provide effective control and are, to varying degrees, effective at improving bradykinesia and rigidity in PD, while tremors may be more refractory (Schippana 2007). Dopamine agonists can be used either as first-line therapy or as an adjunct to levodopa (Schippana 2005). Dopamine agonist monotherapy can control dopaminergic symptoms effectively for a while, but patients will inevitably require levodopa supplementation (Holloway et al., 2004, Rascol et al., 2000). Moreover, dopamine agonists do not target all PD symptom domains, and they are accompanied by significant adverse effects of their own such as nausea, neuropsychiatric effects and sedation (Olanow et al., 2001, Shapiro et al., 2007). It has been stated that one of the reasons for agonist failure during early disease is that they are often used at sub-optimal doses and are abandoned before an appropriate level is reached. If used correctly, they can produce symptom control comparable to that achieved with levodopa (Schippana 2005).

MAO-B inhibitors are also effective as monotherapy in early disease and as adjuvant therapy in more advanced disease. The primary rationale for using selective MAO-B inhibition in PD is that it enhances striatal dopaminergic activity by inhibiting the metabolism of dopamine (Samii et al., 2004, Riederer et al., 1978). Two isoforms of MAO have been identified: MAO-A and MAO-B. The B type is the predominant isoform in the human brain. MAO-B inhibitors such as selegiline and rasagiline both improve motor function in early and advanced PD (Schippana 2005, Rabey et al., 2000). They may also have neuroprotective and potentially even disease-modifying effects in experimental models and clinical studies (Fernandez and Chen, 2007, Palhagen et al., 2006, Olanow et al., 2009).

As stated already, dopamine agonists and MAO-B inhibitors offer effective relief of the motor features of PD in early and more advanced disease and are associated with a low risk of motor complications. However, they are not as potent as levodopa.

2.3.2 Pharmacotherapy beyond the dopamine system

As mentioned earlier (2.1.1), PD involves the degeneration of non-dopaminergic neurons, and treatment of the resulting predominantly non-motor features remains a challenge. However, recent advances in the understanding of the transmitter systems involved in basal ganglia–related motor control have focused attention on multiple non-dopaminergic pathways and their receptors as potential new therapeutic targets (Poewe 2009).

Anticholinergic drugs The cortico-striato-thalamic loop and the nigrostriatal system are innervated largely by cholinergic afferents coming from the tegmentum and the septum and by cholinergic interneurons. Most cholinergic systems, such as muscarinic receptors (Perry et al., 1990, Asahina et al., 1995), nicotinic receptors (Perry et al., 1990, Quik 2004) and choline transporters (Rodriguez-Puertas et al.,
1994) are affected in PD. Anticholinergics were among the first drugs used in PD and were intended to correct the imbalance between dopamine and acetylcholine levels. These drugs do produce some beneficial effects on PD symptoms, but they are also associated with adverse parasympatholytic and cognitive effects (Katzenschlager et al., 2003).

**GABAergic and anti-glutamatergic drugs**
Because the vast majority of pathways in the basal ganglia utilize glutamate and GABA as their respective excitatory and inhibitory neurotransmitters (Schapira et al., 2006), these neural systems are potential drug targets. Enhanced GABA release in the GPe (Fig. 3) via activation of adenosine A$_{2A}$ receptors has been suggested to contribute to the overactivity in the indirect pathway in PD, and adenosine antagonists would be expected to exert anti-parkinsonian effects. Several distinctive features of the adenosine A$_{2A}$ receptor have made its antagonism a leading candidate strategy for the improved treatment of PD (Jenner 2005). Indeed, perhaps uniquely among currently pursued non-dopaminergic targets in PD research, A$_{2A}$ receptors are relatively selectively expressed in the striatum (Dixon et al., 1996). In addition, A$_{2A}$ receptor antagonists consistently reverse parkinsonian motor deficits in preclinical models of PD (Grondin et al., 1999). A placebo-controlled clinical trial with the A2A antagonist istradefylline has shown significant reductions in OFF time in fluctuating PD (LeWitt et al., 2008). However, a recent random double-blind trial, which studied the effect of istradefylline as monotherapy in patients with PD, failed to demonstrate efficacy in improving motor symptoms (Fernandez et al., 2010). Though, additional studies are still needed.

Alpha-adrenergic receptors are also located on striatal GABAergic neurons, and they have been suspected to contribute to overactivity in the direct pathway, which is associated with dyskinesia in PD. The selective α2 antagonist idazoxan has been in preclinical and clinical trials, but the results have been inconsistent (Rascol et al., 2001, Manson et al., 2000). Fipamezole, another member of this class of agents, is currently in clinical development (Poewe 2009, Savola et al., 2003).

Also, glutamate antagonists have been studied that target cortico-striatal glutamatergic input that is abnormally enhanced in response to levodopa and that drives direct pathway neurons that contribute to dyskinesia. The efficacy of amantadine is believed to involve such mechanisms, and more selective agents are being investigated, such as a metabotropic glutamate receptor antagonist (Samadi et al., 2008). Indeed, memantine, an NMDA receptor antagonist, has shown therapeutic potential in various CNS disorders (Parsons et al., 1999). In conclusion, targeting non-dopaminergic pathways in PD seems to be a promising area for future drug development; however no breakthrough has yet emerged in clinical trials.

All of the therapies for PD described above are symptomatic and do not halt the ongoing neurodegeneration. In addition, the efficacy of these treatments wanes over time. The progressive degenerative process in PD provides a basis for the development of neuroprotective therapies aimed at preventing or slowing down the degenerative process and stimulating the recovery and regeneration of the remaining dopamine neurons. One emergent therapeutic approach is the use of neurotrophic factors to halt, reverse or even restore the loss of dopaminergic neurons.
2.4 Experimental animal models for studying Parkinson's disease pathology and therapy

Animal models mimicking the PD pathology are an important tool for studying pathogenic mechanisms and therapeutic strategies in PD. Through the use of an animal model, striatal dopamine deficiency was associated with symptoms of PD (Carlsson et al., 1957). Agents, such as reserpine, methamphetamine, 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) that selectively disrupt or destroy catecholaminergic systems have been used to develop PD models (Table 1). In addition, Betarbet and colleagues have suggested that agricultural chemicals such as rotenone and paraquat, when administered systemically, can reproduce specific features of PD in rodents, apparently via oxidative damage (Betarbet et al., 2002). However, in contrast to the original report this has not been detected in later studies. Moreover, the bacterial endotoxin lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, which acts as a glial activator and induces inflammatory dopamine neurodegeneration (Liu and Hong, 2003, Bronstein et al., 1995), is known to cause significant loss of nigral dopamine neurons and is also used as an animal model of PD (Dutta et al., 2008). The most commonly used toxins to generate animal PD models are 6-OHDA and MPTP, which will be discussed in detail in chapters 2.4.2 and 2.4.3.

2.4.1 Criteria for a good animal model of human disease

Appropriate disease models should be based on possible causative agents or risk factors of human disease or by other means exhibit a substantial degree of behavioral or neuropathologic signs that corresponds conclusively to human disease. Many useful animal models do not reproduce all the key features of the human condition, and some do not reproduce any. Instead of judging a model how exclusively it mimics the human disease, models are traditionally judged by two main criteria, reliability and validity (Geyer and Markou 1995).

Reliability refers to the ability of the model to provide consistent results under different conditions. Validity refers to the framework underlying the model. Animal models for movement disorders achieve validity in one of three ways. The most natural model is one that has face validity denoting that a model recapitulates important anatomical, neuropathological, biochemical or behavioral features of the human disease. Many PD animal models mimic the motor features but fail to achieve validity of other features of PD (non-motor symptoms, Lewy bodies) (Van der Worp et al., 2010). Another way in which a model can achieve validity is etiology. Etiologic or construct validity refers to the disease relevance of the methods by which a disease model is created. In the ideal situation etiologic validity could be achieved by recreating in an animal the etiologic process (either by creating transgenic animal model or through exposure of an animal to a well known disease-causing agent or environmental risk factor) that cause disease in humans and hence replicate behavioral and neural features of the illness. For PD, the LRRK2 transgenic mouse models seem to achieve this well (Zimprich et al., 2004). Though the predictive validity models, which are useful for predicting treatment response may resemble their human disease the least, have the potential to be the most useful for guiding therapy (LeDoux 2005).
2.4.2 6-hydroxypamin (6-OHDA)

6-OHDA was first shown to cause noradrenergic depletion of sympathetic nerves to the heart (Porter et al., 1963) and producing chemical sympathectomy in peripheral nervous system (Thoenen and Tranzer 1968). In addition, 6-OHDA was the first chemical agent discovered that had specific neurotoxic effects on catecholaminergic pathways (Ungerstedt 1968, Sachs and Jonsson, 1975). 6-OHDA is hydroxylated dopamine, and it uses the same catecholamine transport system as dopamine and norepinephrine (DAT and NET, respectively) to enter neurons and produce specific degeneration of catecholaminergic neurons. Because 6-OHDA cannot cross the blood-brain barrier, it must be administered by local stereotaxic injection into the MFB, SN or striatum to target the nigrostriatal dopaminergic pathway (Ungerstedt 1968, Ungerstedt 1971b, Javoy et al., 1976). The specificity of 6-OHDA for dopaminergic neurons can be increased with noradrenaline transport blockers, which inhibit 6-OHDA uptake by noradrenergic neurons (Fuchs and Coper, 1980). Following 6-OHDA injection into the SN or MFB, dopaminergic cells start degenerating within 24 hours and die without clear apoptotic morphology (Jeon et al., 1995). Striatal dopamine depletion commences two to three days later (Faulk and Laverty, 1969). When injected into the striatum, however, 6-OHDA produces a more protracted retrograde degeneration of nigrostriatal neurons that lasts for 1-3 weeks. The axon terminals of dopamine neurons are destroyed first, and the axonal degeneration gradually proceeds toward cell bodies in the SNpc (Sauer and Oertel, 1994, Przedborski et al., 1995).

After partial lesion of the nigrostriatal neurons, there is a compensatory increase in the synthesis and release of dopamine in the remaining dopaminergic terminals (Agid et al., 1975, Hefti et al., 1980). In addition, a reduction in the number of dopamine uptake sites is observed after 6-OHDA administration (Javitch et al., 1985, Marshall et al., 1989). The magnitude of the lesion is dependent on the amount of 6-OHDA injected, the site of injection and inherent differences in sensitivity between animal species (Betarbet et al., 2002). Unlike the striatum, the degree of dopamine depletion in the SN is less severe, and no compensatory increase in dopamine activity in the remaining dopamine soma has been detected after striatal 6-OHDA administration (Schwarting and Huston, 1996). Possible physiological changes in other targets of dopamine innervation, such as the ventral striatum, amygdala or cortical projections, have received less attention than the striatum. Noradrenergic and serotoninergic mechanisms are not consistently affected by a unilateral 6-OHDA lesion (Costall et al., 1976, Saavedra et al., 1978). As stated earlier, 6-OHDA can also be taken up into and can destroy other catecholaminergic neurons, and in the case of mesencephalic 6-OHDA lesions, damage to noradrenergic elements can be expected. If noradrenaline reuptake inhibitors have not been used, noradrenaline tissue levels were found to reduced in several brain areas, including neostriatum, SN, NAcc, septum, cortex and hypothalamus (Costall et al., 1976, Saavedra et al., 1978, Wooten and Collins 1981, Dunnet and Björklund 1983). In addition, neostriatal tissue levels of 5-HT, or its metabolite 5-HIAA were either unchanged (Costall et al., 1976, Iwamoto et al 1976, Shapiro et al., 1987 or even decreased (Costall et al., 1976, Scwartner et al., 1991). Cholinergic neurons, which are located as interneurons in the striatum, show increased activity after dopamine
Table 1. Selected toxin and transgenic rodent models of Parkinson's disease

<table>
<thead>
<tr>
<th>Toxin model</th>
<th>Symptoms</th>
<th>Pathology</th>
<th>Pathogenic relevance</th>
<th>Applications</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPTP (mice/monkeys)</td>
<td>Akinesia, Rigidly, tremor (not in rodents)</td>
<td>SN:TH+cells ↓ STR:TH-IR ↓ STR:DA, LC neurons ↓ α-synuclein aggregation</td>
<td>Oxidative stress, inhibition of mitochondrial complex I</td>
<td>Preclinical testing of PD therapies</td>
<td>Acute, non-progressive or reversible; inclusions bodies are rare</td>
</tr>
<tr>
<td>LPS (rats/mice)</td>
<td>Progressive PD-related behavioral impairment</td>
<td>SN:TH+cells ↓ STR:TH-IR ↓ STR:DA ↓</td>
<td>Chronic inflammatory responses oxidative stress and impaired mitochondrial function prior DAergic neurodegeneration</td>
<td>Screen therapeutics for PD and study mechanisms of inflammation-induced neurodegeneration</td>
<td>Intracerebral injection needed, optimal dose needed to get progressive cell loss</td>
</tr>
<tr>
<td>Reserpine (Mouse)</td>
<td>Akinesia, catalepsy</td>
<td>None</td>
<td>Depletes DA at the nerve terminals</td>
<td>Preclinical testing to improve symptoms</td>
<td>Nonspecific liberation of monoamine transmitters, hypothermia</td>
</tr>
<tr>
<td>Met-amphetamine</td>
<td>No clear parkinsonian symptoms</td>
<td>At high doses loss of TH in STR, loss of DA cells in SNpc</td>
<td>DA-related oxidative stress, DA depletion at the level of DAergic nerve terminals</td>
<td>Screen antioxidant therapies to protect dopamine cells</td>
<td>Acute: limited histopathological change</td>
</tr>
<tr>
<td>Parquat</td>
<td>Locomotor activity ↓</td>
<td>STR: TH-IHR ↓ SN: TH+cells ↓</td>
<td>Multiple environmental toxins/pesticide exposure; oxidative stress</td>
<td>Screen therapies to protect DA cells</td>
<td>Toxic for the whole organism, not well described, low construct validity</td>
</tr>
<tr>
<td>Rotenone</td>
<td>Akinesia, rigidity, tremor, flexed posture, piloerection</td>
<td>SN:TH+cells ↓ STR:TH-IR ↓ Locus ceruleus neurons ↓ Inclusions of Levy bodies</td>
<td>Chronic “environmental” toxin; chronic oxidative stress and inhibition of mito-chondrial complex I</td>
<td>Screen therapies to protect DA cells</td>
<td>Labor and time intensive, substantial morbidity and mortality</td>
</tr>
</tbody>
</table>

Table 1. cont.

<table>
<thead>
<tr>
<th>Genetic models</th>
<th>Symptoms</th>
<th>Pathology</th>
<th>Pathogenic relevance</th>
<th>Applications</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic a-synuclein (mice/ drosophila)</td>
<td>Reduced or abnormal motor activity</td>
<td>a-synuclein-inclusions, change in TH-IHC</td>
<td>Known pathogenic mutations</td>
<td>Screen therapies to protect DA cells</td>
<td>Expensive and time-consuming; mice do not have characteristic PD phenotype/pathology</td>
</tr>
<tr>
<td>Transgenic tau</td>
<td>No change in locomotor activity</td>
<td>No change in DA neurons, TAU inclusions</td>
<td></td>
<td></td>
<td>Expensive, no change in DA</td>
</tr>
<tr>
<td>PINK1 (Park 6)</td>
<td>No change in locomotor activity</td>
<td></td>
<td>Mitochondrial kinase</td>
<td></td>
<td>No parkinsonian phenotype</td>
</tr>
<tr>
<td>DJ-1 (Park 7)</td>
<td>Locomotor activity ( \downarrow )</td>
<td>No degeneration of DA neurons, STR DA ( \downarrow )</td>
<td>Mitochondrial protein, antioxidant activity</td>
<td>Testing gene therapeutic strategies, study DJ-1 in oxidative stress</td>
<td></td>
</tr>
<tr>
<td>Parkin (Park 2)</td>
<td>Behavioural deficits associated with basal ganglia function</td>
<td>No degeneration of DA neurons, decreased DA release to Amp</td>
<td>E3 ubiquitin-protein ligase activity, belongs in a pathway involved in the elimination of damaged mitochondria</td>
<td>Role of Parkin in protein turnover, oxidative stress, mito-chondrial dysfunction</td>
<td>No parkinsonian phenotype</td>
</tr>
<tr>
<td>LRRK2 knock out mice</td>
<td>No motor deficits up to 24 months of age</td>
<td>No neuropathological abnormalities or loss of DAergic neurons up to 12 months of age</td>
<td></td>
<td></td>
<td>Lack of LRRK2 function may be compensated</td>
</tr>
<tr>
<td>BAC transgenic mice expressing h. LRRK2 R 1441G</td>
<td>Age-dependent slowness of movement</td>
<td>Degeneration of TH+ axons and tauopathy, TH+ cell atrophy, reduction of extracellular DA.</td>
<td>LRRK2: the causative gene for PARK8 type PD with autosomal dominant inheritance</td>
<td>Promising model for the investigation of PD pathogenesis and therapeutics</td>
<td>No loss of midbrain TH+ cells</td>
</tr>
<tr>
<td>Mitopark (TFam conditional knockout in mDA neurons)</td>
<td>Decreased locomotor Activity</td>
<td>Degeneration of DA neurons, STR:DA ( \downarrow ), intraneuronal inclusions</td>
<td>Respiratory chain deficiency</td>
<td>Learn secondary changes in degeneration</td>
<td>Complex breeding scheme</td>
</tr>
</tbody>
</table>

The most commonly used toxins are 6-OHDA and MPTP. They are used especially in preclinical studies of novel therapeutic agents. Table modified from (Kitamura et al., 2000; Betarbet et al., 2002; Schober et al., 2004; von Bohlen and Halbach 2005; Harvey et al., 2008; Ekstrand et al., 2009; Hunter et al., 2009; Li et al., 2009; Yue 2009).
denervation. Another striatal interneuron transmitter, GABA, is also affected.

When 6-OHDA is injected unilaterally, the contralateral side can be used as control (Ungerstedt 1971a). Unilateral injections of 6-OHDA produce a dopaminergic imbalance between the hemispheres. When animals are challenged with drugs acting on the dopamine system, an asymmetric circling behavior can be observed, the magnitude of which depends on the severity of the nigrostriatal lesion (Przedborski et al., 1995, Hefti et al., 1980, Ungerstedt and Arbuthnott, 1970). Administration of dopamine-releasing agents such as d-amphetamine creates a dopaminergic imbalance that favors the non-lesioned nigrostriatal projection and thus produces ipsilateral turning behavior, whereas the postsynaptic agonist apomorphine induces rotation contralateral to the lesioned side because of stimulation of denervation-induced upregulated D₂ receptors in the denervated striatum (Ungerstedt 1971b) (Fig. 4). A unilateral lesion can be quantitatively assayed and thus a notable advantage of this model is the ability to assess the anti-parkinsonian properties of new drugs. There are also other clinically relevant tests, such as limb asymmetry (cylinder test) and forelimb placing test that do not require the administration of dopaminergic drugs to detect abnormalities as in the rotation test (Meredith and Kang, 2006).

Mechanism of 6-OHDA neurotoxicity The exact cellular mechanism of 6-OHDA-induced neurodegeneration is not well defined, but a role for oxidative stress has been firmly established (Sachs and Jonsson, 1975). Inside the neurons, 6-OHDA accumulates in the cytosol, generating ROS and inactivating biological macromolecules.

![Figure 4](image-url)  
**Figure 4.** Turning behavior can be quantified and it correlates with lesion severity. LS (left striatum), RS (right striatum), L SN (left substantia nigra), R SN (right substantia nigra). Figure modified from Deumens et al. (2002).
by generating quinones that attack nucleophilic groups (Cohen 1984). It has been reported that 6-OHDA-induced degeneration involves the generation of hydrogen peroxide and hydroxyl radicals in the presence of iron (Sachs and Jonsson, 1975). The fact that intranigral injection of iron (Fe++) produces neurotoxic effects similar to those of 6-OHDA may suggest a role for iron in 6-OHDA-induced degeneration (Ben-Shachar and Youdim, 1991). Several lines of evidence suggest that 6-OHDA impairs mitochondrial function by various mechanisms. 6-OHDA has been shown to impair mitochondrial function in cells by inhibiting mitochondrial respiratory chain complex I, creating more ROS, reducing ATP levels and finally leading to cell death (Sachs and Jonsson, 1975, Blum et al., 2001). Although 6-OHDA induces its neurotoxic effect mainly by inhibiting mitochondrial complex I (Sachs and Jonsson, 1975) and producing oxidative species, it also induces endoplasmic reticulum (ER)-stress (Chen et al., 2004), e.g., in SH-SY5Y cells where it activates glycogen synthase kinase 3β (GSK3β), an ER stress-responsive component (Chen et al., 2004). In PC12 cells, 6-OHDA induces ER stress and activates UPR (Ryu et al., 2002).

Both necrotic and apoptotic mechanisms of cell death occur in response to 6-OHDA (Ochu et al., 1998, Walkinshaw and Waters, 1994, Hanrot et al., 2006). Several studies have suggested a role for the mitochondrial caspases in 6-OHDA-induced apoptosis, which initiates the activation of the main effector caspases 3 and 7 (Han et al., 2003). However, the crucial downstream targets of caspase 3 activation have not been clearly identified. It has been shown that PKCδ has a role in 6-OHDA induced neurotoxicity (Hanrot et al., 2006).

It is not clear how well the 6-OHDA-induced dopaminergic toxicity corresponds to the neurodegeneration observed in PD (Dauer and Przedborski, 2003). The 6-OHDA model does not mimic all the clinical and pathological features characteristic of PD and therefore the face validity is not optimal (see chapter 2.4.1). 6-OHDA does not affect other brain regions (such as the LC), nor does it result in the formation of the cytoplasmic inclusions (Lewy bodies) seen in PD. On the other hand, predictive validity is high for 6-OHDA, since it has been a significant experimental paradigm in animal studies demonstrating efficacy of virtually all antiparkinsonian medication used today in clinic. The acute nature of the experimental models differs from the progressive degeneration of the dopaminergic neurons in PD. 6-OHDA model (when 6-OHDA is given intrastriatally) represents PD better than MPTP model since it causes a progressive degeneration of dopaminergic neurons. However, whereas recent genetic discoveries have lead to a number of different genetic models of PD, none of these show the degeneration of dopaminergic neurons typical of PD, so the 6-OHDA model remains a useful and important tool for non-clinical research of PD.

2.4.3 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

MPTP was discovered in the early 1980’s as a by-product of attempted pethidine synthesis. It caused severe PD-like symptoms in drug abusers (Langston et al., 1983). In humans and monkeys, MPTP produces an irreversible and severe parkinsonian syndrome characterized by all of the motor features of PD, including tremors, rigidity, slowness of movement, postural instability and freezing. Levodopa has beneficial effects in MPTP-treated monkeys. MPTP crosses the blood-brain
barrier and is metabolized in astrocytes to its active metabolite, 1-methyl-4-phenyl-2,3-dihydropyridium ion (MPP⁺), by MAO-B. The MPTP model is mainly used in non-human primates and mice, because rats are almost completely insensitive to MPTP-induced neurodegeneration due to a lack of the MAO-B isoform (Blum et al., 2001, Boyce et al., 1984, Sahgal et al., 1984). The resistance of rats to MPTP toxicity may also be related to the different sequestration of MPP⁺ by rats in comparison to mice and monkeys (Schmidt and Ferger, 2001). In contrast to humans and non-human primates, dopamine terminals are destroyed only transiently in mice that are exposed to single dose of MPTP. Spontaneous recovery occurs weeks to months after lesioning (Date et al., 1993). MPP⁺ is selectively taken up into dopaminergic neurons via its affinity for the DAT, and it is thus selective for dopamine neurons (Javitch et al., 1985). Once in the neuron, MPP⁺ blocks mitochondrial respiration by binding to mitochondrial enzyme complex I (Nicklas et al., 1985, Tipton and Singer, 1993). The blockade of mitochondrial respiration causes an energy crisis, which leads to cell death (Blandini et al., 2000).

MPTP damages the dopaminergic pathway in a topographical pattern similar to that seen in PD, including greater loss of neurons in the SNpc than the VTA and preferential loss of neurons in the ventral and lateral segments of the SNpc (Varastet et al., 1994). As in human PD pathology, dopaminergic neurons that contain neuromelanin are more susceptible to MPTP-induced degeneration in monkeys (Herrero et al., 1993). However, the monkey MPTP model lacks two characteristic features of PD. Neurons are not consistently lost from other monoaminergic nuclei (such as the LC), which is typical for PD (Forno et al., 1986), and Lewy bodies have not been found in the brains of MPTP-treated patients or monkeys (Forno et al., 1993).

2.4.4 Genetic parkinsonian models

Human genetic linkage studies have identified several genes in familial forms of PD. Transgenic models have been created in which the mechanisms of neuropathology in PD and the function of PD-linked genes such as α-synuclein, LRRK2, DJ-1, Parkin and PINK1 (Table 1) (Polymeropoulos et al., 1997, Kruger et al., 1998, Devine and Lewis, 2008, Goldberg et al., 2005, Harvey et al., 2008, von Bohlen and Halbach, 2005) have been studied (see also 2.1.1). Several of the genes found to cause familial parkinsonism have been associated to mitochondrial function. Moreover, toxins that inhibit the mitochondrial respiratory chain have been discovered to cause dopamine cell death. Furthermore, mitochondrial dysfunction is established in the pathophysiology of PD (Bender et al., 2006). The MitoPark mouse model of PD is based on the inactivation of mitochondrial transcription factor A (Tfam) in dopamine neurons (Ekstrand et al., 2007). MitoPark mice, which have respiratory chain-deficient dopamine neurons, express the degeneration of dopamine nerve terminals, progressive loss of dopamine neurons and motor impairments (Ekstrand et al., 2007, Ekstrand and Galter, 2009, Terzioglu and Galter, 2008). Transgenic models offer an opportunity to study mutant genes, (or combination of mutant genes) and their roles in the pathophysiology of the disease. They also provide a platform for evaluating potential genetic and pharmacological therapies.

Although mice with a mutation in several parkinsonism-related genes have been generated, many of them do not develop a parkinsonian phenotype (Table 1). Because the etiology of PD likely involves a combination of environmental
and genetic factors, the mice with known genetic mutations associated with PD in combination with a toxin treatment (e.g. MPTP) may provide better models for understanding the etiology and progression of PD (Kitamura et al., 2000).

2.5 Neurotrophic factors and midbrain dopaminergic neurons

Neurotrophic factors are secreted proteins that produce their trophic effects by activating specific receptors on the neuronal cell surface. The first neurotrophic factor, NGF, was discovered in the early 1950s by Rita Levi-Montalcini, Stanley Cohen and Victor Hamburger (Levi-Montalcini 1987). According to the original neurotrophic factor hypothesis, target tissues produce trophic molecules in limited amounts, and thus only neurons that have successfully established synaptic contacts with their target cells are able to survive. Neurons lacking this trophic support are presumed to die by apoptosis. Neurotrophic factors secreted by target tissues are taken up by nerve terminals and retrograde transport transmits them to the cell bodies (Oppenheim 1991). After the activation of survival-promoting signal pathways, apoptotic death is inhibited. In addition to survival promoting effects, neurotrophic factors stimulate neurite outgrowth, differentiation of neurons, maintenance of neuronal contacts and adult plasticity. Although the target-derived neurotrophic factor hypothesis is well established in the peripheral nervous system, whether NTFs participate in the development and maintenance of the CNS is less clear.

Trophic factors in the CNS are grouped into families based on structural homology, receptors and common signal transduction pathways. The neurotrophin family characterized in mammals includes NGF, brain-derived neurotrophic factor (BDNF) (Barde et al., 1982), neurotrophin-3 (NT-3) (Ernfors et al., 1990, Hohn et al., 1990) and neurotrophin 4/5 (NT-4/5) (Berkemeier et al., 1991, Hallbook et al., 1991). These factors, which are similar in sequence and structure, all derive from a common ancestral gene. Neurotrophins are important regulators of neural survival, development, function and plasticity (Huang and Reichardt, 2001).

GDNF family includes GDNF (Lin et al., 1993), neurturin (NRTN), artemin (ARTN, also known as neublastin or enovin) and persephin (PSPN). Similarly to the neurotrophin family, these factors are synthesized as precursor proteins that are cleaved into mature proteins. The members of the GDNF family belong to the TGF-β superfamily, because they contain seven cysteine residues in the same relative spacing and have a similar three-dimensional structure as other members of the superfamily (Ibanez 1998).

Dopamine neuron development

Dopamine neurons located in the ventral mesodiencephalon emerge halfway during embryonic development. The development of mesodiencephalon dopamine (mDA) neurons involve both extrinsic and intrinsic signals needed to initiate the dopaminergic phenotype. Among the extrinsic signals, instructive signals such as the growth factors Shh (Sonic Hedgehog), FGF-8 (fibroblast growth factor 8) and TGF-β are involved in determining the site of mDA neuron generation in the midbrain area in mice (Smidt and Burbach, 2007). Cross-talk between extrinsic signals and transcription factors induces mitotic neuronal precursor cells in the midbrain region to become post-mitotic dopaminergic neurons (Andressoo and Saarma, 2008).

It has been suggested that the target-derived factor concept could explain dopaminergic neuron survival and death.
in the SN during development and in degeneration (Krieglstein, 2004). During development, most neurons are initially overproduced, and their final number is determined by an ontogenetic programmed cell death process that is regulated by target-derived neurotrophic factors during target tissue innervation (Andressoo and Saarma, 2008). It seems that neurotrophic factors do regulate the number of dopaminergic neurons (Krieglstein, 2004). Five established neurotrophic factors can be counted as candidate target-derived neurotrophic factors for nigrostriatal dopaminergic neurons: GDNF, NRTN, BDNF, neurotrophin-4 (NT-4) and FGF-2 (Krieglstein, 2004). However, in vivo analysis of the respective NTF knockout mice have shown that only GDNF and its receptor RET regulate the number of dopamine neurons during development (Kramer et al., 2007, Mijatovic et al., 2007, Pascual et al., 2008).

In addition, other neurotrophic factors and growth factors such as vascular-endothelial growth factor A (VEGF-A), TGF-beta and erythropoietin have been shown to support the survival of embryonic dopamine neurons in vitro and to protect these neurons in animal models of PD (Andressoo and Saarma, 2008). BDNF was the first protein identified that directly supports the survival of dopamine neurons in vivo. However, only GDNF and NRTN have well-established neurorestorative properties in the nigrostriatal dopaminergic system in animal models of PD (I, II).

2.6 The GDNF family of neurotrophic factors

GDNF was originally purified from cell culture medium of a rat glioma cell line (B49), and it was shown to increase dopamine uptake and promote the survival and morphological differentiation of dopaminergic neurons in embryonic midbrain cultures (Lin et al., 1993). The GDNF family ligands (GFLs) include GDNF and its structurally related factors NRTN (Kotzbauer et al., 1996), ARTN (Baloh et al., 1998) and PSPN (Milbrandt et al., 1998).

All members of the TGF-β family function as homodimers. GFLs belong to the cysteine-knot protein family and they are synthesized as a prepro-form of 211 amino acids from which the signal peptide (pre) is cleaved upon secretion. The mature protein consists of 134 amino acids. The molecular mass of a glycosylated GDNF monomer is 16 KDa (Lin et al., 1993).

GFLs signal through a transmembrane receptor tyrosine kinase (RET) by first binding to their cognate GDNF family receptor α (GFRα) receptor. GFRα proteins are attached to the plasma membrane by a glycosyl phosphatidylinositol (GPI) anchor and have three globular cysteine-rich domains (except for GFRα4, which only has two). GFRα receptors determine the ligand specificity of the GFRα-RET complex (Airaksinen and Saarma, 2002). GDNF binds to GFRα1, NRTN binds to GFRα2, ARTN binds to GFRα3 and PSPN activates RET by binding to GFRα4 (Fig. 5). Moreover, cross binding can occur. Following binding of a dimeric GFL to a GFRα co-receptor, the GFL-GFRα complex dimerizes and activates RET. The activation of RET induces further downstream signaling via multiple pathways including the MAP kinase, Src-kinase, PI3K-Akt and phospholipase Cγ pathways. In addition to RETs, neural cell adhesion molecules (NCAMs) are alternative receptors for GFLs (Paratcha et al., 2003).

GDNF is the most potent survival-promoting factor for dopamine neurons both in vitro and in vivo. In addition to its effects on midbrain dopaminergic neurons, GDNF supports the survival of several different neuronal populations in both the central and the peripheral nervous
systems (Airaksinen and Saarma, 2002). GDNF is a potent trophic factor for spinal motoneurons (Henderson et al., 1994), central noradrenergic neurons (Arenas et al., 1995) and central serotonergic neurons (Cass 1996). In addition, GDNF promotes the survival and regulates the differentiation of many peripheral neurons, such as sympathetic, parasympathetic, sensory and enteric neurons (Airaksinen and Saarma, 2002). The expression patterns of the mRNAs for GDNF and its receptors (GFRα1 and RET) in the striatum and SN respectively suggested a role for GDNF as a target-derived neurotrophic factor for dopamine neurons (Choi-Lundberg and Bohn, 1995, Trupp et al., 1997). Thus, although the neurotrophic effects of GDNF are by no means limited to the mesencephalic dopamine system (Lapchak 1996), GDNF and its close relative NRTN are of particular interest because of their potent neuroprotective and neurotrophic effects on the lesioned adult dopamine system.

NRTN was originally identified through its ability to promote the survival of cultured sympathetic neurons (Kotzbauer et al., 1996). NRTN has also been shown to promote the survival of retinal ganglion cells (Koeberle and Ball, 2002), enteric neurons (Heuckeroth et al., 1998), sensory and parasympathetic neurons (Rossi et al., 1999) and forebrain cholinergic neurons (Golden et al., 2003). In addition, NRTN has been shown to have neuroprotective (Heger et al., 1998, Rosenblad et al., 1999, Akerud et al., 1999) and neurorestorative (Rosenblad et al., 1999, Oiwa et al., 2002) effects on dopaminergic neurons in in vivo models of PD. In dopaminergic neurons, NRTN signals to RET via GDNF cognate co-receptor GFRα1 (Cacalano et al 1998).

PSPN has been shown to promote the survival of ventral midbrain and motor neurons, but in contrast to GDNF and

---

*Figure 5* Communication of GDNF family ligands with their receptors. A homodimeric GFL binds a GPI-linked GFRα co-receptor, and the GFL-GFRα complex induces dimerization of two RET molecules leading to autophosphorylation of their tyrosine residues in the intracellular domain. All ligands activate RETs via specific GFRα co-receptors. Arrows indicate the preferred ligand-receptor interactions that are known to exist physiologically in vivo. Modified from Airaksinen and Saarma (2000).
NRTN, it appears to have no effect on the peripheral nervous system (Milbrandt et al., 1998). Dopaminergic neurons and motoneurons do not express the principal PSPN receptor GFRα4 (Lindahl et al., 2000, Lindahl et al., 2001) and PSPN may activate RET via GFRα1 (Sidorova et al., 2010). ARTN promotes the survival of sympathetic and sensory neurons (Balogh et al., 1998) and has no effects on CNS neurons.

2.6.1 Expression of GDNF in the brain

GDNF mRNA is widely expressed in various regions of the developing and adult brain as well as outside the nervous system (Choi-Lundberg and Bohn, 1995, Trupp et al., 1995). GDNF mRNA and protein are expressed in the globus pallidus, striatum, nucleus accumbens, olfactory tubercle, cerebellum, hippocampus, thalamus and olfactory bulb in rat (Choi-Lundberg and Bohn, 1995, Schaar et al., 1993, Springer et al., 1994) (see Table 2).

Whether GDNF is a target-derived neurotrophic factor for midbrain dopaminergic neurons is unclear (Andressoo and Saarma, 2008). Oo and Burke (1997) have shown in rats that the death of dopaminergic neurons is mainly postnatal. In fact, programmed cell death (PCD) of midbrain dopaminergic neurons occurs largely postnatally in two phases, peaking at postnatal day 2 (P2) and P14 (Oo and Burke, 1997). Intrastriatally injected GDNF is transported in a retrograde manner to the dopaminergic cell bodies in the SNpc (Tomac et al., 1995a). Furthermore, GDNF mRNA is expressed in the targets of nigral dopamine neurons. GDNF mRNA is expressed at higher levels in the early postnatal striatum than in the adult striatum (Choi-Lundberg and Bohn., 1995, Schaar et al., 1993). However, expression studies of GDNF mRNA in the SN have been controversial, as some groups have found GDNF mRNA expression in the SN and suggested autocrine and paracrine role for GDNF (Oo et al., 2005, Cho et al., 2004, Golden et al., 1998), while others have not detected GDNF mRNA at all in the SNpc (Nosrat et al., 1997). Oo and colleagues (2005) showed that medium-sized striatal neurons express GDNF mRNA and protein during the first postnatal week. They further indicate that these neurons are the source of the striatal GDNF that regulates the natural cell death event in SNpc dopamine neurons during this developmental period (Oo et al., 2005, Oo et al., 2003). These authors also found that GDNF was localized in the striatal neuropil, in line with Lopez-Martín et al., (1999). Many groups have found GDNF protein in axon terminals and in fibers in principal striatal efferent target nuclei such as the SNpc, GP and EP. This expression suggests the possibility that GDNF of striatal origin provides trophic support not only in a retrograde fashion to afferent projection systems but also in an anterograde fashion to striatal efferent targets. Consistent with this idea, anterograde transport of GDNF from the STR to the SNpc and into the GP has been reported (Georgievská et al., 2004, Kordower et al., 2000).

There have been contradictory results concerning GDNF expression in animal models of PD. Some have observed increase in the striatal levels of GDNF protein and mRNA after 6-OHDA lesions (Nakajima et al., 2001, Zhou et al., 2000), whereas others have not detected any changes in either 6-OHDA- (Smith et al., 2003) or MPTP models (Inoue et al., 1999). It has been speculated that altered GDNF expression levels may be involved in the pathology of PD. Indeed, upregulation of GDNF mRNA levels in the putamen of PD patients with a loss of nigral neurons...
has been detected, but no changes in GFRα1 or RET mRNA levels were observed. This suggests that the extensive loss of dopaminergic neurons in the SN may induce compensatory increase in the expression of target-derived GDNF, but it does not alter the GDNF receptor system (Backman et al., 2006).

2.6.2 Physiological role of GDNF in the midbrain dopaminergic system

Homozygous GDNF knockout mice die shortly after birth, thus only the prenatal development of these mice can be studied (Airaksinen et al., 1999). At birth, GDNF−/− mice show normal numbers of catecholaminergic neurons in the SN and LC, thus suggesting that endogenous GDNF is not necessary for the embryonic development of dopaminergic systems in the brain (Sanchez et al., 1996, Granholm et al., 1997, Moore et al., 1996, Boger et al., 2006, Pichel et al., 1996). Similar to GDNF knockout mice, neither RET nor GFRα1- homozygous knockout mice (who also die at birth) show any reduction in the number of dopaminergic neurons at birth (Airaksinen and Saarma, 2002). Heterozygous (GDNF+/−) mice are fertile and develop normally (Boger et al., 2006). However, GDNF deficit seems to have an impact on adults, as at 20 month of age, the GDNF−/− mice show fewer TH positive SN neurons (Boger et al., 2006) and increased susceptibility to neurotoxins (Boger et al., 2007). Moreover, Airavaara and colleagues have found increased striatal extracellular dopamine levels in GDNF−/− mice, suggesting compensatory alterations in their dopamine system (Airavaara et al., 2004).

The development of conditional knockout mice has enabled the physiological roles of RET (Kramer et al., 2007) and GDNF (Pascual et al., 2008) to be studied. Studies of conditional RET knockout mice showed that Ret is not necessary for the embryonic or postnatal development and maturation of the nigrostriatal system (Kramer et al., 2007). Compensatory neuroprotective mechanisms may have been activated during embryonic development (Sanchez et al., 1996, Granholm et al., 1997, Moore et al., 1996, Boger et al., 2006, Pichel et al., 1996). On the other hand, in aged conditional RET knockout mice, midbrain dopamine neurons are lost and dopamine terminals degenerate, suggesting that RET has a role in the long term maintenance of the nigrostriatal system (Kramer et al., 2007). Pascal and colleagues (2008) generated an inducible GDNF knockout mouse to suppress GDNF expression in adulthood, hence avoiding the establishment of developmental compensatory modifications that may mask the true physiological role of GDNF in the adult nervous system (Pascual et al., 2008). The adult conditional GDNF knockout mice showed progressive hypokinesia and a selective decrease of brain TH mRNA accompanied by catecholaminergic neuronal death, affecting especially the LC, SN and VTA. GABAergic and cholinergic pathways appeared to be unaffected (Pascual et al., 2008). These data are in line with previous findings (Oo et al., 2003) that GDNF is essential for the postnatal survival of dopaminergic neurons. However, the actual role of endogenous GDNF in the adult midbrain remained largely unclear. Analysis of inducible GFRα1- and RET knockout mice should clarify the discrepancy.

2.6.3 The effect of exogenous GDNF on intact dopaminergic system

GDNF administration causes an increase in tissue dopamine and dopamine turnover in intact rodents after a single bolus
injection at doses of 1-100 µg, into either the SN (Hudson et al., 1995, Hebert et al., 1996, Martin et al., 1996b, Hebert and Gerhardt, 1997), the striatum (Horger et al., 1998, Martin et al., 1996b) or the lateral ventricle (Martin et al., 1996a, Lapchak et al., 1997). These effects are associated with enhanced spontaneous and amphetamine-induced locomotor activity (Horger et al., 1998, Hudson et al., 1995, Martin et al., 1996b, Hebert and Gerhardt, 1997, Martin et al., 1996a, Kobayashi et al., 1998). In microdialysis experiments, GDNF increases the striatal release of dopamine and its metabolites (Hoffer et al., 1994, Lapchak et al., 1997, Beck et al., 1995). GDNF also induces sprouting in dopaminergic neurons (Hudson et al., 1995). However, the molecular mechanisms whereby GDNF increases dopamine function and/or levels have not been elucidated.

Multiple studies have addressed the effects of GDNF on TH expression. Rosenblad et al. (2003) showed that TH levels decrease (without effects on other dopaminergic markers) in vivo as a result of viral delivery of high levels of GDNF into the intact rat nigrostriatal system. Palfi and colleagues reported an increased number of TH-positive neurons in striatum after GDNF delivery to the primate striatum (Palfi et al., 2002). Acute striatal GDNF administration (100 µg) was shown to increase TH phosphorylation at Ser31 in the rat STR and SN (Salvatore et al., 2004), indicating that GDNF-induced increases in dopamine function are associated with a sustained increase in TH phosphorylation. The authors speculated that the molecular changes occurring both in the STR and in the SN suggest that dopamine biosynthesis capacity increases in both regions, particularly in the SN (Salvatore et al., 2004).

On the other hand, down-regulation of TH expression and aberrant sprouting of dopaminergic fibers were seen after sustained lentiviral expression of GDNF in the STR (Georgievsk et al., 2002). Down-regulation of TH protein levels has been suggested to reflect a compensatory effect in response to continuous GDNF stimulation of dopamine neurons that is mediated by a combination of overactivity at dopamine synapses and direct GDNF-induced action on TH gene expression. This compensatory mechanism has been proposed to maintain long-term dopamine neuron function (Georgievsk et al., 2002).

Taken together, the available experimental data show that exogenously administered GDNF may, in addition to promoting neurite survival and outgrowth, have additional effects on intact dopamine neuron function, either by stimulating dopamine release and function or by regulating the expression of the TH enzyme.

2.6.4 The effects of exogenous GDNF on the lesioned dopaminergic system

GDNF injections in animal PD models

The most likely mechanism by which GDNF attenuates toxin-induced behavioral impairments is through its trophic actions on midbrain dopamine neurons in the SN.

The initial studies with GDNF utilized direct bolus injection of the trophic factor either into the STR, the lateral ventricle or directly into the SN. Most of these studies were carried out in the 6-OHDA model in rats or in the MPTP model in monkeys or mice. If administered before or around the time of dopamine neuron injury, GDNF prevents the loss of nigral DA neurons, the reduced of dopamine nerve terminal density, the depleted dopamine levels in the striatum and the lesion-induced motor deficits (Tomac et al., 1995b, Beck et al.,
1995, Kearns and Gash, 1995, Sauer et al., 1995). Indeed, GDNF has been shown to protect nigrostriatal dopaminergic neurons in animal models of PD when delivered either to the SN, if administered before or around the time of neural injury (Kearns and Gash, 1995, Winkler et al., 1996, Kearns et al., 1997) or to the STR (Shults et al., 1996, Kirik et al., 2000). Kirik and colleagues have postulated that the site-dependence of the GDNF-induced effect suggests local action, because the protective effect is seen only when both GDNF and the toxin are injected in the same location (Kirik et al., 2000a). GDNF protects dopaminergic axon terminals only if it is applied in the STR (Tomac et al., 1995b, Kirik et al., 2004). Thus, when GDNF is administered to the STR, both cell bodies in the SN and TH-positive fibers in the STR are protected (Kirik et al., 2000a, Kirik et al., 2004, Kirik et al., 2000b). This neuroprotection is accompanied by enhanced motor function, which is a more relevant barometer of trophic factor efficacy. When GDNF is administered directly to the SN, it protects nigral cell bodies and causes some local axonal sprouting, but it does not preserve striatal TH levels or motor function (Kirik et al., 2000a).

In the treatment of PD, neurotrophic factors could be used as neurorestorative therapy in patients with severely compromised nigrostriatal dopamine nerves. Only two neurotrophic factors of the GDNF family, i.e., GDNF (Hoffer et al., 1994, Bowenkamp et al., 1995, Aoi et al., 2000, Rosenblad et al., 1998) and NRTN (Rosenblad et al., 1999), have well-established neurorestorative effects when they are given after intrastrital 6-OHDA injection in rats. Recovery of the nigrostriatal dopamine system after 6-OHDA lesioning has been reported (Hoffer et al., 1994, Bowenkamp et al., 1995, Schneider and Peacock, 1998), when GDNF was injected into the SN. However, only intrastrital injection of GDNF induces functional recovery of the dopaminergic system and sprouting of dopaminergic fibers after striatally injected 6-OHDA (Aoi et al., 2000, Rosenblad et al., 1998). Rosenblad and colleagues administered GDNF (10 μg) by repeated injections into the lesioned striatum for three weeks starting four weeks after 6-OHDA lesioning. This intrastrital GDNF treatment restored striatal dopamine innervation (as monitored by autoradiography of dopamine uptake sites), prevented the slow degeneration of nigral neurons and completely normalized the motor impairments (Rosenblad et al., 1998). These observations are in accordance with other observations (Tomac et al., 1995b) in MPTP-treated mice, in which the MPTP-induced degeneration of dopamine terminals in the STR was unaffected by injection of GDNF over the SN, whereas GDNF injected directly into the STR induced significant recovery of striatal dopamine levels and TH-positive innervation in MPTP-treated animals.

Single injections of GDNF have also been studied in unilateral and bilateral non-human primate MPTP models of PD (Gash et al., 1995, Gerhardt et al., 1999, Costa et al., 2001). GDNF administered either intranigrally, intracaudally or intraventricularly induced functional recovery. GDNF treatment elevated tissue levels of dopamine and its metabolites, enhanced dopamine turnover and stimulated dopamine release in the SN and GP. In addition to its trophic actions on midbrain dopamine neurons in the SN, exogenous GDNF may also affect some other system within the basal ganglia. In a recent study (Xin et al., 2008), it was reported that the globus pallidus is critical
in GDNF-induced functional changes in PD monkeys.

**GDNF infusions in animal PD models**

GDNF protects and restores the nigrostriatal dopaminergic system in non-human primate PD models, many of which have involved intraventricular GDNF administration (Gash et al., 1996, Gerhardt et al., 1999, Costa et al., 2001). Recognizing that the effect of bolus injections of GDNF either into the SN, STR or lateral ventricle was relatively short-term, more long-term ways of getting GDNF into target regions have been explored. Catheters attached to subcutaneous pumps have been implanted in the putamen and programmed to deliver GDNF over several weeks in aged monkeys (Ai et al., 2003). This delivery method was efficient at diffusing GDNF to several areas away from the injection site such as the rostral putamen, the caudate nucleus and the globus pallidus. Significant improvement in the overall motor performance of the aged monkeys compared to controls was seen after GDNF administration (Maswood et al., 2002). Levels of dopamine significantly increased in the caudate nucleus and the globus pallidus in response to GDNF administration. Similarly, encouraging results were seen with GDNF in a PD model using MPTP, where GDNF was continuously infused to either the lateral ventricle or putamen (Grondin et al., 2002). GDNF infusion improved motor function and promoted the survival of dopaminergic neurons.

Long-term sustainable ways of getting GDNF into target regions have been explored also in rats. Chronic GDNF infusion has been shown to restore nigrostriatal dopaminergic neurons in rats (Kirk et al., 2001, Lu and Hagg, 1997). Lu and Hagg (1997) showed that intranigral infusion of GDNF prevents 6-OHDA-induced death of SNpc neurons at a dose of 3 µg/24 h, and a half-maximal effect was seen with a dose of 1 µg/24 h. Neither of these doses was able to reverse the lesion-induced reduction in TH immunoreactivity. In Kirik et al. (2001), intrastriatal GDNF (3 µg/24 h) administered for four weeks starting two weeks post-lesion modified motor behavior only during the infusion, and poor motor performance returned when the infusion ended (Kirik et al., 2001). In contrast, intraventricular infusion of GDNF completely blocked the degeneration of neurons in the SN and also had a long-lasting effect on behavior (Kirik et al., 2001). In the study of Kirik et al. (2001), GDNF at 3 µg/24 h was given for four weeks starting two weeks post-lesion, but the dose of 6-OHDA was 28 µg divided into four deposits. The lesion was thus larger than in the (Rosenblad et al., 1998) study, which may partially explain why intrastriatal GDNF infusion had only a weak and transient effect.

**2.6.5 GDNF administration in Parkinsonian patients**

Translating successful animal studies into effective clinical therapy for PD has proven difficult. As neurotrophic factors do not cross the blood-brain barrier, they need to be administered directly to the brain. Because the human brain is almost 1000 times larger than the rat brain, the distribution properties of growth factors are more important. These considerations place high demands on the delivery method utilized in neurotrophic factor therapy. To date, only a few clinical trials have been undertaken in which GDNF was directly delivered to PD brains (Ramawamy et al., 2009). These trials have produced mixed results. An initial double-blind clinical trial examining the effects of GDNF injected
into the cerebral ventricles failed to show a clinical benefit and produced serious side effects (Nutt et al., 2003), but further analysis suggested that GDNF may not have properly diffused into the target tissues (the SN and putamen), because of poor trophic factor penetration through the ventricular wall into the parenchyma of the basal ganglia (Kordower et al., 1999). Intraputaminal infusions resulted in clear clinical improvement in two open-label studies (Gill et al., 2003, Slevin et al., 2005, Patel et al., 2005), but in a randomized, double-blind clinical trial no symptomatic improvement compared to a placebo was reported (Lang et al., 2006). In addition to questions regarding the efficacy of GDNF therapy, two important safety concerns have arisen surrounding GDNF administration. About 20% of patients who participated in the double-blind study developed neutralizing antibodies to GDNF, likely occurring due to leakage of GDNF from the pump upon refilling (Slevin et al., 2007). Cerebellar toxicity was also detected in nonhuman primates after high doses of GDNF (100 μg/putamen/day infused unilaterally) (Sherer et al., 2006). The inconclusive outcomes of the clinical trials listed above may be due to limited distribution of GDNF in the brain (Salvatore et al., 2006), because in an experimental model GDNF was shown to bind to heparin sulphate proteoglycans on the cell surface or in the extracellular matrix (Piltonen et al., 2009). Based on current studies, three factors are critical for successful therapy of PD: site-specific delivery of GDNF into the nigrostriatal dopaminergic system, its distribution to the target tissues and the dose of GDNF.

In a phase I, open-label clinical trial, the NRTN gene was delivered into the STR using an adeno-associated virus 2 (AAV2) vector (Marks et al., 2008). However, after 12 months, no significant clinical improvement was observed. Recent study assessed the efficacy and safety of stereotaxic surgery with injections of AAV2-NRTN versus sham surgery in 58 patients with advanced PD in a double-blind, randomised trial. There was no significant difference in the primary endpoint in PD patients treated with AAV2-NRTN as compared to the sham operated controls (Marks et al., 2010). Moreover, three patients in the AAV2-NRTN group and two in the sham control group developed tumours. Also, serious adverse effects occurred in one third of AAV-NRTN treated PD-patients. Although the intraputaminal AAV-NRTN gene delivery was not better than sham surgery according to the UPDRS motor score at 12 months, continued study of a gene therapy that primarily targets the dopaminergic system is worthwhile. Future studies will clarify the effects of AAV2-NRTN in a new clinical study where SN will be targeted directly and higher dose of AAV2-NRTN will be injected to the putamen, and patients will be followed longer time periods (Marks et al., 2010).

In a recent study, convection-enhanced delivery of GDNF and NRTN was used to determine the tissue clearance from the rat STR and the response of the dopaminergic system to a single infusion (Hadaczek et al., 2010). Convection-enhanced delivery is an interstitial drug delivery technique that can produce drug concentrations within brain tissue and brain tumor orders of magnitude higher than that of systemic administration (Bobo et al., 1994). Infused GDNF (both 3 and 15 μg) resulted in a sharp increase in striatal GDNF levels followed by a rapid decrease between days three and seven (Hadaczek et al., 2010). However, IHC revealed GDNF in the brain even 14 days after administration. Moreover, dopamine turnover was enhanced for more than seven days after a GDNF infusion,
revealing that GDNF is a very stable protein. The effects of NRTN were similar. These persistent effects of a single dose of GDNF and NRTN on dopamine metabolism suggest that intermittent administration by means of a convection-enhanced delivery may not only ensure accurate and efficient spread of trophic factors within the target, but may also provide more uniform tissue levels (Hadamzec et al., 2010). This technique may eliminate the need for a continuously active implantable pump and allow infusions to be conducted at intervals. Indeed, this approach may offer one alternative approach to the delivery of NTFs in PD patients.

2.7 The CDNF/MANF family of neurotrophic factors

2.7.1 Mesencephalic astrocyte-derived neurotrophic factor (MANF)

The founding member of the CDNF/MANF family, MANF, was originally derived from the culture medium of rat mesencephalic type-1 astrocyte cell line 1 (VMCL1) (Petrova et al., 2003). The active protein was found in part homologous to a predicted human arginine-rich protein (ARP) of 234 amino acids (Petrova et al., 2003, Shridhar et al., 1996). MANF was initially named ARMET (arginine-rich, mutated in early stage tumors) because it was thought to contain an arginine-rich region at its N-terminus (Shridhar et al., 1996). ARP (or ARMET) is highly conserved and encodes a novel, ubiquitously expressed arginine-rich protein from chromosomal band 3p21.1. The human ARP gene contains an amino-terminal arginine-rich region, and mutations within it are commonly observed in pancreatic cancer as well as many other cancers (Shridhar et al., 1996, Shridhar et al., 1997). However, subsequent studies have shown that MANF protein does not contain the arginine-rich region, apparently because this region is not synthesized in vivo (Petrova et al., 2003, Lindholm et al., 2008). The protein encoded by ARP has been renamed MANF (Petrova et al., 2003), reflecting its survival promoting activity.

Human preMANF consists of 179 amino acids (aa). A 21-amino acid long signal sequence is cleaved off resulting in the mature MANF protein of 158 amino acids (Petrova et al., 2003). Human MANF is a 20-kDa glycosylated protein; however secreted glycosylated MANF has not been detected in later studies (Lindholm et al., 2008). MANF was shown to selectively support the survival of embryonic rat nigral dopaminergic neurons over that of GABAergic or serotoninergic neurons in vitro (Petrova et al., 2003). This selectivity may be due to MANF’s ability to maintain the stable expression of TH in dopaminergic neurons but not that of glutamic acid decarboxylase (GAD) and tryptophan hydroxylase in GABAergic and serotoninergic neurons, respectively (Petrova et al., 2003). Recombinant human MANF was also tested in cerebellar granule, superior cervical ganglion (SCG) sympathetic neuron, nodose ganglion and dorsal root ganglion (DRG) sensory neuron cultures, but it had a modest survival-promoting effect only on DRG neurons (Petrova et al., 2003). Zhou and colleagues showed that exogenous MANF increases the frequency of GABA A receptor-mediated inhibitory postsynaptic currents (IPSCs) and decreases the paired pulse ratio in dopamine neurons in vitro (Zhou et al., 2006). MANF also increases the frequency of spontaneous and miniature IPSCs. GABA-mediated inhibition of dopamine cell firing may contribute to the neuroprotective effect of MANF on dopaminergic neurons. MANF protein has also recently been shown to protect
neurons against cerebral ischemia in rats. Administration of MANF into the cerebral cortex before middle cerebral artery occlusion (MCAO) reduces the infarction volume and rescues markers of apoptotic cell death in the ischemic cortex, possibly by inhibiting cell necrosis/apoptosis in the cerebral cortex (Airavaara et al., 2009). Moreover, pretreatment with AAV-MANF reduce the volume of cerebral infarction and facilitates behavioral recovery in a rat model of stroke (Airavaara et al., 2010). In line with these findings, MANF protein prevents cell death in cardiac myocyte cultures (Tadimalla et al., 2008). MANF is transiently upregulated in the adult rat brain after global forebrain ischemia and status epilepticus (Lindholm et al., 2008). MANF may thus regulate neuronal survival and synaptic plasticity in the hippocampus and the cortex (Lindholm et al., 2008).

2.7.2 Cerebral dopamine neurotrophic factor (CDNF)

CDNF was discovered using bioinformatic tools and then biochemically characterized (Lindholm et al., 2007). The human and mouse CDNF protein consist of 161 amino acids, while preCDNF is 187 amino acids long (Fig. 6). Human CDNF is 18 kDa, a glycosylated protein, and like MANF, it is evolutionarily highly conserved. Together, CDNF and MANF proteins form a novel family of conserved secreted factors with eight cysteine residues of similar spacing, suggesting a similar protein fold.

Vertebrates have separate CDNF and MANF genes, whereas invertebrates, including the nematode Caenorhabditis elegans and the fruit fly Drosophila melanogaster, have a single, homologous CDNF/MANF gene (Palgi et al., 2009). Human CDNF shows 59% amino acid identity with human MANF, 49% identity with D. melanogaster and 46% identity with C. elegans MANF proteins (Lindholm et al., 2007), whereas human MANF shows 53% amino acid identity with D. melanogaster and 50% identity with the C. elegans MANF proteins (Lindholm et al., 2008). Exogenous CDNF has no effect on cultured sympathetic neurons or motoneurons in vitro.

Both CDNF and MANF are secreted from transiently transfected cells (Lindholm et al., 2007, Palgi et al., 2009, Lindholm et

![Figure 6](image_url). Schematic illustration of amino acid sequences of CDNF and MANF. Pre corresponds to the signal sequence, and the numbers show the length of the polypeptides in amino acids. Conserved cysteine residues are shown by yellow vertical bars. Modified from Lindholm and Saarma (2010): this material is reproduced with permission of John Wiley & Sons, Inc.
Table 2. mRNA and protein expression of CDNF, MANF and GDNF in selected areas of developing and adult rodent brains.

<table>
<thead>
<tr>
<th>Developing brain region</th>
<th>CDNF mRNA P1, P10 (a)</th>
<th>CDNF protein P10 (a)</th>
<th>MANF mRNA P10 (b)</th>
<th>MANF protein P1 (b)</th>
<th>GDNF mRNA Pre- and postnatal (f, g, h, i)</th>
<th>GDNF protein P1-P21 (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cpu</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SNpc</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNpr</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAc</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hipp</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>GP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thalamus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Adult brain region</th>
<th>CDNF mRNA P1</th>
<th>CDNF protein P1</th>
<th>MANF mRNA P1</th>
<th>MANF protein P1</th>
<th>GDNF mRNA P1-P21</th>
<th>GDNF protein P1-P21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cpu</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SNpc</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNpr</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAc</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Hipp</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>GP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thalamus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

“-” indicates not detected, “±” indicates barely detected, “+” indicates detected; SNpc = substantia nigra pars compacta, SNpr = substantia nigra pars retcullate, Cpu = caudate putamen, NAc = nucleus accumbens, Hipp = hippocampus, GP= globus pallidus. Table combined from the following references: a: (Lindholm et al., 2007), b: (Lindholm et al., 2008), c: (Golden et al., 1998), d: (Ikeda et al., 1999), e: (Trupp et al., 1997), g: (Nosrat et al, 1996), h: (Strömberg et al., 1993), f: (Golden et al., 1999).
Unlike neurotrophins and GDNF family ligands, CDNF and MANF proteins seem to lack a prosequence, suggesting that enzymatic cleavage is not necessary for their activation (Lindholm and Saarma, 2010).

2.7.3 Expression of CDNF and MANF

CDNF is expressed in several neuronal and non-neuronal mouse and human tissues, including the mouse embryonic and postnatal brain (I). Based on RT-PCR and western blot analyses, relatively high levels of CDNF transcripts were observed in the mouse heart and in the mouse and human skeletal tissue and testis. Moreover, CDNF transcripts were detected in embryonic and postnatal stages and in adult brain regions, including striatum (I). By immunohistochemical analysis, CDNF specific signal was observed in the hippocampus, cerebral cortex, striatum and in the Purkinje cells of cerebellum. The expression of CDNF is discussed further in the results-chapter (5.2). The expression pattern of MANF resembles that of CDNF, being widely expressed in the brain and non-neuronal tissues. MANF transcripts have been detected in the embryonic (E13, E15), postnatal (P9, P14) and adult (P60) mouse brain and in all human brain regions analyzed, including the SN and STR. In adult mice, MANF mRNA is highly expressed in the cerebral cortex, hippocampus, cerebellar Purkinje cells and the midbrain, as well as in some dopaminergic neurons (Lindholm et al., 2008). In the brain, MANF mRNA is localized mainly in cells with large nuclei, suggesting neuronal expression. MANF mRNA has also been detected in non-neuronal cells of the choroid plexus. Immunohistochemical and in situ hybridization analyses of MANF are in

Figure 7. Crystal structure of CDNF-ΔC (A) and mature MANF (B). Cartoon illustration of CDNF-ΔC and MANF color coded from blue to red. Disulphide bridges are shown as sticks with carbon in magenta and sulfur in yellow. Alpha-helices are numbered starting from the N-terminus. Modified from Parkash et al. (2009) with permission of the publisher.
accordance with the mRNA analysis. MANF protein is expressed in cerebral cortex, hippocampus and cerebellar Purkinje cells of adult mice. MANF protein has also been detected in the SN and in STR of P1 and P10 and adult mice (Lindholm et al., 2008). In the adult SN, MANF protein has been detected in TH positive neurons. MANF protein and mRNA are expressed at high levels in the developing cerebral cortex of E12.5 and E14 mouse embryos, respectively. In addition, MANF protein has also been detected in the developing midbrain of the E16 mouse embryos. MANF protein has also been detected in the embryonic PNS, including dorsal root ganglia, trigeminal and superior cervical ganglia of E17 mouse embryos. CDNF and MANF are also expressed in several non-neuronal tissue such as in testis, liver and heart (Lindholm et al., 2007, Lindholm et al., 2008).

2.7.4 Structure of CDNF and MANF

Although MANF and CDNF are structurally related, they resemble none of the known neurotrophic factors and display no sequence homology to other neurotrophic factors such as GDNF and BDNF (Parkash et al., 2009). Parkash and colleagues have solved the crystal structures of both the mature human MANF protein and the N-terminal domain of CDNF (Parkash et al., 2009). Structural analysis revealed that CDNF and MANF consist of two domains: an N-terminal domain that is structurally similar to a saposin-like lipid-binding domain, which suggests that MANF and CDNF may bind to lipids or membranes and a presumably unstructured C-terminal domain, with an intradomain cysteine bridge in a CXXC motif (Parkash et al., 2009) (Fig. 7). Specific roles of the N-terminal and domains and C-terminal domains of CDNF and MANF remain to be studied.
3. AIMS OF THE STUDY

Growth factors have attracted much attention as a potential way of prevent ongoing neurodegeneration and to restore degenerating dopaminergic neurons in Parkinson’s patients. The use of growth factors with neurotrophic capacity has opened a new era of exploration of restorative therapies for Parkinson’s disease. Because GDNF and NRTN have shown modest and contradictory results in clinical trials, it is important to search for novel growth factors. The purpose of the present study was to characterize the distribution and in vivo effects of two novel neurotrophic factors (CDNF and MANF) in a 6-OHDA model of PD. There was no information about their in vivo effects when we started this study.

The specific aims of this study were:

1. To characterize the neuroprotective and restorative effects of single intrastriatal injections of CDNF and MANF proteins in the rat 6-OHDA PD model.

2. To study the effects of chronic intrastriatal infusions of CDNF and MANF in the rat 6-OHDA PD model.

3. To investigate the distribution and transportation profiles of intrastriatally injected CDNF and MANF in rats.
4. MATERIALS AND METHODS

4.1 DNA and RNA methods

4.1.1 RNA isolation and reverse transcription

Human RNAs derived from post-mortem brain regions and mouse RNAs were isolated as described in detail in (I). Human RNAs of peripheral tissues and total brain were purchased from Clontech. First-strand cDNAs were synthesized with SuperScriptII reverse transcriptase (Invitrogen), according manufacturer’s instructions.

4.1.2 PCR, cloning and DNA sequencing

RT-PCR analysis of CDNF mRNA expression in mouse and human tissues was carried out using primers described in (I). CDNF cDNA was cloned into vector (pCRII-TOPO, Invitrogen), and verified by sequencing. Human CDNF was further subcloned into expression vectors: pCR3.1 (without tags) and pcDNA3.1 vector (with carboxy-terminal V5 and 6xHis tags, Invitrogen).

4.1.3 In situ hybridization

Mouse Cdnf cDNA comprising nucleotides 409-823 of GenBank sequence Acc. No. NM_177647 cloned to pCRII was used to prepare antisense and control-sense cRNA probes. Probes were generated by in vitro transcription using 32P-labelled UTP (Amersham Biosciences). Hybridization temperature was 52°C, washing was performed at 55°C. Hybridized sections were exposed in NTB-2 emulsion (Kodak) for 5-6 weeks.

4.2 Cell culture methods

4.2.1 Primary neuronal cultures

Cultures for and the survival assays with rat E14 motoneurons and mouse P1 SCG neurons or mouse E14 and E15 DRG neurons were performed essentially as described in (I).

4.2.2 Transfection

Transfection of CDNF vector into mammalian cell lines with Lipofectamine 2000 reagent (Invitrogen) was carried out according to manufacturer’s instructions (I). Mammalian expression vectors are described in detailed in (I).

4.3 Animals

Male Wistar rats were used in all experiments. The rats were purchased from the Harlan Laboratories, Netherlands. The rats weighed 250-280 g at the beginning of the experiments. The animal room was kept at an ambient temperature of 20-23°C. Food pellets (Harlan Teklad Global diet, Netherlands) and tap water were available throughout the experiment ad libitum. The rats were housed in groups of three to four under a 12 hour light-dark cycle. The animals were housed separately during the days of surgery (I-III) and installation of osmotic minipumps (III). The animals were returned to their home cages 24 hour after lesioning or minipump detachment. The animals received tramadol at 1 mg/ml (4.2) after each surgery for post-operative pain relief. The experimental design was approved by either the Committee for Animal Experiments at the University of Helsinki or by the chief veterinarian of the
county administrative board (permissions HY 1406 and ESLH-2007-06679/Ym-23)

4.4 Drugs and treatments

D-amphetamine hydrochloride (2.5 mg/kg, University Pharmacy, Helsinki, Finland) and tramadol hydrochloride (Tramal, Orion Pharma, Finland) were dissolved in 0.9% NaCl solution (saline). Desipramine hydrochloride (Sigma-Aldrich, MO, USA) was dissolved in sterile water. 6-OHDA hydrochloride (Sigma-Aldrich, MO, USA) was dissolved in ice-cold saline containing 0.02% ascorbic acid. Tramadol was administered subcutaneously (s.c.), amphetamine and desipramine were given intraperitoneally (i.p.), whereas 6-OHDA and neurotrophic factors (4.2.1) were given intrastriatally. Sodium pentobarbital (90 mg/kg, i.p, Orion Pharma, Finland) was used to anesthetize rats in perfusions. The doses refer to the base form, and the injection volume of 1 ml/kg was used.

4.4.1 Production and purification of rhCDNF and MANF

Proteins were produced and purified as described: CDNF (I) and MANF (Lindholm et al., 2008, II). GDNF was purchased for the neuroprotection and neurorestoration studies from PreproTech Inc. (Rocky Hill, NJ, USA) (I, II) and from Amgen Inc. (Thousand Oaks, CA, USA) (III). For the diffusion studies (III), GDNF was purchased from ProSpec-Tany TechnoGene Ltd., (Rehovot, Israel).

4.4.2 Protein stability (III)

Protein stability (0.25 μg/μl) was assessed by incubating proteins for 2-14 days in buffer solution at 37°C (III: Fig. 1). After the incubation, the proteins were analyzed with Western blot. Western blotting was performed essentially as described (Gyrfas et al., 2010). The protein samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blotting with anti-CDNF (1:750), anti-MANF (1:750) or anti-GDNF antibodies (1:1000) or stained with coomassie blue.

4.4.3 Preparation of 125I-labeled neurotrophic factors (II, III)

GDNF (1.5 μg), CDNF (1.0 μg) and MANF (1.0 μg) were iodinated with 125I-1Na using the lactoperoxidase method as described in detail (II).

4.5 Stereotaxic surgery

4.5.1 Stereotaxic operation

For the neuroprotection studies (I, II), stereotaxic surgery was performed in two sessions ((I, Kearns et al., 1997), Fig. 8) using isoflurane anesthesia (4.5% during induction and 3% during maintenance). The animals were placed in a stereotaxic frame (Stoelting). The skull was exposed and burr holes were made using a high-speed dental drill. Injections were performed using a 10 μl Hamilton microsyringe at a rate of 1 μl/min. At the completion of each injection, the needle was kept in place for 2 min to minimize backflow of the solution. All control animals were administered 4 μl of phosphate buffered saline (PBS) or 10 mM citric acid in the left STR using coordinates relative to the bregma and dura [anterior/posterior (A/P), +1.0; lateral/medial (L/M), +2.7; dorsal/ventral (D/V), -5] according to the atlas of Paxinos and Watson (Paxinos G. and Watson C., 1997). Trophic factor-treated animals received either 1, 3 or 10 μg of recombinant human CDNF (I), 3, 10 or 30 μg of human MANF (II) or 10 μg of recombinant human GDNF (I, II)
(PeproTech) in 4 µl of buffer delivered to the striatum. CDNF and MANF were dissolved in PBS and GDNF in citrate buffer. In the second surgery 6 h later, all animals were given 8 µg of 6-OHDA in 4µl in the left striatum. Before the 6-OHDA injections in neuroprotection (I, II), neurorestoration (I, II) and chronic infusion studies (III), desipramine (15 mg/kg, i.p.; 1 ml/kg) was administered to prevent the uptake of 6-OHDA into noradrenergic nerve endings in order to protect these nerve terminals from destruction.

In the neurorestorative studies (I, II), the rats received a unilateral injection of 20 µg/4 µl of 6-OHDA. Four weeks later, the rats were given an intrastriatal injection of CDNF (I), MANF (II), GDNF (10 µg in 4 µl of buffer), or vehicle. The time course of the neurorestorative experiments is given in Figure 8.

Iodinated trophic factors (125I-MANF, 125I-CDNF and 125I-GDNF) were injected into the left striatum to coordinates (A/P, +1; L/M, +2.7; D/V, -4) according to the rat brain atlas (Paxinos and Watson, 1997). Transport of the proteins was assessed 24 h after the injections based on autoradiograph and radioactive (counting with gamma spectrometer) detection of 125I-labeled trophic factors in brain sections.

To study the distribution of MANF and GDNF after a single injection (II), 10 µg of protein (MANF or GDNF) diluted in 4 µl of PBS/citric acid was injected unilaterally into the rat STR according to the same brain coordinates as mentioned above.

In the chronic infusion paradigm (III), animals received unilateral stereotaxic injections of a total of 20 µg of 6-OHDA in two deposits (10 µg/ 4 µl each) in the left STR using coordinates relative to the bregma and dura (A/P, +1.6, L/M +2.2, D/V -5 and A/P, -0.4, L/M +4, D/V, -5). A lesion-control group served as a reference for the analysis of the extent of dopamine neuron degeneration following the lesion at the time when the infusion pumps were implanted. At two weeks post-lesion, the rest of the animals were stereotaxically implanted with a brain infusion cannula (Alzet brain infusion kit, DirecCorporation, Cupertino, CA, USA) connected to a mini-osmotic pump (Alzet model 2002) via 2.5-cm-long catheter tubing. The pumps were filled with recombinant human CDNF or MANF (0.125, 0.25 or 0.375 µg/µl), recombinant human GDNF (0.25 µg/µl) or phosphate buffered saline (PBS) pH 7.4 (vehicle-treated group). Before insertion, the pumps were allowed to reach a steady-state infusion rate (0.5 µl/h) by incubation in sterile saline at 37°C overnight. The tip of the infusion cannula was placed in the left STR between two 6-OHDA injection sites. The infusion coordinates were: A/P +1, L/M +2.7, D/V -5. The infusion cannula was secured to the skull with two stainless steel screws and zinc polycarboxylate cement (Aqualox; VOCO, Germany). The pumps were placed into a subcutaneous pocket between the shoulder blades. The pumps were removed under anesthesia two weeks later. The design of the experiment is presented in Fig 8.

In order to study the brain tissue penetration of the NTFs and their effect on non-lesioned brain, CDNF, MANF and GDNF (3 µg/24 h) was infused into intact rat striatum (same coordinates as above) for 3, or 14 days (Alzet osmotic pumps, model 1007D or 2002). After the infusion period, the rats were perfused and their brains were processed for immunohistochemistry. The diffusion volume was assessed following three and fourteen days of infusion in brain sections cut in series of six and immunohistochemically stained with antibodies for CDNF, MANF or GDNF. The area of diffusion in each section was
measured and the diffusion volume was estimated using the Cavalier Estimator function in the Stereo Investigator platform according to (II).

4.6 Rotational behavior

D-amphetamine (2.5 mg kg⁻¹, i.p.) was used to induce rotational activity in rats with a unilateral lesion of nigrostriatal dopaminergic nerves. In the neuroprotection studies (I, II) the rotational behavior was measured for 2 h at two and four weeks post-lesion, and in the neurorestoration studies (I, II), behavioral tests were carried out 1 week before (that is, 3 weeks after 6-OHDA injection) and 2, 4, 6 and 8 weeks after the growth

**Figure 8.** Experimental design for neuroprotection (A), neurorestoration (B) and continuous infusion studies (C) (I, II, III).
Materials and methods

factor injections, as described previously (Ungerstedt and Arbuthnott., 1970) (I, II).
In the chronic infusion studies, rotational behavior was recorded 1, 4, 6, 8, 10 and
12 or 14 weeks post-lesion. Drug-induced rotational activity of the rats was monitored
in automatic rotometer bowls [Colbourn Instruments (I, II); MED Associates (II,
III)]. Following a habituation period of 30 min, a single dose of d-amphetamine was
injected intraperitoneally. The rotation sensor recorded full (360°) clockwise and
clockwise uninterrupted turns for a period of two hours, and ipsilateral rotations were assigned a positive value.

4.7 Immunohistochemistry

4.7.1 Tissue preparation

The rats (or mice for CDNF expression studies) were anesthetized with sodium
pentobarbital and perfused transcardially with PBS followed by 4% paraformaldehyde
in 0.1 M sodium phosphate buffer (pH 7.4) as described in detail in (I, II). The brains
were removed, post-fixed for 4 h and stored in sodium phosphate buffer containing
20% sucrose at 4°C. Serial coronal frozen sections at 40 μm depth (or 17 μm for
CDNF expression studies) were cut on a sliding microtome. Six sets of sections were
collected in a cryoprotectant solution (0.5 M phosphate buffer pH 7.4, 30% glycerol and
30% ethylene glycol) and stored at -20°C until immunohistochemical processing.
Free-floating sections were processed for immunohistochemistry as described
previously (I-III).

4.7.2 Immunohistochemistry

Tyrosine hydroxylase immunohistochemistry. In brief, after endogenous
peroxidase activity quenching and rinses in PBS, the sections were pre-incubated with
normal horse serum/0.3% Triton X-100 in PBS to block nonspecific staining. Thereafter,
the sections were incubated overnight at room temperature with a 1:2,000 dilution
of biotinylated mouse anti-TH (Millipore Bioscience Research Reagents). This was
followed by incubation with a 1:200 dilution of biotinylated horse anti-mouse (Vector
Laboratories; BA2001) and by incubation with an avidin–biotin peroxidase complex
using the Elite ABC Vectastain kit (Vector Laboratories). The reactions were visualized
using 3,3-diaminobenzidine (DAB) as a chromogen.

CDNF, MANF and GDNF immunohistochemistry (II, III). Whole rat brains were cut
into crossections (40 μm) and stained with anti-MANF (1:300) anti-CDNF (1:10000,
ProSci, Poway, CA, USA) or anti-GDNF (1:3000, R&D systems, Minneapolis, MN, USA)
antibodies using the Vectastain Elite ABC kit and DAB. The CDNF and MANF antibodies
were produced as described earlier (I, II).

Neu N immunohistochemistry (I).
Adult male NMRI mice brain paraffin sections (8μm) were used. For double
immunofluorescence, mouse anti-NeuN
(1:500, MAB377, Chemicon) or mouse
anti-TH (1:2000, MAB318, Chemicon), goat
anti-mouse 569 Alexia and donkey anti-
rabbit 488 Alexia (1:400, Molecular probes)
antibodies were used with antibodies to
CDNF.

4.7.3 Quantification of immunoreactivity

The analysis of TH-positive cells, OD analysis
and volume of distribution measurements
were performed under blinded conditions
on coded slides.
**Stereological analysis of TH-positive cells.**
The optical fractionator method was used to estimate the number of TH-positive cells in the SNpc (West, Slomianka and Gundersen, 1991) (II). The entire SNpc was analyzed with the Stereo Investigator platform (MicroBrightField, Inc. VT, USA) attached to an Olympus BX51 microscope as described in detail (I, II). Cell numbers were expressed as the mean number per section. Cells were counted using the optical fractionator method in combination with the dissector principle and unbiased counting rules.

**Striatal fiber density measurements.** The optical densities (ODs) of the TH-positive fibers in the STR were determined from three coronal STR sections from each rat as described (I, II). The data is presented as a percentage of the intact side, which was defined as 100%.

**Volume of distribution.** The diffusion volume (II, III) was assessed in brain sections cut in series of six and immunohistochemically stained with antibodies for CDNF, MANF or GDNF. The area of diffusion in every section was measured and the diffusion volume was estimated using the Cavalier Estimator function on the Stereo Investigator platform according to (II).

4.8 Neurotrophic factor transportation studies (II,III)

4.8.1 Quantification of ²²⁵I-neurotrophic factors in CNS

The amount of intrastriatally administered neurotrophic factors in different brain structures was determined after perfusion. The brain was removed from the skull; the hippocampus, SN, STR, and cortex were dissected out; and the wet tissue was weighed. The results are expressed as counts per minute per milligram of wet weight.

4.8.2 Emulsion autoradiography

Coronal paraffin sections of the STR, frontal cortex and SN were dipped in emulsion (Kodak) and exposed for eight weeks. The slides were developed and counterstained with hematoxylin.

4.8.3 Autoradiographic analysis of the distribution of ²²⁵I-neurotrophic factors

Rats receiving intrastriatal injections of ²²⁵I-MANF (5–10 ng), ²²⁵I-CDNF (5–10 ng) or ²²⁵I-GDNF (5–10 ng) were perfused for 24 h after stereotaxic injections. Coronal paraffin sections (7 μm thick) were juxtaposed against autoradiography film (Kodak Biomax MS) for four weeks.

4.9 Statistical analysis

The results were analyzed using one-way ANOVA followed by Tukey–Kramer’s post hoc test. A one-way ANOVA for repeated measures followed by Tukey–Kramer’s post hoc test was used to analyze the results of the behavior studies (xy-graphs) and the diffusion profile studies. Student’s t test was used to statistically analyze total protein diffusion volume. The results are expressed as mean± SEM and are considered significant at p < 0.05.
5. RESULTS

5.1 CDNF- an evolutionarily conserved protein

CDNF was found by bioinformatic tools. Human and mouse expressed sequence tag (EST) sequences homologous to MANF cDNA, which encoded a novel protein of 187 amino acids, were discovered. This previously unknown protein was originally named conserved dopamine neurotrophic factor, and the protein was later re-named to cerebral dopamine neurotrophic factor, CDNF. Full-length CDNF cDNAs from human and mouse brain RNA were cloned by using PCR with reverse transcription (RT-PCR). It was found that MANF and CDNF form a novel CDNF/MANF family of conserved secreted factors with eight cysteine residues of similar spacing (I). Additionally, it was discovered that predicted secondary structure of human and mouse CDNF and MANF is dominated by \( \alpha \)-helixes.

Analyses of genome and EST sequences from various organisms revealed that vertebrates, including the zebrafish *Danio rerio* and frog *Zenopus ternationalis*, have orthologous genes for both CDNF and MANF, whereas the invertebrates, such as *D. melanogaster* and *C. Elegans* have a single ancestral MANF/CDNF gene orthologue (I). This suggests that MANF and CDNF encoding genes are highly concerved in evolution. Indeed, human CDNF shows 59% amino acid identity with human MANF, 46% identity with *C. elegans* and 49% identity with *D. melanogaster* MANF proteins (I).

Secretion and post-translational modification of CDNF protein was studied by cloning human CDNF full length cDNA into a mammalian expression vector. CDNF was secreted from transiently transfected HEK 293T cells (I).

5.2 CDNF expression in neuronal and non-neuronal tissue

CDNF is expressed in several tissues of mouse and human, including the mouse embryonic and postnatal brain (I). CDNF mRNA was detected in the caudate nucleus, SN and putamen (STR) of adult humans and mice (I) (Table 2). CDNF mRNA was also expressed in these areas in postnatal (P1, P10) mice (I). CDNF mRNA levels in the embryonic brain were low and not easily detectable by in situ hybridization techniques. Additionally, relatively strong CDNF mRNA signals were detected in the hippocampus and thalamus of P1 and P10 mice and in the olfactory bulb of P1 mice. CDNF was also found in the thalamus of P10 mice but not in other brain regions at P10. Interestingly, rather high levels of CDNF transcripts were found in the testis and skeletal muscle of human and mouse and in mouse heart.

In line with the in situ hybridization data, immunohistochemical analysis indicated widespread expression of CDNF protein in P1, P10 and adult brains. In the adult cerebellum cortex, CDNF protein was expressed in neurons. In the SN, CDNF did not co-localize with TH, suggesting that CDNF is not expressed in the dopaminergic neurons. Relatively high levels of CDNF protein were detected in the Purkinje cells of the cerebellum and in regions of the LC (I). In agreement with the results of *Cdnc* mRNA expression in non-neuronal tissues, high amounts of CDNF protein were detected in the adult mouse skeletal muscle, heart and testis by western blotting (I).
5.3 Testing of survival promoting activity of CDN on primary neuronal cultures (I)

CDNF (100ng/ml) did not promote the survival of E14 and E15 mouse dorsal root ganglion (DRG) neurons or mouse P1 superior cervical ganglion (SCG) sympathetic neurons in culture. NGF had a clear survival promoting effect on DRG neurons at concentration of 100 ng/ml and on SCG neurons at concentration 10 ng/ml. CDNF (0.1-100 mg/ml) had no survival promoting activity on the E14 rat motoneurons in vitro.

5.4 Neuroprotective and restorative effects of CDN (I)

In the neuroprotection studies, a single intrastriatal injection of CDN (10 μg) significantly reduced amphetamine-induced turning behavior at two (p < 0.01) and four weeks (p < 0.05) post-lesion (Study I, Fig. 9a). Consistent with this result, the number of TH-positive cells in the SN and the density of TH-positive fibers in the striatum were significantly higher four weeks post-lesion in 6-OHDA-lesioned rats pretreated with CDN (96% of the intact side, p < 0.001 for TH-positive cells and; 75% of the intact side p < 0.001 for TH-positive fibers) compared to rats treated with vehicle (65% for the TH-positive cells and 55% for TH-positive fibers) (Table 3, Fig. 9b). GDNF (10 μg) significantly reduced amphetamine-induced rotation at two weeks post-lesion (p < 0.01) but not at four weeks post-lesion. The neuroprotective effect of GDNF was similar to that of CDN. GDNF also significantly protected TH-positive cells (93%, p < 0.01) and TH-positive fibers (69%, p < 0.01). There was a dose-response in the neuroprotective effects of CDN. Clear neuroprotection was observed with 3 μg but not with 1 μg.

In the neurorestoration studies, intrastriatal injection of CDN (10 μg) restored the function of dopaminergic neurons in the SNpc and prevented their degeneration. CDN significantly reduced amphetamine-induced rotational behavior at 12 weeks post-lesion compared with the control group (Table 5.2, Study I, Fig. 9c). Furthermore, when the rotations at 3, 6, 8, 10 and 12 weeks post-lesion were summed, treatment with CDN or the positive control GDNF differed significantly from vehicle treatment (1604 rotations, p < 0.001 for
Results

CDNF; 1673 rotations, \( p < 0.001 \) for GDNF and 2942 rotations for the vehicle-treated controls. The number of TH-positive cells in the SNpc (measured as a percentage of the cell count of the intact side) was higher in rats treated with CDNF (58%, \( p = 0.0629 \)) and in rats treated with GDNF (57%, \( p < 0.05 \)) than in rats treated with vehicle only (26%) (Table 4).

5.5 Neuroprotective and restorative effects of MANF (II)

In neuroprotection studies, MANF dose-dependently prevented 6-OHDA-induced destruction of dopamine neurons as evaluated by TH immunohistochemistry in the SNpc and STR and by measuring rotational behavior at two and four weeks post-lesion. There were no statistical differences at two weeks post-lesion; the maximum protective effect was observed with 10 \( \mu \)g MANF at four weeks post-lesion \( (p < 0.05) \). The effect was less prominent at MANF concentrations of 3 and 30 \( \mu \)g and with GDNF at 10 \( \mu \)g (Fig. 10a). The behavioral and morphological measurements correlated well with each another. At 10 \( \mu \)g, MANF significantly protected TH-positive cells in the SNpc compared to the vehicle treated controls (14% loss vs. 34% loss of TH-positive cells, \( p < 0.01 \)). MANF also protected TH-positive cells at 3 \( \mu \)g and at 10 \( \mu \)g, but there were no statistical differences (Table 3, Fig. 10b). MANF at 10 \( \mu \)g was also able to protect fibers in the STR (26% loss in OD), but this did not differ statistically from vehicle treatment (36% loss in OD). GDNF (10 \( \mu \)g) protected TH-positive cells in the SNpc (12%, loss of TH-positive cells, \( p < 0.01 \)) and TH-positive fibers in the STR (32% loss in OD). Neither the MANF (3 or 30 \( \mu \)g) nor GDNF (10 \( \mu \)g) treated groups showed statistical differences in OD analyses (Table 3).

In neurorestoration studies, intrastriatal MANF (10 \( \mu \)g) was able to restore the functional activity of the nigrostriatal dopaminergic system when

Table 3. Neuroprotective effects of CDNF and MANF in a partial lesion model of PD. The number of TH-positive cells in the SNpc and the density of TH-positive fibers in the STR in the lesioned side relative to the intact side (=100%)

<table>
<thead>
<tr>
<th>Pretreatment 6h before 6-OHDA (8 ( \mu )g)</th>
<th>TH-positive cells (per cent of the intact side)</th>
<th>TH-positive fibers (per cent of the intact side)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDNF 1 ( \mu )g</td>
<td>↔</td>
<td>—</td>
</tr>
<tr>
<td>CDNF 3 ( \mu )g</td>
<td>↑↑↑↑</td>
<td>↑↑↑↑</td>
</tr>
<tr>
<td>CDNF 10 ( \mu )g</td>
<td>↑↑↑↑↑</td>
<td>↑↑↑↑</td>
</tr>
<tr>
<td>MANF 3 ( \mu )g</td>
<td>↑↑</td>
<td>↔</td>
</tr>
<tr>
<td>MANF 10 ( \mu )g</td>
<td>↑↑↑</td>
<td>↑</td>
</tr>
<tr>
<td>MANF 30 ( \mu )g</td>
<td>↔</td>
<td>↔</td>
</tr>
</tbody>
</table>

- indicates not measured, ↔ indicates no change in comparison with vehicle-treated rats (60-70% of the intact side), ↑ indicates 70-80% of the intact side, ↑↑ indicates 80-90% of the intact side, ↑↑↑ indicates 90-100% of the intact side. \( *p<0.05, **p<0.01, ***p<0.001 \) as analyzed using one-way ANOVA and a Tukey-Kramer post hoc test.
administered four weeks after 6-OHDA in the STR. The maximum effect was evident at 12 weeks post-lesion. Nonetheless, there were no statistical differences between the treatments. However, when the cumulative rotations at 3, 6, 8, 10 and 12 weeks post-lesion were summed, MANF and the positive control GDNF significantly differed from vehicle treatment (1926 rotations for MANF, \( p < 0.001 \); 2875 rotations for GDNF, \( p < 0.01 \) and 3860 rotations for the vehicle-treated controls) (Table 4, Fig. 10c). Consistent with results from the behavioral studies, MANF and GDNF were able to partially restore TH-positive cell bodies in the SNpc (59% for MANF and 59% for GDNF, measured as a percentage of the cell count of intact side) as compared to the vehicle-treated controls (47%) (Table 4; II).

### 5.6 Stability of the neurotrophic factors in buffer solution

The stability of CDNF, MANF and GDNF (0.25 \( \mu \)g/\( \mu \)l each) was studied by incubating proteins for 2, 4, 6, 8, 10, 12 and 14 days in PBS at 37°C (III: Fig.1). After incubation, protein samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and either analyzed by Western blotting or stained with Coomassie blue. No signs of protein degradation during the 2–14 days of incubation at 37°C were observed (III).

### 5.7 Chronic infusion of CDNF reduce amphetamine-induced ipsilateral rotations and protects TH-positive cell bodies in the SNpc and their fibres from degeneration (III)

In neurorestoration paradigm (see Fig. 8), continuous infusion of CDNF was able to protect nigrostriatal dopaminergic nerves from 6-OHDA-induced degeneration and restore the functional balance of the nigrostriatal neural circuit as assessed by morphological and behavioral analyses. The maximum functional effect of CDNF was observed with the dose of 3 \( \mu \)g/24 h (40 \( \mu \)g of protein over two weeks), while doses of 1.5 \( \mu \)g/24 h (20 \( \mu \)g of protein over two weeks) and 4.5 \( \mu \)g/24 h (60 \( \mu \)g of protein over two weeks) had no effect. A 14-day

![Figure 10](image_url)

**Figure 10.** The effect of intrastrital MANF on 6-OHDA-induced degeneration of dopaminergic neurons in a rat model of PD. (A) MANF (3, 10 or 30 \( \mu \)g) injected six hours before intrastrital 6-OHDA reduced amphetamine-induced rotation at two and four weeks postlesion. (B) In the same experimental setting, MANF also protected TH-positive cells in the SNpc. (C) MANF administered four weeks after 6-OHDA restored dopaminergic function as reflected in combined cumulative amphetamine-induced ipsilateral rotations measured at 3, 6, 8, 10 and 12 weeks post-lesion. \( * p < 0.05, ** p < 0.01, *** p < 0.001 \) as analyzed using one-way ANOVA and a Tukey-Kramer post hoc test.
Table 4. Neurorestorative effects of CDNF and MANF in a 6-OHDA model of PD. The number of TH-positive cells in the SNpc and the density of TH-positive fibres in the STR are presented.

<table>
<thead>
<tr>
<th>Treatment four weeks after 6-OHDA (20 µg)</th>
<th>TH-positive cells (per cent of the intact side)</th>
<th>Reduction of cumulative rotations during 12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDNF 10 µg</td>
<td>↑↑</td>
<td>↓↓.</td>
</tr>
<tr>
<td>MANF 10 µg</td>
<td>↑</td>
<td>↓.</td>
</tr>
</tbody>
</table>

↑ indicates a 10-20% increase in TH+ cells compared to the corresponding control, ↑↑ indicates a 20-40% increase in TH+ cells compared to the corresponding control. ↓ indicates a 50% reduction in cumulative rotations compared to the corresponding control, ↓↓ indicates a 55% reduction in cumulative rotations compared to the corresponding control.

* p < 0.05 analyzed using one-way ANOVA and a Tukey-Kramer post hoc test.

Continuous infusion of CDNF was also able to restore 6-OHDA-induced destruction of TH-positive neurons. The maximum effect of CDNF was observed with the dose of 3 µg/24 h (67% of the intact side, p < 0.01) but also the dose of 4.5 µg/24 h (58% of intact side p < 0.05) had a clear effect as compared with rats treated with vehicle (25% of the intact side). The effect with the lowest dose (1.5 µg/24 h) was less prominent (26% of the intact site), respectively. Consistently, CDNF at 3 µg/24 h was the most effective dose tested at protecting TH-positive fibers (10% loss in OD; p < 0.01, Study III Fig. 5A) as compared with vehicle-treated rats (38% loss in OD). Moreover, CDNF at 1.5 µg/24h (19% loss in OD, p < 0.01) and 4.5 µg/24 h (15% loss in OD, p < 0.01) differed significantly from the vehicle-treated control. The effect of GDNF in behavioral analyses and the ability of GDNF to restore TH-positive cells (to 52% of intact side in the SN) or fibers (17% loss in OD) was inferior to that of CDNF at 3 µg/24 h.

5.8 Lack of neurorestorative effect of chronic infusion of MANF in 6-OHDA model of PD (III)

Given as a continuous infusion, MANF, did not clearly reduce amphetamine-induced ipsilateral rotations. The maximum effect of MANF infusion was observed at 4.5 µg/24 h. However, the difference in rotations between the vehicle-treated group was not statistically significant. Although the dose of 4.5 µg/24 h was the most effective in behavioral experiments, this dose was inferior at protecting TH-positive cells in the SNpc and fibers in the STR (Study III Fig. 3C-D). The lowest dose of MANF (1.5 µg/24 h) was the most effective at protecting TH-positive cells in the SNpc (52% of the intact side) and fibers in the STR (35% loss in OD) as compared with vehicle-treated rats (40% for TH-positive cells, 40% loss OD). In rats treated with other doses of MANF (3 µg/24 h and 4.5 µg/24 h), the number of TH-positive cells in the lesioned side of the SN was reduced to 46% and 33% of the intact side, respectively. In the GDNF-infused groups, the number of TH-positive cells was reduced to 52% in the lesioned side of the SN as compared to intact side in the SN. There were no statistical differences
between the treatment groups and control groups in the TH-positive cell counts (Study III, Fig 4B). Neither the GDNF 3 μg/24 h, the MANF 3 μg/24 h nor the MANF 4.5 μg/24 h treatment groups (36%, 41% or 48% loss in OD, respectively) showed any differences with respect to the vehicle-treated group.

5.9 Effect of chronic infusion of CDNF and MANF on TH-immunoreactive neurons in SNpc and fibers in striatum in non-lesioned animals (III)

In non-lesioned rats treated with chronic infusion of CDNF, MANF or GDNF for two weeks (3 μg/24 h), the number of TH-positive cells relative to intact side in the SN was 113%, 101% or 79%, respectively (III: Fig 7A). The OD of TH-immunoreactive fibers in the striatum was identical after the chronic infusion of CDNF, MANF or GDNF in the non-lesioned rats (100%, 103%, 100%, respectively, III: Fig. 7B). There were no statistically significant differences in TH-immunoreactivity in non-lesioned rats treated with NTFs.

5.10 Distribution of intrastrial CDNF and MANF (II, III)

The distribution of MANF (10 μg) injected into the STR was studied 24 and 72 h after a single intrastrial injection. MANF protein was distributed throughout the STR and the frontal cortex after 24 h. The volume of distribution of MANF in the STR was significantly larger than that of GDNF; (49.5 ± 3.2 vs. 30.1 ± 5.8 mm³, respectively; P < 0.05, Student’s t-test). After 72 h, MANF protein was distributed to most of the ipsilateral STR and cortex, but the staining intensity was lower than after 24 h (data not shown). GDNF was detected throughout the STR 24 h after injection. No signal was detected in the cortical section. GDNF immunoreactivity was still present in the STR after 72 h (data not shown), although the staining intensity was lower than at 24 h.

The volume of distribution of CDNF, MANF and GDNF was measured after 3- and 14-day continuous infusions in the STR by IHC. Assessment of the volume of tissue containing CDNF, MANF or GDNF immunoreactivity revealed differences in their penetration through brain tissue after a 3-day continuous infusion but not after a 14-day infusion. After a 3-day continuous infusion, MANF protein was distributed through a larger volume than GDNF (p<0.05) or CDNF, and CDNF was distributed through a larger volume than GDNF. The distribution volumes were 29.2 ± 2.8 mm³ (MANF), 21.2 ± 3.7 mm³ (CDNF) and 15.3 ± 1.9 mm³ (GDNF) (III, Fig. 6A-E). The distribution profiles of the proteins differed significantly after the 3-day infusions (distance*treatment effect one-way ANOVA F (2,48) = 4.585, p<0.01). Following the 14-day continuous infusions, the distribution volumes were equal for all three neurotrophic factors (CDNF: 24.7 ± 2.7, MANF: 26.4 ± 1.8 and GDNF 25.6 ± 4.0). There were no significant differences in the distribution profiles after the 14-day infusions.

5.11 Transportation experiments (II, III)

Intrastrially injected of ¹²⁵I-CDNF was transported in a retrograde manner from the STR to the SN. The film autoradiography revealed signals in the ipsilateral cortical (olfactory bulb), striatal and SN section. Counting of radioactivity in brain tissue samples 24 h after the striatal injection of ¹²⁵I-CDNF confirmed the transport of ¹²⁵I-CDNF to the SN. The transport of ¹²⁵I-CDNF was dose-dependently inhibited by a 100-, 1,000- and
20,000-fold molar excesses of unlabeled CDNφ competitor (Study III, Figure 8). Like $^{125}$I-CDNF, $^{125}$I-GDNφ was also transported to the SN after intrastriatal injection. According to the direct radioactivity measurements, which were normalized to radioactivity in the STR (100%) about 1.9% of the intrastriatally injected $^{125}$I-CDNF and about 1.5% of intrastriatally injected $^{125}$I-GDNφ was detected in the SNpc. There were no significant differences between the iodinated protein groups or between the iodinated protein and unlabeled protein groups. The transport of neither $^{125}$I-CDNF nor $^{125}$I-GDNφ to the frontal cortical area was markedly affected by unlabeled trophic factor.

The transportation of $^{125}$I-MANφ was different than that of CDNφ. After intrastriatal injection, MANφ was preferentially transported to cortical areas (II). Results from emulsion autoradiography were in accordance with film autoradiography. Both film and emulsion autoradiography analyses revealed radioactive MANφ signals in the ipsilateral striatum and in the cortex but not in the SN. When radioactivity was measured in brain samples that were removed immediately after the sacrifice of rats, radioactivity in the SN was at background levels. There was five times more radioactivity in the frontal cortical area after $^{125}$I-MANφ injection than after $^{125}$I-GDNφ injection.

GDNφ was used as a positive control in the transport studies (II, III). In film and emulsion autoradiography, a strong signal was detected in the ipsilateral striatum and in the ipsilateral SN, demonstrating GDNφ transport from the striatum into the SN. By radioactivity counts, $^{125}$I-GDNφ was transported in a retrograde manner to the SN, and this transportation was partially inhibited in the presence of 100–400 fold molar excess unlabeled GDNφ, as reported previously (Tomac et al., 1995b, Lapchak et al., 1997).
6. DISCUSSION

The purpose of these studies was to clarify the neuroprotective and restorative effects of human CDNf and MANF in a rat unilateral partial 6-hydroxydopamine (6-OHDA) PD model using both behavioral (amphetamine-induced rotation) and immunohistochemical analyses. Also the distribution and transportation profiles of intrastratally injected CDNf and MANF in rats were investigated.

6.1 CDNf: a promising trophic factor in 6-OHDA PD model

In the present study a novel evolutionarily conserved protein, CDNf, was identified and biochemically and pharmacologically characterized (I). CDNf and MANF are secreted proteins that constitute a novel, evolutionarily conserved neurotrophic factor family expressed in vertebrates and invertebrates. The expression of Cdnf mRNA and protein was detected in adult and postnatal mouse striatum and SN, where axon terminals and cell somas of midbrain dopaminergic neurons are located. It can be speculated that CDNf might provide a local trophic support for dopaminergic neurons in the midbrain with paracrine mechanism. Striatal expression indicates that CDNf may support the function and survival of dopaminergic axon terminals.

In order to study the neuroprotective and neurorestorative effect of CDNf (I) on dopaminergic neurons, we used a partial lesion of the nigrostriatal pathway induced by an intrastriatal injection of 6-OHDA (Sauer and Oertel, 1994, Cadet and Zhu, 1992). This model provides an opportunity to investigate not only the ability of the treatments to protect dopamine neurons during the acute cell death phase, but also their effects on recovery and regeneration during the chronic phase of protracted degeneration (Björklund et al., 1997, Lee et al., 1996). The partial lesion model may also mimic the slow neurodegenerative process that occurs over a long time in the brains of Parkinson’s patients. MPTP is another neurotoxin that affects nigrostriatal dopamine neurons in mice, primates and humans and causes symptoms of PD (Jackson-Lewis and Przedborski, 2007). Although MPTP is also widely used toxin in PD research, it should be noted that the effects of single injection of MPTP on dopamine terminals are transient, and spontaneous regeneration occurs after weeks or months (Date et al., 1993). 6-OHDA creates a progressive and more stable lesion than MPTP, and thus we decided to use the 6-OHDA model.

The effects of NTFs were first studied in the neuroprotection paradigm, in which a trophic factor was injected 6 hours before 6-OHDA into the striatum. In the neurorestoration paradigm, the NTFs were injected into the same location in striatum as was 6-OHDA, which was given four weeks prior the NTF. In the neuroprotection paradigm, CDNf dose-dependently prevented the loss of TH-positive cells in the SN. The immunohistochemistry correlated well with behavioral results. Dose-response studies revealed that CDNf at 10 μg was the most effective, and therefore this dose was selected for the neurorestoration studies. In the treatment of PD, neurotrophic factors would be used in patients with severely degenerated nigrostriatal dopamine nerves. Thus, we also evaluated the effects of CDNf in a neurorestoration model of PD. In the neurorestoration experiment, CDNf was able to increase the number of TH-positive cells in the SN. The restorative effect of CDNf probably reflects the
recovery of neurons not fully destroyed at four weeks after the 6-OHDA injection. In both paradigms, CDNF induced behavioral recovery from unilateral lesion by restoring balance of movements in response to amphetamine and restored the function of midbrain dopaminergic neurons *in vivo* as efficiently as GDNF.

**6.2 MANF: neuroprotective and neurorestorative properties in the 6-OHDA PD model**

In the neuroprotection paradigm, MANF (10 µg) was able to reduce amphetamine-induced ipsilateral rotations even more efficiently than GDNF (II). MANF showed a bell-shaped dose–response with the most potent effect at the dose of 10 µg/rat. Similar dose-response properties have also been reported with GDNF (Shults *et al.*, 1996, Hou *et al.*, 1996). According to the morphological analyses, 10 µg of MANF protected TH-positive cells in the SN as effectively as GDNF, and it also protected TH-immunoreactive fibers in the STR.

In the neurorestoration paradigm MANF (10 µg) reduced amphetamine-induced turning behavior, but in contrast to the results of the neuroprotection studies, there was only a small increase in the number of TH-positive nerve cells in the SN. This may reflect indirect effects of MANF on other neural circuits controlling movement and/or circuits controlling the dopaminergic activity of the nigrostriatal tract. This will be discussed in more detail in Chapter 6.5.

All NTFs tested were more effective in the neuroprotection paradigm than in neurorestoration paradigm. This may be due to at least two reasons. First, the dose of 6-OHDA was 8 µg and 20 µg in the neuroprotection and neurorestoration experiments, respectively. Second, there is a high concentration of NTF (in the neuroprotection paradigm) during the time 6-OHDA is inducing its toxic effects. Indeed, $^{125}$I-CDNF and $^{125}$I-MANF were mostly found in the STR for at least 24 hours after injection. Under these conditions the neurons may be optimally protected and recovered from toxicity. The situation is different in the neurorestoration paradigm: 6-OHDA is no longer present and the degeneration has progressed significantly (Björklund *et al.*, 1997).

In principle, NTFs might compete with 6-OHDA in binding to DAT in the neuroprotection paradigm. It has been shown that GDNF increases the uptake of $^{[3]H}$dopamine in primary cultures of embryonic rat mesencephalon (Hou *et al.*, 1996), and thus it is unlikely that GDNF would block the function of DAT. CDNF and MANF do not presumably either prevent 6-OHDA to enter the dopamine cells since they were effective in the neurorestoration experiments. The NTFs may produce neuroprotective effect, because they prevent the ER-stress and complex I respiratory chain damages caused by acute 6-OHDA (Tadimalla *et al.*, 2008, Apostolou *et al.*, 2008). In addition to protection from the toxic effect of 6-OHDA, NTFs possess long-term effects on neuronal plasticity (Chen *et al.*, 2008). It is known that GDNF induces sprouting of dopaminergic neurons (Hudson *et al.*, 1995). Moreover, intrastratal GDNF has both acute and long-lasting pharmacological effects on dopamine neurons in adult animals (Kobayashi *et al.*, 1998). It stimulates locomotor behavior by activating dopamine D$_1$ and D$_2$ receptors as shown by studies when selective dopamine antagonists were used. Our results suggest that CDNF may restore axons and induce axon sprouting, induce synapse formation and stimulate the function of dopamine neurons. Whether or not CDNF induces sprouting, will be determined in the future.
6.3 Continuous infusion of CDNF and MANF

Continuous intracranial infusion of GDNF has been effective against 6-OHDA induced damage to the dopaminergic system in previous studies (Kim et al., 2001, Lu and Hagg, 1997). A two-week infusion of CDNF was able to halt the neurotoxin-induced degeneration of TH-positive cell bodies in the SNpc and the loss of TH-positive fibers in the STR (III). CDNF also restored the function of neural circuits controlling movement. The maximum functional effect was observed at the end of the observation period. In CDNF-treated rats at 14 weeks post lesion, the number of nigrostriatal TH-immunoreactive cells was identical as in rats analyzed at the time, when NTF treatments were started. This is in line with our results from the neuroprotection paradigm, where a pretreatment with CDNF almost totally prevented 6-OHDA-induced reduction in TH-immunoreactivity (I). It can be further speculated that a decrease in TH-positive cells between weeks 4 and 14 reflects at least in part a loss of TH-phenotype within cells rather than cell death. It may be that only cells that have lost their TH-phenotype, but have not died, are able to recover after the NTF treatment. In conclusion, our experiments with continuous infusion show that CDNF has strong behavioral effects in the 6-OHDA model.

In contrast to CDNF, MANF and GDNF had no significant effect. As discussed earlier, protective effect of GDNF is seen only when both GDNF and the toxin are injected in the same location (Kim et al., 2000a). We showed the same in the continuous infusion studies (III). Unexpectedly, the same seems to apply also MANF. Indeed, chronic infusion of MANF or GDNF, administered between two lesions created by 6-OHDA, did not significantly restore TH-positive cells or induce behavioral recovery differently to the single injection studies (I, II) where both MANF and GDNF was injected in the same location as the toxin.

6.4 Methodological consideration

Both CDNF and MANF had clear neuroprotective and neurorestorative effects in the present studies (I-III). Based on our results (I-III), CDNF may be slightly more effective in the neuroprotection model than MANF, because 10 µg of CDNF protected TH-positive cells and fibers better than 10 µg of MANF (see Table 3 and studies I, II). The effects of single injections of CDNF and MANF were rather similar in the neurorestoration paradigm (I, II). Interestingly, CDNF was more effective than MANF when given as a continuous infusion in the neurorestorative paradigm (III).

This difference may result from the dose selected; the dose of CDNF was optimal whereas the dose for the chronic MANF infusion studies may have been either too low or too high. The possible therapeutic significance of the neuroprotective effect is less important than the neurorestorative effect of CDNF and MANF, because the latter would be initiated in PD patients who already have severe degeneration in the nigrostriatal system. It took many weeks to see the neurorestorative effects of CDNF and MANF after their injections, whereas their neuroprotective effects were already evident at two weeks post-lesion. This may be a consequence of the less severe lesions induced by the small dose of 6-OHDA (8 µg) used in the neurorestoration paradigm compared with the larger dose (20 µg) used in the neuroprotection study. Also, in the neurorestoration paradigm, the lesion was almost fully developed before NTF was administered, whereas in the neuroprotection study, the trophic factor
was administered as a pretreatment and the follow-up was only up to four weeks. In any case, apparently only those dopaminergic nerves not fully destroyed may recover after neurotrophic factor treatment.

The effects of chronic infusion of CDNF and MANF were tested using a slightly different experimental setting than was used for the single injection studies (I, II), as the continuous NTF infusion was given between the two 6-OHDA injection sites. In a magnetic resonance imaging (MRI) study in rats, it was shown that a dose of 8 μg of 6-OHDA in the striatum produces a hyperintense area with a radius of ca. 1 mm (Kondoh et al., 2005). Thus, the lesion in the chronic infusion study (10 μg in two places) was larger and more challenging for the NTFs to reach than in our earlier studies in which the NTFs were applied at the same location as 6-OHDA (I, II).

6.5 Distribution properties of CDNF and MANF

Striatically injected 125I-CDNF was transported in a retrograde manner from the STR to the SNpc (III). Similar pattern of retrograde transport of GDNF has been reported previously by several groups (Tomac et al., 1995a, Lapchak et al., 1997). In contrast to GDNF and MANF, MANF was transported to frontal cortical areas (II). Because CDNF and GDNF have similar transportation profiles, CDNF may be assumed to be transported via the same nerve tracts as GDNF. The transportation profile of MANF may reflect a distinct mechanism of neurorestorative action. The functional recovery of the lesioned brain may result from the indirect effects of MANF on other neural circuits controlling movement and/or controlling the dopaminergic activity of the nigrostriatal tract. Thus, MANF might modify glutamatergic activity and produce neuroprotection similar to that afforded by glutamate receptor antagonists (Turski et al., 1991). MANF has been shown to increase GABAergic activity, thereby regulating dopaminergic firing in tissue slices (Petrova et al., 2004) and in dissociated cells in culture (Zhou et al., 2006). Therefore, an indirect protective effect on dopaminergic cells via GABAergic neurons is possible. Furthermore, MANF may have a dual neuroprotective effect with additional direct effect on dopaminergic nerves, as indicated by in vitro data (Petrova et al., 2003).

The brain tissue distributions of the different NTFs varied. After three days of continuous infusion into the striatum, the volume of tissue distribution of MANF (as measured by the intensity of IHC signal) was 38% larger than that of CDNF, which in turn was 39% larger than that of GDNF (III). At the end of a 14-day continuous infusion, the volume of distribution of MANF was not increased, further indicating that the spread of MANF had already reached a plateau after three days, but this process took longer for CDNF and GDNF. Interestingly, after a three-day infusion, the distribution of MANF was not greater than that observed after a single 10 μg injection of MANF measured 24 h after injection (II). Although it is not possible to compare these two experiments, the ratio of the distribution volumes of MANF and GDNF in the study (III) was similar to that in study (II) (1.9 after chronic infusion and 1.7 after a single injection). Both experiments show that MANF and CDNF penetrate better than GDNF into brain tissue.

After a 14-day infusion, there were no differences between the distribution volumes of the three NTFs. This may not directly reflect the distribution ability of the compounds, because during continuous infusion the volume of distribution is
a balance between distribution and elimination of the protein. We have insufficient data about the degradation rate of the growth factors in brain tissue, but this may vary also. Although the stability of the proteins in vitro was good and comparable, the situation may be different in brain tissue where the proteolytic protein degradation occurs. It is also possible that the concentration of MANF and CDNF at the edge of the diffusion rim is already so low that it is not detectable with the current IHC.

The chemical characteristics of the NTFs correlate only partially with the observed volumes of distribution. GDNF is a more basic protein than MANF and the pI of MANF and GDNF is 8.6 and 9.6, respectively. As the basic protein GDNF binds to heparan sulfate in the ECM and therefore the diffusion of MANF during the first days may well be better than that of GDNF. However, it was unexpected that CDNF (with a pI of 7.7) did not diffuse better than MANF. As Piltonen et al. (2009) previously showed after a single injection, a non-heparin-binding truncated GDNF mutant (Δ38N-GDNF) was more widely distributed than wtGDNF, but the neuroprotective effect of the truncated GDNF in vivo was weaker (Piltonen et al., 2009). Taken together, it seems plausible that a good penetration is an important property for a trophic factor, but it is not the only property relevant to its efficacy.

6.6 Neuroprotective mechanisms of neurotrophic factors in PD models

As discussed earlier (chapter 2.4.2), 6-OHDA is thought to induce its neurotoxic effect mainly by inhibiting mitochondrial complex I (Sachs and Jonsson, 1975) and increasing ROS production, but it also induces apoptotic cell death and inflammation (Schober 2004). MANF and CDNF may activate signaling pathways that counteract some of these phenomena. 6-OHDA has also been reported to induce ER stress and the UPR (Chen et al., 2004). Several lines of evidence suggest that at least part of the neuroprotective effect of MANF and CDNF against 6-OHDA-induced neurotoxicity may be attributable to inhibition of ER stress.

6.6.1 Endoplasmic reticulum-stress

The accumulation of misfolded proteins in the ER causes stress that initiates the UPR. The UPR activates both adaptive and apoptotic pathways, which contribute differently to disease pathogenesis. The ER is an organelle responsible for the synthesis, initial post-translational modification, folding, export and secretion of membrane proteins (Kim et al., 2006). It also serves as a site for Ca2+ storage and sterols and lipids synthesis (Malhotra and Kaufman, 2007). These functions are susceptible to perturbation by various pathogenic insults, such as genetic mutation, aging, oxidative stress, hypoxia and viral infection. A consequence of these perturbations is protein misfolding and accumulation in the ER (Marciniak and Ron, 2006). Cells remove misfolded proteins by utilizing a protective mechanism called ER-associated degradation (ERAD) that prevents proteins from accumulating (McCracken and Brodsky, 2003). If the accumulated proteins exceed the capacity of ERAD, ER stress is induced that initiates the UPR which restores ER homeostasis (Malhotra and Kaufman, 2007). Three ER transmembrane proteins (pancreatic ER kinase, PERK; inositol-requiring enzyme 1, IRE1 and activating transcription factor 6, ATF6), respond to accumulated unfolded protein (Zhang and Kaufman, 2004). The expression of UPR target genes is regulated
Discussion

by the transcription factor X-box binding protein (XBP1) (Szegedi et al., 2006). They sense the misfolded proteins and transduce information to elicit responses.

The expression of MANF is enhanced after ER stress in cell lines (Apostolou et al., 2008, Mizobuchi et al., 2007) and after brain injury in vivo. Immunoreactivity for MANF increases in the ischemic cerebral cortex after brain ischemia, a condition that induces ER stress. Similarly, MANF mRNA increases after brain ischemia and epileptic insults in the hippocampus and cerebral cortex. Up-regulation of MANF after injury may be a result of the activation of endogenous neuroprotective processes during insults. In cultured U2OS cells, overexpression of MANF by lentivirus-mediated transfection increases resistance to glucose-free conditions and to ER stress induced by tunicamycin (Apostolou et al., 2008).

Previous studies have shown that exogenous MANF reduces ER stress, and it can thus be called an ER stress-response protein (Tadimalla et al., 2008, Apostolou et al., 2008). MANF is a soluble protein that is secreted and/or resides in the endoplasmic reticulum and Golgi apparatus (Apostolou et al., 2008, Mizobuchi et al., 2007). Moreover, MANF was shown to inhibit ER stress-induced cell death in HeLa cells in vitro (Apostolou et al., 2008). It should be noted however, that in all these experiments, MANF was overexpressed in cells.

6.6.2 Oxidative stress

Oxidative stress (2.1.1) is implicated as either a major cause or a result of the neurodegeneration associated with PD (Maguire-Zeiss et al., 2005). Because increases in oxidative stress can be detected in PD models before signs of neuronal degeneration, oxidative stress may be an early component of neuronal loss (Venero et al., 1997). GDNF reduces oxidative stress-induced cell death in vitro (Toth et al., 2002), but only few groups have studied the effect of GDNF on oxidative stress in vivo. A recent study showed that intrastratal GDNF reduces the generation of oxidative stress in a PD model. Oxidative stress markers were significantly reduced in animals treated with GDNF as compared to vehicle-treated animals (Smith and Cass, 2007).

Most of the biological effects of GDNF and NRTN are triggered by activating of Akt, Srk and MAP kinases. Although the effects of GDNF are well established, the mechanisms of its neuroprotection have not been completely elucidated. However, the protective effects of GDNF against 6-OHDA seem to involve a reduction of oxidative stress. Whether CDNF and MANF reduce oxidative stress will be revealed in the future.

6.7 Two putative activities of CDNF and MANF

The transportation profile of MANF and the results from MANF infusion studies imply that the mechanism of action of MANF in 6-OHDA model is different from those of CDNF and GDNF (II). Thus, MANF may partially elicit its in vivo effects via non-dopaminergic neurons, whereas CDNF acts more directly on dopaminergic neurons. This difference is reflected in our observations of amphetamine-induced rotational behavior and in the immunohistochemical analyses.

The structural analysis suggests that CDNF and MANF may have a dual mechanism of action at the cellular level. Crystal structure analysis revealed that CDNF and MANF consist of two domains (Parkash et al., 2009). The amino-terminal domain is a saposin-like putative lipid-
binding domain, suggesting that MANF and CDNF may bind lipids in membranes. Prosaposin and saposin C have shown neurotrophic activity in cultured neurons (O’Brien et al., 1994, Misasi et al., 2004). The carboxy-terminal domain of CDNF and MANF may protect cells against ER stress (Lindholm and Saarma, 2010, Parkash et al., 2009). Through their C-terminal C-X-X-C motif, CDNF and MANF may facilitate the formation of cysteine bridges during protein folding in the ER and thereby reduce the ER stress caused by unfolded or incorrectly folded proteins. Interestingly, a recent study showed that the C-terminal domain of MANF (C-MANF) is homologous to the SAP domain of Ku70, a well-established inhibitor of pro-apoptotic BCL-2-associated X protein (Bax) (Hellman et al., 2010). Authors further conclude that MANF is an NTF that can protect the cells both intracellularly (Hellman et al., 2010, Apostolou et al., 2008) an in vivo extracellularly (I, II, Airavaara et al., 2009). Small terminal C-domain of MANF accounts for the intracellular protection against the BAX-dependent apoptosis (Hellman et al., 2010). These structural properties of MANF and CDNF suggested that their neurotrophic activity may reside in their N-terminal domains and their ER-stress-response activity in the C-terminal domains. Both mechanisms may have a significant role in the neuroprotective and neurorestorative effects of MANF and CDNF.

6.8 CDNF and MANF as potential therapeutic proteins

Because CDNF and MANF have both neuroprotective and restorative effects in a rat model of PD (I,II), they can be included in the list of promising trophic factors for the treatment of PD.

Our results from neuroprotection and restoration studies (I, II and III) in the 6-OHDA model of PD are encouraging because CDNF and MANF were generally effective in all experimental settings. Consistently with the detected neurotrophic activities, CDNF and MANF are secreted from transiently transfected cells (I, Palgi et al., 2009, Lindholm et al., 2008). This is in line with the finding, where the secretion of endogenous MANF has been demonstrated in vitro (Apostolou et al., 2008). Human CDNF contains one potential N-linked glycolysation site, and both glycosylated (Apostolou et al., 2008) and unglycosylated (I) form of human CDNF is secreted from transiently overexpressing cells. Glycosylation seem not to be crucial for the neurotrophic activity of CDNF, because the unglycosylated form of CDNF is biologically active (I,III). Both MANF and CDNF are monomeric and soluble at neutral pH 7 (I).

No apparent toxicity of CDNF or MANF was observed in non-lesioned and lesioned animals during or after continuous infusions of MANF or CDNF (animal weight was not changed; posture and movements were normal). Compared to singe injections, continuous or repeated infusions appear preferable for the delivery of NTFs to PD patients. Using long-term infusions, it is possible to fine-tune the dosage and also to stop infusions if adverse-effects occur.

CDNF, given as a single injection to non-lesioned animals, did not increase the number of TH-positive cells in the SNpc or the fiber density in SNpr as compared to the control side (Airavaara et al., unpublished). Moreover, the chronic infusion of CDNF in the non-lesioned animals did not increase the number of TH-positive cells in the SNpc or fibers in the striatum (III). Whether the secretion of endogenous MANF or CDNF is regulated by physiological stimuli or injury in vivo is currently unclear. As discussed
earlier, recent studies demonstrate that MANF expression is upregulated by ischemia (Lindholm et al., 2008, Tadimalla et al., 2008, Apostolou et al., 2008, Yu et al., 2010) as well as by endoplasmic reticulum (ER) –stress (Apostolou et al., 2008, Mizobuchi et al., 2007, Lee et al., 2003). Furthermore, as CDNF did not increase the number of TH-positive cells in the SNpc or fibers in the striatum in non-lesioned animals after single or chronic infusion but had clear neuroprotective and restorative effects in 6-OHDA model of PD, it can be speculated that MANF and presumably also CDNF may be activated upon physiological stimuli or injury.

The trophic effects of CDNF have also been detected by others when given either by gene therapy or as a protein injection. In a recent unpublished study, CDNF had neuroprotective effects in a rat 6-OHDA PD model where it was delivered by a gene therapy approach using an AAV vector (Bäck et al., unpublished). GDNF injection into the STR or SN before MPTP or into the STR after MPTP increases dopamine levels and TH-positive fiber density and also improves motor behavior (Tomac et al., 1995b). However, GDNF injection into the SN does not induce sprouting of dopaminergic fibers in the striatum when given after MPTP. Airavaara and colleagues have shown that CDNF has neuroprotective and neurorestorative properties in the MPTP mouse model (Airavaara et al., unpublished). Moreover, studies with Drosophila MANF (DmMANF) null mutant flies have shown that DmMANF acts as a neurotrophic factor in the fly. Interestingly, ubiquitous expression of HsMANF or HsCDNF can rescue the larval lethality of DmMANF mutants ((Palgi et al., 2009), R. Lindström and T. Heino, personal communication). These results are promising, but more efficacy and toxicology studies on rodents and primates are needed before clinical trials can be initiated. As the receptors for CDNF and MANF are unknown (Lindholm and Saarma, 2010), it remains unclear whether they use the same signalling pathways. Moreover, as Manf and Cdnf are expressed in the developing nigrostriatal system of the mouse at P1 and P10 (I, (Lindholm et al., 2008), it is plausible that they have a role in the development of midbrain dopaminergic system. Whether CDNF or MANF function as target-derived NTs for midbrain dopaminergic neurons is unknown.

GDNF was originally established as a specific NT for dopamine neurons (Lin et al., 1993), but nowadays it is well known that GDNF has actions also on other cell populations. Low specificity of NTf and potential adverse effects might be problematic in NTf therapy. Studies on MANF and CDNF knockout mice will presumably reveal the roles of MANF and CDNF in vivo. Also, even if CDNF and MANF have effects on dopaminergic and cortical neurons, further studies are needed to explore do they affect PNS neurons or other neuronal types in vivo.

The delivery of NTs to patients is challenging because the factors do not penetrate the blood-brain barrier, thus they need to be injected intracranially. In clinical trials, GDNF protein has been administered both intraventricularly (Nutt et al., 2003) and intraputamenally (Gill et al., 2003, Lang et al., 2006, Slevin et al., 2005, Patel and Gill, 2007), whereas NRTN has been delivered into putamen using a gene therapy technique by AAV2 vector (Marks et al., 2008). These trials have produced varying results. One likely reason for failure in GDNF clinical trials is its low penetration into brain parenchyma. However, some patients have shown a sustained clinical response to this treatment that correlates
with evidence of increased dopaminergic activity in the brain at the site of delivery, as seen in a post-mortem study (Love et al., 2005) and using an 18F-fluorodopa PET method. The NTF approach clearly works at least in animal models, but further studies are needed to optimize therapeutic effects, solve the technical delivery problems and minimize adverse effects.

Transplantation of encapsulated NTF-secreting cells is one potential strategy for therapy (Lindvall and Wahlberg, 2008). The development of small molecules that specifically activate NTF receptors or growth factor genes is also a fascinating approach (Bespalov and Saarma, 2007). Furthermore, intravenous NTF plasmid DNA and non-viral gene therapy using liposomes that are targeted with a monoclonal antibody to the rat transferrin receptor is a potential non-invasive technique (Zhang and Pardridge, 2009).

Animal studies evaluating restorative and protective effects in the settings such as of impaired mitochondrial metabolism, α-synuclein fibrillization and alterations in the ubiquitin-proteasome system will continue to be useful in guiding different treatment and delivery approaches for PD (Kotzbauer and Holtzman, 2006).

However, several challenges remain for future studies, since PD is a complex disease of largely unknown etiology and affects also other than nigrostriatal system. Thus, better understanding of both PD pathogenesis and the mechanisms of action of novel therapies (such as CDNF and MANF) seems essential for developing new therapies for PD.
7. CONCLUSIONS

The neuroprotective and restorative properties CDNF and MANF were studied in a partial rat 6-OHDA lesion model of Parkinson’s disease. Generally, this study showed that both CDNF and MANF protect midbrain dopaminergic neurons against 6-OHDA-induced toxicity and restore the function of the nigrostriatal dopaminergic system.

The more specific conclusions drawn from this study are:

1. Intrastriatal CDNF and MANF protected midbrain dopaminergic neurons when administered before 6-OHDA. More importantly, CDNF and MANF restored the function of the nigrostriatal dopaminergic system when administered four weeks after 6-OHDA administration in the striatum.
2. A 14-day continuous infusion of CDNF (but not of MANF) restored the function of neural circuits controlling movements when initiated two weeks after unilateral injection of 6-OHDA.
3. Studies concerning the retrograde transport of MANF and CDNF revealed that CDNF and GDNF have similar transportation profiles from the striatum to the SNpc; therefore CDNF may act via the same nerve tracts as GDNF. In contrast to CDNF, intrastriatal MANF was transported to cortical areas, which may reflect a neurorestorative mechanism that is different from those of CDNF and GDNF.
4. The midbrain tissue distribution of CDNF and MANF was better than that of GDNF.

These results imply that CDNF and MANF have potential as neurorestorative therapy of PD.
8. ACKNOWLEDGEMENTS

This study was carried out at the Division of Pharmacology and Toxicology, Faculty of Pharmacy, University of Helsinki in collaboration with Institute of Biotechnology, University of Helsinki during the years 2004-2010.

I wish to express my deepest gratitude to the following persons:

Professor Raimo K. Tuominen, the head of the Division of Pharmacology and Toxicology, for his support and guidance throughout these years. His experience in pharmacology, optimism and trust in me made this work possible. I am grateful for the excellent research facilities and for the given opportunity to study and learn a number of pharmacological methods in the division of Pharmacology and Toxicology.

Professor Mart Saarma, from Institute of Biotechnology, for his guidance in the neurotrophic field. I am grateful for his interest and a significant contribution to my work. His enthusiasm and deep understanding on science has been most impressive and inspiring.

Professor Pekka T. Mannistö, for his help, constructive criticism and valuable comments especially during the preparation of my latest manuscript and thesis.

The reviewers of the thesis, Docent Matti Airaksinen and Professor Deniz Kirik, for having devoted their time to thorough reading of the thesis and for valuable comments how to improve it. Docents Pekka Rauhala, Seppo Kaakkola and Petteri Pieponnen are acknowledged for valuable comments when defending my research plan.

Professor Susan Wonnacott from University of Bath, UK, for welcoming me as M.Sc. student to her lab and for introducing the fascinating field of neuropharmacology to me. Her enthusiasm for science inspired me to start the PhD studies in the division of Pharmacology and Toxicology.

All the co-authors of these studies. I wish to thank PhD Päivi Lindholm for her excellent comments on the manuscripts and all the advices during the project and for producing CDNF protein, Docent Johan Peränén for providing essential research material, Sanna Janhunen for the help concerning the methods especially in the beginning of the project. I am grateful for Liisa Toppinen M.Sc. (Pharm), Eeva Pörsti M.Sc. (Pharm) and Docent Atso Raasmaja for their contribution to the studies. In particular, I wish to thank Susanne Bäck, M.Sc. (Pharm) for excellent collaboration and contribution to the studies. Also, I am thankful to Docent Petteri Pieponnen for his help with statistics and for scientific conversations.

I wish to thank Anna Niemi, Kati Rautio, Marjo Vaha, Minna Baarman and Ritva Ala-Kulju for excellent technical assistance.

I wish to thank Timo ”Tinde” Päivärinta for the help in layout design of this book.

My colleagues and friends at the Division of Pharmacology and Toxicology for their kindness and support. In particular, I would like to thank Tanja Kivinummi, Kati Rautio, Susanne Bäck, Jelena Mijatovic and Virpi Talman for their friendship.
Acknowledgements

I wish to thank the following foundations which have supported this study with grants: the Association of Finnish Pharmacies, the Emil Aaltonen foundation, the Finnish Concordia Foundation, the Finnish Cultural Foundation, the Finnish Parkinson foundation, The Finnish Pharmaceutical Society, Michael J. Fox Foundation, The Otto A Malm Foundation, the Sigrid Jusélius Foundation and the University Pharmacy.

During all these years, I have especially valued the support from my friends, relatives and family. I wish to thank especially my dear friends Anna Sauso, Jenni Syrjänen, Jenni Setälä, Eevi Rikkonen, Johanna Palmen, Ninna Leimu, Anu Maasalmi for their continuous support and sharing the joys and sorrows of life with me.

I wish to thank my beloved parents and my sister for their enduring love and support. I particularly value the encouragement and support of my mother. Without you this project would not have been possible. I wish to thank Marjo and Taito Rantola for their support and hospitality. Finally, my warmest thanks belong to my husband Jukka, for all the love, support and understanding during this work, and to our son, Elias, for bringing joy and laughter into my life and keeping in mind the priorities of life.

Helsinki, November 2010

Merja
9. REFERENCES


References


Boyce, S., Kelly, E., Reavill, C., Jenner, P., Marsden, C.D., 1984. Repeated administration of N-methyl-4-phenyl 1,2,5,6-tetrahydropyridine to rats is not toxic to striatal dopamine neurons. Biochem. Pharmacol. 33, 1747-1752.


References

Hanrott, K., Gudmunsen, L., O’Neill, M.J., Wonnacott, S., 2006. 6-hydroxydopamine-
induced apoptosis is mediated via extracellular auto-oxidation and caspase 3-dependent
dysfunction in experimental animals. Nucl.
Med. Biol. 25, 721-8
Hardy, J., 2010. Genetic analysis of pathways to
Parkinson disease. Neuro 68, 201-206
Harvey, B.K., Wang, Y., Hoffer, B.J., 2008
Transgenic rodent models of Parkinson’s
and neurochemical effects of intranigral
administration of glial cell line-derived
neurotrophic factor on aged Fischer 344 rats.
J. Pharm. Exp. Ther. 282, 760-768.
Hebert, M.A., Van Horne, C.G., Hoffer, B.J.,
Gerhardt, G.A., 1996. Functional effects of
GDNF in normal rat striatum: presynaptic
studies using in vivo electrochemistry and
microdialysis. J. Pharm. Exp. Ther. 279, 1181-
1190.
Hefti, F., Melamed, E., Wurtman, R.J.,
1980. Partial lesions of the dopaminergic
nigrostriatal system in rat brain: biochemical
characterization. Brain Res. 195, 123-137.
Hefti, F., (1997) Pharmacology of neurotrophic
factors Annu Rev Pharmacol Toxicol. 37:239-
67.
Hellman, M., Arumäe, U., Yu, L-Y., Lindholm,
Neurotrophic factor MANF has a unique
mechanism to rescue apoptotic neurons. J. Biol.
Chem. Doi 10.1074/jbc.M110.146738.
Henderson, C.E., Phillips, H.S., Pollock, R.A.,
Davies, A.M., Lemeulle, C., Armanini, M.,
Simmons, L., Moffet, B., Vanden, R.A.,
Simpson LC [corrected to Simmons, L.],
1994. GDNF: a potent survival factor for
motoneurons present in peripheral nerve and
muscle. Science 266, 1062-1064.
Herrero, M.T., Hirsch, E.C., Kastner, A.,
Ruberg, M., Luquin, M.R., Laguna, J., Javoy-
neuromelanin contribute to the vulnerability of
catecholaminergic neurons in monkeys
intoxicated with MPTP? Neuroscience 56, 499-
511.
Heuckeroth, R.O., Lampe, P.A., Johnson, E.M.,
promote proliferation and survival of enteric
200, 116-129.
Hoffer, B.J., Hoffman, A., Bowenkamp, K.,
Huettl, P., Hudson, J., Martin, D., Lin, L.F.,
neurotrophic factor reverses toxin-induced
injury to midbrain dopaminergic neurons in
Hohn, A., Leibrock, J., Bailey, K., Barde, Y.A.,
1990. Identification and characterization of a
novel member of the nerve growth factor/
brain-derived neurotrophic factor family.
Nature 344, 339-341.
Holloway, R.G., Shoulson, I., Fahn, S., Kieburtz,
K., Lang, A., Marek, K., McDermott, M., Seibyl,
J., Weiner, W., Musch, B., Kamp, C., Welsh, M.,
Shinaman, A., Pahwa, R., Barclay, L., Hubble, J.,
LeWitt, P., Miyasaki, J., Suchowersky, O., Stacy,
M., Russell, D.S., Ford, B., Hammerstad, J.,
Riley, D., Standaert, D., Wooten, F., Factor, S.,
Jankovic, J., Atassi, F., Kurlan, R., Panisset, M.,
Rajput, A., Rodnitzky, R., Shults, C., Petsinger,
G., Waters, C., Pfeiffer, R., Biglan, K., Borchert,
L., Montgomery, A., Sutherland, L., Weeks, C.,
DeAngelis, M., Sime, E., Wood, S., Pantella, C.,
Harrigan, M., Fussell, B., Dillon, S., Alexander-
Brown, B., Rainey, P., Tennis, M., Rost-Ruffner,
E., Brown, D., Evans, S., Berry, D., Hall, J.,
Shirley, T., Dobson, J., Fontaine, D., Pfeiffer,
B., Brocht, A., Bennett, S., Daigneault, S.,
Hodgeman, K., O’Connell, C., Ross, T., Richard,
Prampoxole vs levodopa as initial treatment for
Parkinson disease: a 4-year randomized
controlled trial. Arch. Neurol. 61, 1044-1053.
mimetics induce aspects of unfolded protein
response in death of dopaminergic neurons. J.
Biol. Chem. 278, 19367-19377.
Hoozemans, J.J., van Haastert, E.S., Eikelenboom,
P., de Vos, R.A., Rozemuller, J.M., Scheper,
W., 2007. Activation of the unfolded protein
Hörger, B.A., Nishimura, M.C., Armanini, M.P.,
Wang, L.C., Poulsen, K.T., Rosenblad, C., Kikir,
D., Moffat, B., Simmons, L., Johnson, E., Jr,
Milbrandt, J., Rosenthal, A., Björklund, A.,
Neurturin exerts potent actions on survival

References
References


References


Parsons, C.G., Danysz, W., Quack, G., 1999. Memantine is a clinically well tolerated N-methyl-D-aspartate (NMDA) receptor antagonist--a review of preclinical data. Neuropharmacology 38, 735-767.


References


References


Shapiro, R.M., Glick, S.D., Camarota N.E., 1987. A two-population model of rat rotational behavior: effects of unilateral nigrostriatal 6-hydroxydopamine on striatal
neurochemistry and amphetamine-induced rotation. Brain. Res. 426, 323-331
Shink, E., Bevan, M.D., Bolam, J.P., Smith, Y., 1996. The subthalamic nucleus and the external pallidum: two tightly interconnected structures that control the output of the basal ganglia in the monkey. Neurosci. 73, 335-357.

References
References


Yue, Z., 2009. LRRK2 in Parkinson’s disease: in vivo models and approaches for understanding pathogenic roles. FEBS J. 276: 6445-6454


