CDNF IN AN EXPERIMENTAL MODEL OF PARKINSON’S DISEASE: STUDIES ON GENE THERAPY AND PROTEIN INFUSION

Susanne Bäck

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy, University of Helsinki, for public examination in Auditorium 1041, Viikki Biocenter 2, on February 7th 2014, at 12 noon.
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Neurodegenerative diseases are characterized by progressive loss of distinct neuronal populations. In Parkinson’s disease (PD) the most prominent cell loss is seen in the dopamine (DA) neuron population in the substantia nigra pars compacta (SNpc). The resulting decrease in striatal DA levels causes dysregulation of neuronal circuits controlling movement and leads to motor symptoms typical to the disease. As for other neurodegenerative diseases, there are no available treatments that would interfere with the degenerative process in PD. The purpose of this work was therefore to test the therapeutic potential of long-term delivery of the neurotrophic factor (NTF) cerebral dopamine neurotrophic factor (CDNF) in the rat partial 6-hydroxydopamine (6-OHDA) lesion model of PD.

When injected unilaterally in the striatum, 6-OHDA causes progressive dose-dependent loss of DA neurons in the SNpc accompanied by asymmetrical motor impairment. The 6-OHDA model used in our NTF studies (2x10 µg 6-OHDA) showed a stable lesion progression with a cell loss at two weeks post-lesion corresponding to that seen in PD at symptom onset. In the 6-OHDA model, the DAergic system is traditionally evaluated using immunodetection methods or measurements of tissue neurotransmitter levels. Imaging methods, such as single-photon emission computed tomography (SPECT), allows in vivo detection of neuronal circuits, and together with the DA transporter (DAT) radioligand 2β-carbomethoxy-3β-(4′-[123]I)iodophenyl)tropane ([123]I-β-CIT), SPECT/CT provided reliable estimations of the DA cell degeneration showing high correlation to immunohistochemical findings. The method is sensitive and selective and provides substantial benefits in pre-clinical research allowing longitudinal studies in living animals.

The neuroprotective effect of CDNF was studied by applying the NTF intrastriatally as two-week protein infusion with osmotic pumps, or as gene therapy with a recombinant adeno-associated viral vector in 6-OHDA-lesioned rats. Both CDNF delivery methods normalized the amphetamine-induced rotational asymmetry and provided partial protection of the tyrosine hydroxylase (TH) –reactive DAergic cells in the SNpc and DA fibers in the striatum. As for GDNF, there were indications of retrograde transport of CDNF, but contrary to what has been reported for GDNF, CDNF did not affect the intact rat DAergic system. In addition, there were differences between the treatments in the capacity to induce sprouting of TH-reactive fibers. Our results confirm that CDNF can be considered as a potential therapy in PD, and that the neuroprotective mechanism of CDNF differs from that of GDNF.
ABBREVIATIONS

AADC  aromatic amino acid decarboxylase
AAV  adeno-associated virus
Aβ  β-amyloid peptide
ACH  acetylcholine
ACHE  acetylcholine esterase
AD  Alzheimer’s disease
ALS  amyotrophic lateral sclerosis
ANOVA  analysis of variances
APP  amyloid precursor protein
ASO  antisense oligonucleotide
α-syn  α-synuclein
β-CIT  2β-carbomethoxy-3β-(4-iodophenyl)tropane
BBB  blood-brain barrier
BDNF  brain-derived neurotrophic factor
BH4  tetrahydrobiopterin
CDNF  cerebral dopamine neurotrophic factor
ChAT  choline acetyl transferase
CNTF  ciliary neurotrophic factor
CSF  cerebrospinal fluid
COMT  catechol-O-methyltransferase
CNS  central nervous system
DA  dopamine
DAT  dopamine transporter
DOPAC  dihydroxyphenylacetic acid
ELISA  enzyme-linked immunosorbent assay
ER  endoplasmic reticulum
ESC  embryonic stem cells
FDG  fluorodeoxyglucose
FG  FluoroGold
FMT  6-fluoro-L-m-tyrosine
FUS  fusion in sarcoma
GABA  gamma aminobutyric acid
GAD  glutamic acid decarboxylase
GDNF  glial cell line-derived neurotrophic factor
GFL  GDNF family ligand
GFP  green fluorescent protein
GP  globus pallidus
GPe  globus pallidus externa
GPi  globus pallidus interna
GCH  GTP cyclohydrolase
HD  Huntington’s disease
5-HIAA  5-hydroxyindoleacetic acid
HSV  herpes simplex virus
5-HT  serotonin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTT</td>
<td>huntingtin</td>
</tr>
<tr>
<td>HVA</td>
<td>homovanillic acid</td>
</tr>
<tr>
<td>i.c.</td>
<td>intracerebral</td>
</tr>
<tr>
<td>i.c.v.</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>i.m.</td>
<td>intramuscular</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>levodopa, 3,4-dihydroxy-L-phenylalanine</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LRRK2</td>
<td>leucine-rich repeat kinase 2</td>
</tr>
<tr>
<td>LV</td>
<td>lentivirus</td>
</tr>
<tr>
<td>MANF</td>
<td>mesencephalic astrocyte-derived neurotrophic factor</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>MFB</td>
<td>medial forebrain bundle</td>
</tr>
<tr>
<td>MPP</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>NCS</td>
<td>neural stem cell</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>3-NP</td>
<td>3-nitropropionic acid</td>
</tr>
<tr>
<td>NTN</td>
<td>neurturin</td>
</tr>
<tr>
<td>NTF</td>
<td>neurotrophic factor</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-induced kinase 1</td>
</tr>
<tr>
<td>PSEN1/2</td>
<td>presenilin 1/2</td>
</tr>
<tr>
<td>QA</td>
<td>quinolinic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>SNpc</td>
<td>substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNpr</td>
<td>substantia nigra pars reticulata</td>
</tr>
<tr>
<td>SOD1</td>
<td>superoxide dismutase-1</td>
</tr>
<tr>
<td>SPECT/CT</td>
<td>single-photon emission computed tomography/computed tomography</td>
</tr>
<tr>
<td>STN</td>
<td>subthalamic nucleus</td>
</tr>
<tr>
<td>TDP-43</td>
<td>TAR DNA-binding protein</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>UPDRS</td>
<td>Unified Parkinson’s Disease Rating Scale</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin-proteasome system</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>vg</td>
<td>virus genome</td>
</tr>
<tr>
<td>VMAT2</td>
<td>vesicular monoamine transporter 2</td>
</tr>
</tbody>
</table>
This thesis is based on the following publications:


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1. INTRODUCTION

Neurodegenerative diseases, such as Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease (HD), and amyotrophic lateral sclerosis (ALS), are characterized by progressive loss of neuronal function and cell death in certain neuronal pathways (Fahn, 2003; Querfurth and LaFerla, 2010; Kiernan et al., 2011; Ross and Tabrizi, 2011). The incidence of these diseases increases with age, suggesting that the continuous rise in life expectancy will further increase the number of afflicted people. Moreover, the prognosis of neurodegenerative diseases is today very grim, and they are not only among the top ten causes of death, but also one of the leading causes of disability and dependency of other people. Present treatments used in these disorders only relieve symptoms and no disease-modifying treatments have yet been discovered.

Because of lack of curative treatments, there is an urgent need for new therapeutic approaches. Neurotrophic factors (NTF) regulate the life of neurons during development, but they also affect plasticity and regeneration in the adult organism (Airaksinen and Saarma, 2002; Lindholm and Saarma, 2010). Therefore, the NTFs constitute an interesting therapeutic opportunity. Indeed, the two NTFs glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN) have both been effective in pre-clinical in vivo models of PD. Nevertheless, results from clinical studies have this far been controversial (reviewed by Barker, 2009). Novel NTFs that have received attention for their potential as neuroprotective agents are mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF) (Lindholm and Saarma, 2010). MANF promotes survival of dopamine (DA) neurons in vitro (Petrova et al., 2003), and the invertebrate analogue DmMANF has been shown to be crucial for maturation and maintenance of the fruit fly (Drosophila melanogaster) nervous system (Palgi et al., 2009). Both MANF and CDNF have also been neuroprotective in rodent models of PD when given as single intrastriatal injections (Lindholm et al., 2007; Voutilainen et al., 2009; Airavaara et al., 2012).

Since the neurodegenerative diseases are chronic progressive diseases, long-term delivery of therapeutic agents is probably a requisite. This can be achieved, e.g., by continuous protein infusions or by gene therapy. In animal models of neurodegenerative diseases, several approaches using gene therapy have proven to be beneficial. In these studies, researches have tried to alter neuronal networks by modulating the synthesis of neurotransmitters (Björklund and Kordower, 2010), or aimed at neuroprotection by removing pathogenic proteins (San Sebastian et al., 2013), or by supplying the diseased tissue with neurotrophic support (Allen et al., 2013). Even though many of these therapeutic interventions have shown great potential in pre-clinical in vivo studies, the results from clinical trials have been rather disappointing.
2. REVIEW OF THE LITERATURE

2.1 NEURODEGENERATION AND NEURODEGENERATIVE DISEASES

In neurodegeneration the structure and function of neurons or neuronal populations are progressively lost. In this chapter I will review four neurodegenerative diseases: PD, AD, HD, and ALS (Table 1). I chose these four diseases since even though they are characterized by vulnerability of distinct neuronal populations giving rise to symptoms typical to each disease, the underlying proposed pathological mechanisms show a great deal of similarities.

Age is considered to be the greatest risk factor for neurodegenerative diseases and many of them show a steep increase in incidence with age (Hirtz et al., 2007). Age itself is thought to contribute to changes in neuronal systems, but it is still debated whether the loss of neuronal structure and function can be attributed purely to processes due to aging, or whether what is considered to be “normal” age-dependent decline in cognition and neuronal function is a result from underlying disease (discussed by Jagust, 2013). Even if age itself is most likely not the cause of neurodegenerative diseases, age seems to be a contributing factor predisposing to certain pathological events and clinical manifestations. Most of the cases of neurodegenerative diseases are classified as sporadic late-onset with unknown etiology. However, the impact of genetic factors is becoming more clear and has lately received a growing amount of interest (Hardy and Orr, 2006; Harvey et al., 2011; Cookson, 2012). Mutations in seemingly unrelated genes have been found to modify the risk of getting a certain neurodegenerative disorder, and have provided new insights into the pathology of the diseases. In addition to age and genes, environmental factors (exposure to toxins, developmental events etc.) have been implicated in the etiology of these diseases (Whalley et al., 2006; Kiernan et al., 2011; Wirdefeldt et al., 2011). For most neurodegenerative diseases (sporadic forms), the disease is most likely multifactorial in which age, genes and environment together modify the risk of getting the disease. The exception is HD which is a monogenic disorder caused by an abnormally long CAG triplet repeat in the huntingtin (HTT) gene (Ross and Tabrizi, 2011).

The pathogenesis in AD, PD, HD, and ALS shows many similarities. In all diseases there are misfolding and aggregation of certain proteins typical to each disorder (Table 1) (Ross and Poirier, 2004). The role of these abnormal protein inclusions is still debated, and their contribution to the pathogenesis is unclear. Other proposed molecular pathological mechanisms common for the diseases discussed below are mitochondrial dysfunction, oxidative stress, dysfunction of the ubiquitin-proteasome system (UPS), chronic inflammation and excitotoxicity (Bossy-Wetzel et al., 2004). All these complex mechanisms are interrelated and may all contribute to the progressive neurodegeneration.
### Table 1. Late-onset neurodegenerative diseases and some of their characteristics.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Etiology; Incidence</th>
<th>Main neuron population affected</th>
<th>Characteristic pathology</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer’s disease</td>
<td>Sporadic 95% Familial ≤5% (including APP, PSEN1/2); 1275/100 000 (&gt;65 years)</td>
<td>Acetylcholinergic neurons; degeneration in the entorhinal cortex, spreading to the temporal lobe and frontal cortex</td>
<td>Extracellular β-amyloid plaques, intracellular neurofibrillary tangles</td>
<td>Cognitive dysfunction with memory loss</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>Sporadic 90-95% Familial 5-10% (including α-syn, parkin, PINK, DJ-1, LRKK2); 160/100 000 (&gt;65 years)</td>
<td>Dopamine neurons in the substantia nigra pars compacta</td>
<td>Lewy bodies containing aggregated α-syn</td>
<td>Resting tremor, postural instability, rigidity, akinesia/bradykinesia</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>Monogenic (huntingtin); 0.3-0.8/100 000 (all ages)</td>
<td>GABAergic medium spiny neurons in the striatum</td>
<td>Aggregated huntingtin</td>
<td>Chorea, psychiatric disturbances, cognitive impairment</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>Sporadic 90-95% Familial 5-10% (including SOD1, C9orf72; TDP-43, FUS, UBQLN2); 1.6/100 000 (all ages)</td>
<td>Lower motor neurons in the spinal cord, upper motor neurons in the cortex</td>
<td>Cytoplasmic ubiquitinated aggregates of SOD1, FUS or TDP-43</td>
<td>Progressive muscle weakness, muscular atrophy, spasticity</td>
</tr>
</tbody>
</table>

APP, amyloid precursor protein; α-syn, α-synuclein; FUS, fusion in sarcoma; GABA, gamma aminobutyric acid; PSEN1/2, presenilin 1 and presenilin 2; SOD1, superoxide dismutase-1; TDP-43, TAR DNA-binding protein; UBQLN2, ubiquitin-like protein-2. Incidences in Europe and North America from Hirtz et al., 2007 and Pringsheim et al., 2012.

### 2.1.1 PARKINSON’S DISEASE

With a prevalence of 9.5 per 1000 in the population aged over 65 years in the Western world (Hirtz et al., 2007), PD is considered to be the second most common neurodegenerative disorder. PD is mainly characterized as a progressive neurodegenerative movement disorder with cardinal symptoms consisting of resting tremor, rigidity, akinesia/bradykinesia, and postural instability (Fahn, 2003). The motor symptoms in PD result from an imbalance in the basal ganglia signaling (Parent et al., 2000). The basal ganglia consist of the striatum (caudate nucleus and putamen), globus pallidus interna (GPI), and externa (GPe), subthalamic nucleus (STN), substantia nigra pars reticulata (SNpr) and pars compacta (SNpc), and the intralaminar nuclei of the thalamus (Figure 1). DAergic neurons in the SNpc innervate the striatum. DA released at the striatal level generally facilitates signaling through the direct pathway (striatum-GPi, action through D₁ receptors), while the indirect pathway is inhibited (striatum-GPe, action through D₂ receptors). In PD, degeneration of the neuromelanin-containing DA neurons in...
the SNpc causes a decrease in DA concentration in the striatum. As the DA concentration decreases, both DAergic and non-DAergic compensatory mechanisms (e.g., increase in DA synthesis and release, loss of DA reuptake sites, supersensitivity of receptors, reduction in activity in the indirect pathway etc.) set in to counteract the loss of DA signaling (Brotchie and Fitzer-Attas, 2009). Because of these compensatory actions, it is estimated that, as the motor symptoms appear, 30-60% of the nigral DAergic cell bodies are lost (Fahn, 2003; Cheng et al., 2010) and the striatum is subjected to an up to 80% decrease in DA concentration (Fahn, 2003). Despite compensatory mechanisms, further loss of DA eventually results in lost control over the striatopallidal neurons causing an imbalance in the signaling through the direct and indirect pathway, and giving rise to the classical symptoms of PD (Figure 1).

![Figure 1. Simplified schematic figure of basal ganglia circuitry in normal (A) and PD brain (B). Decrease in striatal dopamine (DA) concentration leads to loss of control of the glutamatergic stimulation of the striatopallidal GABAergic neurons, causing an imbalance in the signaling equilibrium through the direct and indirect pathways (according to Parent et al., 2000). ENK, encephalin; GABA, gamma-aminobutyric acid; GP_e, globus pallidus externa; GP_i, globus pallidus interna; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulate; SP, substance P; STN, subthalamic nucleus.](image)

In addition to the classical motor symptoms, many PD patients suffer from a range of non-motor symptoms, such as fatigue and sleep disturbances, depression, cognitive deficits, and autonomic dysfunction (Wolters, 2009). These symptoms are attributed to dysfunctions in other extranigral neuronal networks, and many of them seem to precede the motor symptoms. This suggests that the disease does not actually start in the SN, but is anticipated by dysfunction in other networks. Braak and co-workers have indeed suggested spreading of PD pathology, and according to their theory, the pathologic changes could be initiated in the autonomic nervous system (Braak et al., 2003b; Kaufmann et al., 2004). From the autonomic nervous system, the pathology would then spread to the brain starting from the lower brain stem and olfactory bulb (stage 1-2), to the basal midbrain and...
forebrain (including SN) (stage 3-4), and eventually to the neocortex (stage 5-6) (Braak et al., 2003a). The suggested pattern of PD pathology spreading coincides with many of the symptoms observed in PD patients, going from anosmia, constipation, depression and sleep disturbances, to motor symptoms, and eventually, in the later stages, cognitive dysfunction.

Braak and co-workers based their theory of spreading of PD pathology on results from immunohistochemical staining of α-synuclein (α-syn) (Braak et al., 2003a). α-Syn is the major constituent of Lewy bodies, which are eosinophilic inclusion bodies found in the cytoplasm of surviving neurons and considered to be a pathological hallmark of PD. The Braak hypothesis of α-syn pathology spread is supported by the findings that human embryonic DA nerve cells implanted into the putamen of PD patients develop PD pathology, including Lewy bodies (Kordower et al., 2008; Li et al., 2008). Furthermore, central spreading of α-syn has also been observed in in vitro and in vivo studies following exposure to α-syn (injections or transgenic mice) (Desplats et al., 2009; Luk et al., 2012; Masuda-Suzukake et al., 2013). However, although the data looks convincing, the α-syn seeding theory has lately been heavily debated and the role of α-syn spreading for the PD pathology is still unclear.

**Figure 2.** The complex network of pathological mechanisms that may underlay neurodegeneration in Parkinson's disease and other late-onset neurodegenerative diseases. A combination of age, genetic factors, and environment is thought to mediate changes that eventually result in injury, dysfunction and loss of neurons (Bossy-Wetzel et al., 2004; Wirdefeldt et al., 2011; Dehay et al., 2013; Kalia et al., 2013).

The normal function of α-syn is not completely understood, but the protein is known to be involved in vesicular trafficking and release (Burre et al., 2010; Nemani et al., 2010). α-Syn is characterized by a high degree of plasticity, and whereas the protein is natively unstructured, it has the capability to undergo conformational changes leading to aggregation of the protein monomers (Kalia et al., 2013). Aggregation of α-syn is thought to contribute to the pathogenesis in PD and especially the intermediate oligomeric aggregates of α-syn are considered to be toxic, whereas sequestration of the protein in Lewy bodies could even serve as a protective mechanism. The pathway of α-syn-mediated toxicity is not known, but abnormal aggregation of the protein could induce neuronal death by causing
disturbances in axonal transport and neuronal function, impairment in mitochondrial activity, UPS, and lysosomal and autophagy function, oxidative stress, and activation of inflammation processes (Figure 2) (Bossy-Wetzel et al., 2004; Dehay et al., 2013; Kalia et al., 2013).

Table 2. Selection of genes associated with Parkinson’s disease (according to Houlden and Singleton, 2012).

<table>
<thead>
<tr>
<th>Loci/Gene</th>
<th>Protein</th>
<th>Function; type of mutation</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK1/SNCA</td>
<td>α-synuclein</td>
<td>Synaptic protein, vesicular trafficking and release; gain-of-function</td>
<td>Autosomal dominant; severe parkinsonism with early onset; dementia common; Lewy bodies</td>
</tr>
<tr>
<td>PARK2</td>
<td>Parkin</td>
<td>Ubiquitin-protein ligase; loss-of-function</td>
<td>Autosomal recessive; heterogeneity in age of onset and neuropathological features but often early onset without Lewy bodies</td>
</tr>
<tr>
<td>PARK4/SNCA</td>
<td>Multiplication of SNCA</td>
<td>Excess of α-synuclein</td>
<td>Autosomal dominant; onset (early-to-late) and symptoms depending on number of copies of SNCA (gene dosage)</td>
</tr>
<tr>
<td>PARK6/PINK1</td>
<td>PTEN-induced kinase 1</td>
<td>Mitochondrial protein, provides protection against several stress factors; loss-of-function</td>
<td>Autosomal recessive; onset in the 40s-50s; slow progression; atypical features (dystonia, sleep benefit, pyramidal symptoms); heterogenic neuropathology</td>
</tr>
<tr>
<td>PARK7</td>
<td>DJ-1</td>
<td>Mediates oxidative stress response; loss-of-function</td>
<td>Autosomal recessive; early onset; neuropathology not known</td>
</tr>
<tr>
<td>PARK8/LRRK2</td>
<td>Leucine-rich repeat kinase 2</td>
<td>Cytoplasmic kinase involved in endocytosis and vesicle function; gain-of-function</td>
<td>Autosomal dominant; most common cause of familial PD; age of onset and neuropathological features depending on type of mutation</td>
</tr>
</tbody>
</table>

Besides the fact that α-syn is the major constituent of Lewy bodies, additional clues to the importance of α-syn in the PD pathology come from the observations that mutations (A53T, A30P, E46K) in the α-syn gene (PARK1, SNCA) causes autosomal dominant forms of PD (Table 2) (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004; Kalia et al., 2013). These mutations seem to mediate a gain-of-function promoting aggregation of α-syn. Also multiplication of the α-syn gene has been linked to familial PD (PARK 4) (Singleton et al., 2003; Chartier-Harlin et al., 2004). Other genes associated with heredity of PD are PARK 2 (parkin), PARK 6 (PTEN-induced kinase 1, PINK1), PARK 7 (DJ-1), and PARK 8 (leucine-rich repeat kinase 2, LRRK2) (Table 2) (reviewed by Houlden and Singleton, 2012). The normal functions of the corresponding proteins indicate association between PD pathology and mitochondrial function, oxidative stress, UPS, vesicular trafficking, and lysosomal dysfunction. These processes, together with inflammation and lysosomal impairment, are also thought to contribute to the pathogenesis in sporadic PD (Figure 2).
Why certain neuron populations (e.g., the DA neurons in the SNpc) are especially vulnerable to these changes in PD is still a puzzle. Features that could promote neuron-specific toxicity are type of neurotransmitter and possible reactive metabolites, the morphology of neurons, and pattern of current flow and excitability (discussed by Sulzer and Surmeier, 2013).

The etiology in sporadic PD is still unknown, but the combined sum of age, genes, and environment (exposure to chemicals and life-style factors) is thought to be behind the development of PD (Figure 2) (Wirdefeldt et al., 2011). There is still no treatment available that would slow down the neurodegenerative process in PD. The keystone in the symptomatic treatment of PD is L-DOPA (levodopa, 3,4-dihydroksy-L-phenylalanine), the precursor to DA, which is generally given in combination with inhibitors of peripheral aromatic amino acid decarboxylase (AADC) and catechol-O-methyltransferase (COMT), to increase the levels of L-DOPA in the brain (Fahn, 2003). The treatment aims to reinforce the impaired DA signaling, and is usually effective in earlier stages of PD when the treatment is initiated. In a long-term use, as the disease progresses, the L-DOPA treatment is, however, accompanied by motor disturbances, such as dyskinesias and motor fluctuation (Obeso et al., 2000). Other treatments used in PD to increase the central DA signaling are DA agonists, inhibitors of monoamine oxidase-B (MAO-B, an enzyme participating in the DA metabolism), and amantadine.

2.1.2 ALZHEIMER’S DISEASE
Alzheimer’s disease (AD) is the most common form of dementia and shows a prevalence of 48 per 1000 in the population over 65 years (Hirtz et al., 2007). The most striking neuropathological change in AD is loss of neurons and projections in the neocortex, hippocampus, amygdala and basal nucleus of Meynert together with a robust reduction in cholinergic activity, which clinically results in progressive memory loss and impairment in cognitive functions (loss of attention, changes in personality, speech difficulties, etc.) (Wenk, 2003). The main symptomatic treatment consists of acetylcholine-esterase (AChE) inhibitors, which can provide temporary improvement of neurotransmission and cognitive functions (Bond et al., 2012).

The common view today is that accumulation of misfolded proteins (β-amyloid (Aβ) peptides, hyperphosphorylated tau) leads to failure in neuronal energy production and synaptic dysfunction and contributes to the pathological changes in the AD brain (Figure 3) (reviewed by Querfurth and LaFerla, 2010). Amyloid precursor protein (APP) is a membrane-bound protein that can undergo cleavage by secretases into soluble non-amyloidogenic APP or Aβ peptides of different lengths (reviewed by Selkoe, 2001). Sequential cleavage of APP with β-secretase (mainly beta-amyloid convertase enzyme, BACE 1) and γ-secretase (a complex of four integral membrane proteins, among them presenilin (PSEN)) renders primarily Aβ peptides consisting of 40 or 42 amino acids (Figure 3) (reviewed by Vassar and Citron, 2000). These Aβ peptides can self-aggregate to form soluble oligomers, intermediate filaments or insoluble fibrils (plaques), of which the soluble oligomers and intermediate filaments are considered to have the greatest neurotoxic
potential (discussed by Walsh and Selkoe, 2007). Aβ peptides are formed under normal circumstances (Shoji et al., 1992), but are cleared away by enzymes, such as neprilysin and insulin-degrading enzyme (Miners et al., 2011), by microglial phagocytosis (Rogers and Lue, 2001), perivascular drainage (Weller et al., 2000) or transportation into blood vessels through the blood-brain barrier (BBB) (Deane et al., 2009). The neurotoxic aggregation of Aβ peptides is thus thought to be mediated either by an increase in production, by dysfunction in clearance, or both. There are now clear indications that in late-onset sporadic AD the Aβ peptides are produced at normal levels, but the clearance is impaired (Mawuenyega et al., 2010). Indeed, the most influential gene risk factor for sporadic, late-onset AD is polymorphism in the apolipoprotein E (ApoE) gene (Corder et al., 1993; Bertram and Tanzi, 2005), a protein thought to be involved in the clearance of Aβ (Fagan et al., 2002; DeMattos et al., 2004). In genetically inherited AD, the production of Aβ peptides is instead often altered, and mutations in APP, or PSEN1 or PSEN2 (subcomponents of γ-secretase), have been linked to the disease (reviewed by Bettens et al., 2013).

![Figure 3](image-url)

**Figure 3** The protein aggregation hypothesis of Alzheimer’s disease. Favoring of amyloid precursor protein (APP) cleavage with β/γ-secretases results in the formation of β amyloid peptides (Aβ). Aβ peptides 40/42 amino acids in length are prone to aggregate into oligomers and finally into amyloid plaques. The Aβ aggregates are thought to contribute to neurotoxicity and cell death. In parallel with Aβ aggregation, formation of intracellular neurofibrillary tangles consisting of hyperphosphorylated tau protein is characteristic to AD. The relationship between Aβ/plaque formation and tau/tangle pathology is still unclear. The most established theory, fuelled by the finding of mutations in APP, PSEN1, or PSEN2 in familial early-onset AD, is that abnormal Aβ aggregation is the primary event in AD pathology (Hardy and Selkoe, 2002). Accumulation of extracellular Aβ would then drive the hyperphosphorylation of tau and be the main cause of the pathologic cellular changes in AD. Another alternative theory states that aggregation of Aβ and hyperphosphorylated tau constitutes two different pathways contributing to the pathology of the disease (discussed by Small and Duff, 2008).
Tau is in normal conditions found in axons where the soluble protein promotes stability and assembly of microtubules and is therefore important for vesicle transport along axons (for reviews, see Iqbal et al., 2005; Himmelstein et al., 2012). When tau is hyperphosphorylated, the protein becomes insoluble and can no longer interact with microtubules. Instead it aggregates into paired helical filament structures and further into intermediate aggregates, and finally neurofibrillary tangles (Figure 3). The aggregation of abnormal hyperphosphorylated tau is cytotoxic to neurons (Khlistunova et al., 2006), and has been shown to induce cell loss and behavioral deficits in vivo (Lewis et al., 2000; Santacruz et al., 2005). Furthermore, in vitro and in vivo results indicate that Aβ toxicity requires the presence of tau (Rapoport et al., 2002; Roberson et al., 2007). Even though mutations in TAU have been detected in frontotemporal dementia (for review, see Goedert and Jakes, 2005), no link between these mutations and AD has been found.

2.1.3 HUNTINGTON’S DISEASE
Unlike AD and PD, HD has a clear genetic background. It is a monogenic, fully penetrant disorder caused by abnormal CAG triplet repeats in the 5’ end of the HTT gene (The Huntington’s Disease Collaborative Research Group, 1993). The length of the polyglutamine stretch in HTT is indicative of disease burden (240 repeats cause nearly full penetrance) and age of onset (longer repeats generally cause earlier onset) (Langbehn et al., 2004). It is still unclear why mutated HTT causes HD, and there are different views on whether the abnormally long polyglutamine stretch mediates a gain of toxic function or if the disease is a result of reduction in the normal HTT activity (discussed by Cattaneo et al., 2005; Ross and Tabrizi, 2011). The normal function of HTT is still not completely understood, but the protein has the ability to interact with several proteins indicating that it functions as a scaffold to control and coordinate other proteins (Cattaneo et al., 2005). Moreover, HTT knock-out mice die in early embryonic stage (Duyao et al., 1995; Nasir et al., 1995), and reduced levels of HTT (<50 %) in mice result in impaired neurogenesis and CNS malformation (White et al., 1997), implying that the protein is crucial during the development.

The N-terminal portion of normal HTT is flexible and can adopt different conformations (Kim et al., 2009). In mutant HTT, the expanded polyglutamine stretch seems to force the N-terminal portion of the protein into a compact conformation consisting of short β strands causing aggregation of the protein (Poirier et al., 2005). In HD, large neuronal inclusion bodies of misfolded HTT can be seen mainly in the nucleus, but also in the cytoplasm, dendrites, and axon terminals in the affected areas (Ross and Tabrizi, 2011; Arrasate and Finkbeiner, 2012). There are some discrepancies between the presence and amount of inclusion bodies and extent of neuronal cell death, which indicates that the inclusion bodies might not, themselves, be toxic. Instead, as for Aβ and α-syn, it is hypothesized that oligomeric intermediate aggregates mediate the toxic activity of HTT, while the formation of inclusion bodies may be a protective mechanism that serves to sequester toxic forms of the protein (Sanchez et al., 2003; Ross and Tabrizi, 2011; Arrasate and Finkbeiner, 2012). The most affected cell population in HD is striatal neurons with massive loss of the basal ganglia GABAergic medium spiny projection neurons projecting from the striatum to the GP
and the SN (Figure 1) (Reiner et al., 1988; Vonsattel, 2008; Ross and Tabrizi, 2011). In addition to the extensive loss of striatal neurons, there are also more global changes in the CNS including atrophy of the cerebral cortex, subcortical white matter, thalamus, and specific hypothalamic nuclei in the later stages of the disease (reviewed by Vonsattel, 2008). The mechanisms behind the pathogenicity due to misfolded HTT and vulnerability of striatal GABAergic neurons are summarized in Figure 4.

**Figure 4** Proposed pathologic mechanisms in HD. Misfolded HTT is thought to mediate toxicity by altering the regulation of transcription, axonal trafficking and synaptic transmission, and by mediating mitochondrial dysfunction (Cattaneo et al., 2005; Ross and Tabrizi, 2011). The reason for the striatal vulnerability in HD is still unclear but it is possibly due to reduced neurotrophic support (reduction in transcription and axonal trafficking of the NTF brain-derived neurotrophic factor (BDNF)) (Ferrer et al., 2000; Gauthier et al., 2004; Cattaneo et al., 2005), and excitotoxicity triggered by glutamatergic projections from the cortex (Roze et al., 2011).

As for the other neurodegenerative diseases already discussed, no disease-modifying therapy for HD exists and the disease usually results in death within 10-15 years after symptom onset (Ross and Tabrizi, 2011). The treatments used are aimed at relieving the symptoms, which consist of progressive motor dysfunction (chorea, bradykinesia,
dystonia), psychiatric disturbances (depression, anxiety, aggression, irritability), and cognitive decline (reviewed by Videnovic, 2013). Genetic testing can identify individuals predisposed to the disease before clinical onset which gives a time window that allow the testing of novel treatments that can modify the disease.

2.1.4 AMYOTROPIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is a fatal disease characterized by progressive motor weakness caused by degeneration of lower motor neurons in the lateral horn of the spinal cord and upper motor neurons of the cerebral cortex (Kiernan et al., 2011). The peak onset for ALS happens in late midlife and the incidence in the population in their 60’s is estimated to be 5 per 100 000 with a slightly higher incidence in men than in women (male/female ratio approximately 1.3) (Hirtz et al., 2007).

ALS is a heterogenic disease showing high variability in phenotype and clinical presentation, but despite these variations, the disease generally progresses to paralysis, respiratory failure and death within 3-5 years of symptom onset (Hardiman et al., 2011; Kiernan et al., 2011). The most common form of ALS constitutes a combination of both lower and upper motor neuron degeneration with symptoms starting in the limbs (limb-onset ALS). Loss of lower motor neurons cause symptoms that typically consist of fasciculation and wasting weakness, while upper motor neuron symptoms include spasticity, weakness, and brisk deep tendon reflexes.

As for the above discussed neurodegenerative diseases, the degeneration of motor neurons in ALS is also thought to be a process involving multiple interrelated pathways including excitotoxicity, ion channel dysfunction, oxidative stress, mitochondrial dysfunction, inflammation, and disrupted axonal transport (Figure 2) (Kiernan et al., 2011). In most cases, the diagnosed ALS is labeled as sporadic, while 5-10% of the ALS patients have familial forms of the disease. Many genes have been associated with familial ALS, among them those encoding superoxide dismutase-1 (SOD1), TAR DNA binding protein (TDP-43, or TARDBP), fusion in sarcoma (FUS), ubiquilin 2 (UBQLN2, ubiquitin-like protein), and angiogenin (ANG) (Al-Chalabi et al., 2012). Until recently, mutations in SOD1, mediating excitotoxicity, mitochondrial dysfunction, and oxidative stress, were thought to be the most common genetic risk factor for ALS, accounting for approximately 20% of familial ALS (Kiernan et al. 2011, Al-Chalabi et al., 2012). However, in 2011 a hexanucleotide repeat expansion in the first intron of the yet unknown gene C9orf72 was identified and reported to underly almost 40% of familial ALS in European and US patients (Renton et al., 2011; Majounie et al., 2012). The function of this gene is not yet known, but binding to RNA seems to be involved. Also TDP-43, FUS, and ANG are involved in regulating RNA processing and gene expression, while UBQLN2 has a role in autophagy and protein degradation (Al-Chalabi et al., 2012). The pathogenic mutations in the different ALS-associated genes are manifested as variations in the ALS neuropathology, age of onset, and clinical presentation.

The neuropathology of ALS is generally characterized by abnormal protein inclusions, but the presence and content of these show great variation depending on the type of ALS.
Review of the literature

(Kiernan et al., 2011; Turner et al., 2013). Cytoplasmic ubiquitinated inclusions containing TDP-43 are found in the lower motor neurons in nearly all cases of sporadic ALS (Mackenzie et al., 2007). However, these inclusions are generally not found in ALS patients carrying pathogenic mutations in SOD1 or FUS. In these cases, the inclusions may instead contain aggregated SOD1 or FUS, respectively (Turner et al., 2013). In addition to the ubiquitinated protein aggregates, ALS is associated with the presence of Bunina bodies, which are small eosinophilic intracellular inclusions found in motor neurons in the spinal cord and brainstem (Okamoto et al., 2008). The specific role of the abnormal protein aggregates in ALS is not known, and it is unclear if the protein aggregates themselves mediate toxicity, or whether the cell is merely trying to protect itself by promoting sequestration of pathogenic proteins.

Effort to provide symptomatic relief and preservation of quality of life is the main focus in the treatment of ALS (Hardiman et al., 2011; Kiernan et al., 2011). Despite numerous clinical studies on potentially neuroprotective agents, the only treatment available for ALS today is the glutamate release inhibitor riluzole, which improves the survival time by three to six months.

2.2 EXPERIMENTALLY INDUCED NEURODEGENERATION

Animal models of human diseases are important tools in pharmacological research on the nature of diseases, in the search for novel treatment strategies, and in the pre-clinical evaluation of new therapeutic interventions. Ideally, an animal model should show comparative symptomatology, etiology, and physiological basis (face validity and construct validity), as well as drug effects (predictive validity) to the human condition. Traditionally animal models of neurodegeneration have been developed using specific or non-specific toxins, or surgical procedures (e.g., axotomy). With increasing knowledge about genes that predispose to certain diseases, models based on these genetic alterations have been designed. These so called etiologic models are important tools for studying the pathological basis of diseases, and the rapid growth in this field has been fueled by the enormous progress in our ability to produce genetically modified animals.

2.2.1 TOXIN-INDUCED ANIMAL MODELS

Parkinson's disease

With the help of specific toxins, researchers have tried to model the PD-associated loss of midbrain DA neurons and projections in animals. The most widely used toxin-induced PD models are the 6-hydroxydopamine (6-OHDA) -treated rat and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) -treated mouse and primate. When intracerebrally (i.c.) injected, 6-OHDA is taken up by monoamine transporters (DA and noradrenaline transporters (DAT and NET)) and damage the cells mainly by induction of oxidative stress and mitochondrial dysfunction (Figure 5) (Schwarting and Huston, 1996; Duty and Jenner, 2011). MPTP is used in both mice (mainly C57BL/6) and primates and is converted to the toxic moiety 1-methyl-4-phenylpyridinium (MPP+) in the brain by the sequential action of
MAO-B and spontaneous oxidation (Duty and Jenner, 2011; Bove and Perier, 2012). MPP+ is taken up into DA neurons via DAT, and once inside the cell it accumulates in mitochondria where it inhibits the complex I of the electron transport chain, leading to a decrease in ATP production and induction of oxidative stress. Another complex I inhibitor that has been used to model PD is rotenone, a pesticide that induces cell death mainly by an increase in reactive oxygen species (ROS) (Duty and Jenner, 2011; Bove and Perier, 2012). Imitation of several PD features has also been done with lactacystin, an inhibitor of the UPS (Zhu et al., 2007b; Xie et al., 2010), and with induction of inflammatory processes using lipopolysaccharide (LPS) (Castano et al., 1998; Choi et al., 2009). Figure 5 summarizes the actions of the different toxins. A review of the toxin-induced PD-like properties can be found in Table 3.

Figure 5 Simplified drawing of the proposed mechanisms of toxins used for induction of neurodegeneration of dopaminergic neurons in animal models of Parkinson's disease (according to Duty and Jenner, 2011; Bove and Perier, 2012). DAT, dopamine transporter; MAOB, monoamine oxidase B; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP, 1-methyl-4-phenylpyridinium; 6-OHDA, 6-hydroxydopamine; ROS, reactive oxygen species.

Huntington’s disease
Toxin models of HD have been developed using substances that induce neuronal cell death by triggering excitotoxicity or mitochondrial dysfunction, two mechanisms that have been implied in the pathogenesis of HD (reviewed by Ramaswamy et al., 2007b). The most frequent toxins used in both rats, mice, and non-human primates are quinolinic acid (QA) and 3-nitropropionic acid (3-NP).

QA binds to N-methyl-D-aspartate (NMDA) receptors and causes excitotoxic cell death of the striatal neurons when injected intrastriatally (Schwarcz et al., 1983; Brouillet et al., 1999; Ramaswamy et al., 2007b). Rats treated with QA acid show striatal
neurodegeneration accompanied with a decrease in GABA concentration. In addition, the model is associated with cognitive deficits (Furtado and Mazurek, 1996), and motor dysfunction that can be monitored as asymmetrical behavior in unilaterally QA-lesioned animals (Borlongan et al., 1995; Vazey et al., 2006). The mitochondrial toxin 3-NP inhibits succinate dehydrogenase causing impairment in the energy metabolism, reduction of ATP production, and oxidative stress (Brouillet et al., 1999; Ramaswamy et al., 2007b). 3-NP causes rather selective degeneration of GABAergic neurons in the striatum when given as systemic injections or infusions, indicating that the striatal neurons are especially vulnerable to mitochondrial damage and dysfunction in the energy metabolism. The extent of the lesion and range of behavioral dysfunction varies depending on the injection schedule (e.g., dose of 3-NP and number of repeated injections). In rats there is also a clear strain-dependent difference in toxin sensitivity (Ouary et al., 2000).

Table 3 Overview of the induced phenotypes in toxin and AAV-α-synuclein animal models of Parkinson’s disease (Schwarting and Huston, 1996; Castano et al., 1998; Zhu et al., 2007b; Choi et al., 2009; Xie et al., 2010; Duty and Jenner, 2011; Bove and Perier, 2012; Lindgren and Dunnett, 2012; Lindgren et al., 2012).

<table>
<thead>
<tr>
<th>Model characteristics</th>
<th>6-OHDA</th>
<th>MPTP</th>
<th>Rotenone</th>
<th>Lactacystin</th>
<th>LPS</th>
<th>AAV-α-synuclein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>Rat (mouse, primate)</td>
<td>Mouse, primate</td>
<td>Mouse, rat</td>
<td>Mouse, rat</td>
<td>Mouse, rat</td>
<td>Rat</td>
</tr>
<tr>
<td>Administration of substance</td>
<td>i.c.</td>
<td>i.p., s.c.</td>
<td>i.v., s.c., i.p.</td>
<td>i.c.</td>
<td>i.c.</td>
<td>i.c.</td>
</tr>
<tr>
<td>Striatal dopamine loss/dysfunction</td>
<td>+</td>
<td>+</td>
<td>+³</td>
<td>+ª</td>
<td>+³</td>
<td>+</td>
</tr>
<tr>
<td>Nigral dopamine cell loss</td>
<td>+</td>
<td>+</td>
<td>+³</td>
<td>+ª</td>
<td>+³</td>
<td>+</td>
</tr>
<tr>
<td>Progressive cell loss</td>
<td>5d-4wk²</td>
<td>no</td>
<td>+/-</td>
<td>4-12 wk</td>
<td>no</td>
<td>8-16 wk</td>
</tr>
<tr>
<td>Extra-nigral dysfunction</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n.k.</td>
<td>-</td>
<td>n.k.</td>
</tr>
<tr>
<td>Protein inclusions</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Motor deficits</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Non-motor deficits</td>
<td>+ª</td>
<td>-/</td>
<td>+/-</td>
<td>n.k.</td>
<td>n.k.</td>
<td>n.k.</td>
</tr>
<tr>
<td>Responsiveness to L-DOPA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n.k.</td>
</tr>
</tbody>
</table>

+ detected in the model; -, not detected in the model; +/-, high variability between studies; n.k., not known; ³ depending on dose and/or injection site; ⁴ generally requires bilateral lesion; AAV, adenovirus; i.c., intracerebral; i.p., intraperitoneal; LPS, lipopolysaccharide; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA, 6-hydroxydopamine; s.c. subcutaneous, i.v., intravenous.

Alzheimer’s disease

The cholinergic aspect of AD has been simulated in rodents using different specific and non-specific toxins (Hanin, 1992). Excitotoxins (excitatory amino acids such as kainic acid) are not specific to cholinergic neurons, but by injection into areas with cholinergic nerve
cell bodies in rats, e.g., the basal nucleus of Meynert, loss of cholinergic cells and projections, accompanied by cognitive changes, can be achieved. Ethylcholine aziridinium, AF64A, is a more specific cholinergic toxin. It is a choline analog that is taken up by the high-affinity choline transport system into cholinergic neurons (Fan and Hanin, 1999). At least part of its toxic actions is thought to be due to alteration in choline acetyl transferase (ChAT) mRNA expression and enzyme activity. Administration of activated AF64A (intracerebroventricularly (i.c.v.) or into the hippocampus) causes reduction in cholinergic markers and decrease in the release of acetylcholine (ACh), and induces cognitive impairments including memory and learning deficits in rats (Gower et al., 1989; Hanin, 1992; Fan and Hanin, 1999). The importance of the AD toxin models have diminished as the transgenic models have been developed, but they are still used to some extent for modeling AD in rats.

2.2.2 TRANSGENIC ANIMAL MODELS

Parkinson’s disease

As gene defects underlying familial PD are being identified (for a list of selected genes, see Table 2), transgenic animal models trying to mimic these changes have been created. This far, α-syn has been the main target and α-syn knock-out, over-expressing and transgenic mice have been developed (Harvey et al., 2011; Crabtree and Zhang, 2012). Overexpression of wild type human α-syn in mice results in accumulation of α-syn into neuronal inclusion bodies and the protein aggregation is generally increased in transgenic mice expressing α-syn with PD-associated mutations (e.g., A30P, A53T). The aggregation of α-syn in mice shows some correlation with age-related loss of striatal DAergic terminals, reduced striatal levels of DA and motor impairment, but consistent reports of specific loss of midbrain DAergic neurons are lacking. Although not a subject of this thesis, it could be mentioned that expression of mutated α-syn in Drosophila mimics several features of PD, including motor dysfunction, Lewy-body formation, and age-dependent loss of DA neurons (Feany and Bender, 2000).

Loss of function mutations or knockout/knockdown of PARK2, PARK6, or PARK7 (parkin, PINK1, or DJ-1) also show variable phenotype and although there are reports of motor impairments, DAergic dysfunction and mitochondrial alterations in these models, the nigrostriatal pathology typical to PD is absent (Duty and Jenner, 2011; Harvey et al., 2011; Crabtree and Zhang, 2012). The same holds true for LRRK2 models in which overexpression of mutant LRRK2 has been reported to cause progressive motor dysfunction and alterations in DA signaling, albeit no degeneration of nigral DAergic neurons has been observed. The lack of clear cell pathology does not support the use of these models in pre-clinical animal studies, but they may be used to shed light on the pathogenesis in neurodegenerative diseases.

To imitate the loss of DA signaling in PD, animals with alterations in DA homeostasis (synthesis, metabolism, transport) have been developed (reviewed by Crabtree and Zhang, 2012). In a short summary, mice with reduced expression of the D2 receptor, tyrosine hydroxylase (TH; catecholamine synthesis enzyme), and vesicular monoamine transporter 2
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(VMAT2; pre-synaptic transporter responsible for the uptake of DA into vesicles), as well as increased expression of MAO-B all show PD-like phenotypes. Even though these genetic alterations are not directly associated with human PD, the models can be important in the development of novel DA replacement therapies.

Another genetic model with non-PD-related genetic alterations is the MitoPark mouse (Ekstrand et al., 2007). Based on the idea that mitochondrial dysfunction would play a role in PD pathogenesis, Ekstrand and co-workers developed a conditional knock-out mouse that allowed disruption of the gene encoding mitochondrial transcription factor A (Tfam) specifically in adult DAergic neurons, causing impairment in mitochondrial DNA transcription and respiratory chain dysfunction. The mice showed progressive motor dysfunction that could partially be reversed with L-DOPA treatment. The model was also characterized by progressive loss of midbrain DA neurons and striatal DA neuron innervation that was in addition preceded by the presence of neuronal protein inclusion bodies.

**Alzheimer's disease**

Transgenic models of AD focus on the aggregation of Aβ and include mutations in APP and PSEN (for review, see McGowan et al., 2006; Harvey et al., 2011). Most of the models show increase in insoluble Aβ and plaque formation with a further accelerated rate of amyloid deposition in animals with combined mutations in both APP and PSEN (Borchelt et al., 1997; Holcomb et al., 1998; Chishti et al., 2001). Even though some neuronal loss has been reported in a few models, the extensive neurodegeneration seen in AD is generally lacking (McGowan et al., 2006). Some models are featured by decline in cognitive functions, but no consistent correlation between Aβ levels and degree of memory loss has been found.

**Huntington's disease**

Transgenic mouse models of HD have naturally been generated with focus on HTT (reviewed by Beal and Ferrante, 2004; Vonsattel, 2008; Crook and Housman, 2011). Three alternative strategies have been applied: 1) Random insertion of an N-terminal fragment of HTT (human or chimeric human/mouse gene); 2) Insertion of full-length human HTT with expanded CAG repeats; 3) Introduction of pathological CAG repeats into the mouse Htt. The resulting phenotype (formation of inclusions, degree of cell loss, motor performance, cognitive abnormalities, and survival) varies depending on the type of manipulation (for review, see Crook and Housman, 2011).

**Amyotrophic lateral sclerosis**

Mutations in SOD1 provided the first genetic linkage to ALS and allowed the development of the first transgenic animal models of ALS (Gurney et al., 1994). Today there are many different transgenic SOD1-based ALS models in use and they generally show similarities to the human disease (mitochondrial and axonal dysfunction, progressive motor neuron loss and reduced lifespan) (reviewed by Harvey et al., 2011; McGoldrick et al., 2013). However, the resulting symptoms vary depending on the type of mutation, level of transgene expression, genetic background, and sex. This has made the translation of results from mouse studies to human clinical trials inadequate and may have contributed to the
continuous failure in proving the effect of novel treatment interventions in ALS (Turner et al., 2013). In addition, TDP-43 inclusions that are found in nearly all ALS cases are absent in SOD1-related ALS, and this may also make the generalization of results from animal studies to the human situation unreliable.

As the genetic background of ALS is revealed, new transgenic models are being developed. This far none of the models, including transgenic TDP-43 and FUS animals, have proven to reproduce the ALS phenotype to the same extent as transgenic SOD1 mutants (McGoldrick et al., 2013). Even though several of these models show deficits in motor behavior, axonopathy, muscle atrophy and denervation, the extensive loss of spinal motor neurons is usually absent.

2.2.3 DELIVERY OF PATHOGENIC PROTEIN

**Parkinson's disease**
The accumulation of α-syn seen in PD has been replicated in adult rats (and mice) by intranigral injections of recombinant viral vectors carrying α-syn. Delivery of α-syn gives rise to intracellular overexpression of α-syn and the formation of α-syn-containing inclusions (not, however, identical with Lewy bodies) accompanied by a generally moderate loss (20-50%) of DA neurons in the rat SN (Kirik et al., 2002b; Klein et al., 2002; Lo Bianco et al., 2002). With the advance in viral vector design, Decressac and co-workers (Decressac et al., 2012) reported the use of a more efficient adeno-associated viral (AAV) vector. With their AAV-α-syn vector, they achieved a progressive impairment in motor behavior in rats reaching its maximum 8-16 weeks after the injection. During this time interval, the rats showed a progressive approximately 70-80% loss of DA neurons in the SN as well as progressive loss of DAergic fibers and striatal DA levels (phenotype compared to toxin-induced animal models of PD in Table 3). The morphologic changes were preceded by impairment in DA release and re-uptake (Lundblad et al., 2012), and reduced expression of proteins participating in DA synthesis, metabolism, and transmission (Decressac et al., 2012).

**Alzheimer’s disease**
Small Aβ oligomers have been used to induce AD-like symptoms in rats and mice. I.c.v. injection of Aβ oligomers proved to inhibit hippocampal long term potentiation (LTP) (Walsh et al., 2002), and caused disruption of learned behavior in rats (Cleary et al., 2005). Also hippocampal injections of Aβ peptides induced delayed cognitive deficits (Cleary et al., 1995; O’Hare et al., 1999) and reduced cortical levels of BDNF in rats (Christensen et al., 2008). AD-like pathology has also been replicated in rats by viral vector-mediated overexpression of Aβ peptides in the hippocampus of adult rats (Lawlor et al., 2007). These rats show increase in Aβ levels (but no plaque formation), accompanied by mild behavioral and cognitive deficits.

**Huntington's disease**
Delivery of mutant HTT into the brain (mainly striatum) of rats, mice and non-human primates to model HD has been done using viral vectors (reviewed by Ruiz and Deglon,
2012). Overexpression of the mutant protein has been shown to induce formation of inclusions, neuronal dysfunction, neuronal loss, as well as motoric dysfunction.

**Amyotrophic lateral sclerosis**
Injection of a recombinant AAV vector carrying the gene for human wild-type TDP-43 into the cervical spinal cord of both rats and non-human primates was done by Uchida and co-workers (2012). Overexpression of TDP-43 resulted in progressive muscle weakness and muscle atrophy in both rats and primates. However, whereas injection of AAV-TDP-43 was associated with cytoplasmic aggregates and motor neuron and axon degeneration in primates, no such changes were observed in rats, indicating species-specific differences in the TDP-43-associated pathology.

### 2.3 GENE TRANSFER METHODS

Gene transfer can generally be used to study the function of a specific gene, to provide a therapeutic gene, or to correct for a mutated malfunctioning endogenous gene (Vannucci et al., 2013). In therapeutic approaches, the advantage of gene therapy is continuous targeting of a specific region of interest while overcoming certain problems associated with administration, uptake and distribution. This can provide substantial benefits when trying to target chronic diseases in the brain (Björklund et al., 2000). Gene therapy can roughly be divided into in vivo and ex vivo gene therapy, and in this chapter I will review these gene delivery methods from the perspective of CNS disorders.

#### 2.3.1 IN VIVO GENE DELIVERY

*In vivo* gene delivery is defined as the direct insertion of a gene into the patient’s or animal’s own cells forcing the endogenous cells to produce the protein of interest (Feigin and Eidelberg, 2007). This can be achieved by the use of recombinant viral vectors, or with liposomes and nanoparticles carrying the transgene of interest.

Viral vectors are created by rearrangement of the viruses’ own genome so that while maintaining the infectious properties, the replication and production of new viral particles are prevented (Vannucci et al., 2013). In terms of CNS disorders, AAV, lentivirus (LV), adenovirus, and herpes simplex virus (HSV) have been the mostly studied vectors (Feigin and Eidelberg, 2007; Lim et al., 2010; Vannucci et al., 2013). These all show different characteristics in terms of the nucleic acid they are carrying (DNA, RNA, single- or double-stranded), the size of transgene that can be incorporated, infectiousness, tropism to certain cell types, as well as onset and duration of the induced expression (Table 4). AAV vectors have been the main viral vectors used to deliver therapeutic genes in clinical trials of neurodegenerative diseases (Lim et al., 2010), and I will continue to discuss the attributes of this vector.

*Wild-type AAV* is a small, icosahedral non-enveloped virus containing single-stranded DNA coding for four *rep* genes (needed for virus replication) and three *cap* genes (coding for
structural proteins), flanked by short inverted terminal repeats (ITRs) (Rose et al., 1971; Samulski et al., 1983; Mendelson et al., 1986; Vonsattel, 2008). Following infection, the wild-type virus genome is integrated into the host genome, but AAV cannot complete its replicative life cycle until a helper virus (e.g., adenovirus, HSV) has activated the cell. In recombinant AAV vectors, the rep and cap genes have been replaced with the transgene (containing promoter, cDNA, and poly-adenylation signal) (Figure 6) (Lim et al., 2010; Vannucci et al., 2013). To produce viral vector particles in vitro, the rep and cap genes will therefore need to be supplied by co-transfection of a rep- and cap-containing plasmid. In addition, the helper function is applied either by co-transduction with a helper virus (e.g., adenovirus) or, more commonly today, by co-transfection with plasmids containing the helper genes, or using cell lines expressing these genes.

### Table 4 The characteristics of viral vectors proposed for use in neurodegenerative disorders (according to Lim et al., 2010; Vannucci et al., 2013).

<table>
<thead>
<tr>
<th>Viral vector</th>
<th>Genome</th>
<th>Transgene max. size</th>
<th>Cell tropism in CNS</th>
<th>Existence in host cell</th>
<th>Onset/duration of expression</th>
<th>Immuno-genicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV, recombinant</td>
<td>ssDNA</td>
<td>2-6kb</td>
<td>Neurons (glia)</td>
<td>Episomal</td>
<td>Days-weeks/Months-years</td>
<td>Low</td>
</tr>
<tr>
<td>Lentivirus, recombinant</td>
<td>ssRNA</td>
<td>6-9kb</td>
<td>Neurons, glia</td>
<td>Integrated</td>
<td>Week/Months-years</td>
<td>Low</td>
</tr>
<tr>
<td>Adenovirus, wild-type or recombinant</td>
<td>dsDNA</td>
<td>8-30 kb</td>
<td>Glia (neurons)</td>
<td>Episomal</td>
<td>Days/Weeks-year</td>
<td>High</td>
</tr>
<tr>
<td>HSV, recombinant or amplicon</td>
<td>dsDNA</td>
<td>40-150kb</td>
<td>Neurons</td>
<td>Episomal</td>
<td>Days/Weeks-year</td>
<td>High</td>
</tr>
</tbody>
</table>

AAV, adeno-associated virus; HSV, herpes simplex virus; ss, single stranded; ds, double stranded

AAV has the ability to transduce both dividing and non-dividing cells, and in the CNS AAV vectors transduce mainly neurons, but also glial cells (Lim et al., 2010; Chtarto et al., 2013b). The specific cell tropism of AAV is determined mainly by the cap genes, since the capsid structure mediates the interaction between host cell and viral vector particle, including binding to host cell membrane receptor, internalization, and genome delivery into the cell nucleus (Chtarto et al., 2013b). By selecting or modifying the rep/cap source (serotype) in the production of recombinant AAV particles, the resulting transduction and transgene expression in a certain cell type (e.g., neurons vs. glial cells in the CNS), or subpopulation of cells can be altered. This far, AAV serotype 2 has been most extensively applied in both clinical and pre-clinical settings. There are, however, indications that other serotypes or hybrid serotypes would provide better spread and more efficient transduction (Burger et al., 2004; Asokan et al., 2012). Selection of transgene promoter can further affect the tropism and cell-specific expression of the transgene (Chtarto et al., 2013b).

Since the AAV DNA genome consists of single-stranded DNA, production of double-stranded DNA is required before the transgene expression can start. This makes the AAV
vectors somewhat slow and limits their use in certain in vivo applications. The development of AAV vectors containing double-stranded (Wang et al., 2003) or self-complementary DNA (McCarty et al., 2003) has therefore provided an important improvement to the AAV technology, allowing earlier onset of transgene expression following AAV transduction. However, a drawback with self-complementary AAV vectors is the lowering of transgene capacity by approximately half of the normal.

**Figure 6** Production of recombinant AAV vectors. Transfection of cells with a plasmid containing necessary rep and cap genes and the recombinant AAV vector plasmid together with a helper plasmid renders AAV particles containing the transgene of interest (Lim et al., 2010; Vannucci et al., 2013).

### 2.3.2 EX VIVO GENE THERAPY

In ex vivo gene therapy, genetically modified cells expressing the protein of interest are implanted/transplanted into the host CNS. Cells used for this purpose are neural stem cells (NSC, from host or embryo), embryonic stem cells (ESC), re-programmed adult fibroblasts, or different cell lines that have been modified to ensure survival and continuous proliferation by fusion with tumor cells or insertion of transforming agent (Mejia-Toiber et al., 2011). The cells are then further modified in vitro to transgene-expressing cells. The transgene can be delivered into the cells by non-viral (e.g., with liposomes, plasmids, electroporation, “gene gun”) or viral vector methods, and once the transgene expression is ensured, the cells can be implanted / transplanted into the host, where they function as small protein pumps, delivering the therapeutic agent to the host tissue.

The implanted / transplanted cells can elicit an immune response in the host, resulting in rejection of the introduced cells (Deierborg et al., 2008; Mejia-Toiber et al., 2011). In addition, there is a risk for overgrowth of stem cells or immortalized cell lines with sequential tumor formation. Both of these concerns can be overcome by encapsulating the transplanted cell population with a semi-permeable material, allowing influx of nutrients and efflux of the therapeutic protein. The encapsulation has also the advantage of allowing retraction of the genetically modified cells in case of any safety concern or when only transient delivery of the therapeutic protein is needed.
2.3.3 RNA INTERFERENCE

In addition to introduction of transgenes into the host cells, the spectra of expressed proteins can also be modified by post-transcriptional sequence-specific silencing of genes by RNA interference (RNAi).

Initiation of RNA interference happens as double-stranded homologues RNA molecules are cleaved by the double-stranded RNA-specific endonuclease Dicer into shorter fragments (around 20 nucleotides) called small interfering RNAs (siRNA) (Raoul et al., 2006). Alternatively, siRNA can be synthesized by Dicer-mediated cleavage of single-stranded short hairpin RNA (shRNA), which in turn can be generated by transcription of a DNA template introduced in experimental settings into the host cell by viral or non-viral vector methods. After cleavage, the siRNA is unwound into single-stranded RNA and one of the strands (guide strand) is incorporated into the RNA-induced silencing complex (RISC) in which it functions as an antisense strand guiding the highly specific degradation of an mRNA.

Another way to target mRNAs is to use specific antisense oligonucleotides (ASO) (Devos and Miller, 2013). ASOs are short nucleic acid sequences made to complement a target mRNA. The result is an RNA:DNA complex that can prevent splicing, processing, or translation of the targeted mRNA. In addition, ASOs can direct RNA cleavage through the action of RNAseH, an enzyme that targets RNA:DNA duplexes and cleaves the RNA strand to release the DNA (Devos and Miller, 2013). Delivery of ASOs to the CNS can be done by direct infusion into the cerebrospinal fluid (CSF), or, alternatively, packed into vectors for delivery.

2.4 STRATEGIES USING GENE THERAPY IN NEURODEGENERATIVE DISORDERS

When examining the potential of gene therapy for the treatment of neurodegenerative diseases, three different strategies could be applied (Figure 7):

1) Symptomatic gene therapy. The neurodegenerative diseases described above are characterized by vulnerability of specific neuronal populations (nigral DAergic neurons in PD, AChergic neurons in AD, striatal GABAergic medium spiny neurons in HD, and motoneurons in ALS) (Table 1) (Fahn, 2003; Wenk, 2003; Kiernan et al., 2011; Ross and Tabrizi, 2011). The loss of these neurons leads to the loss of specific neurotransmitters and decrease in signaling resulting in the symptoms typical to each disorder. To re-establish the lost signaling, the production or availability of neurotransmitters should be increased. This could be done by addition of genes encoding enzymes needed for the synthesis of neurotransmitters, or other proteins important for the synaptic transmission (e.g., storage, re-uptake).

2) Elimination of pathogenic protein. The four neurodegenerative diseases described are all featured by deposition of protein aggregates (Ross and Poirier, 2004). Even if the
role of these protein inclusions is not entirely known, they are thought to contribute to the pathogenesis. Mutations in the proteins forming the main constituents of the inclusions have also a strong connection to familial forms of the diseases (APP, PSEN1, PSEN2 in AD; α-syn in PD; HTT in HD; TDP-43, FUS, and SOD1 in ALS) (Kiernan et al., 2011; Ross and Tabrizi, 2011; Houlden and Singleton, 2012; Bettens et al., 2013). To decrease the formation of inclusions, different approaches can be applied. The expression of the mutated protein can be silenced using siRNA. The clearance of the protein can be increased by adding metabolic enzymes, or by enhancing the function of chaperon-mediated protein refolding, UPS or autophagy-lysosome system.

3) Addition of a therapeutic endogenous or exogenous gene. Of all the substances that could be considered to belong to this category (e.g., anti-apoptotic proteins, proteins that affect the division, migration or differentiation of neural progenitor cells), the NTFs are the most interesting. NTFs have received attention because of their survival-promoting effects on neurons (Bespalov and Saarma, 2007), but also since dysfunction in NTF signaling has been reported in several neurodegenerative disorders (Connor et al., 1997; Strand et al., 2007). Gene therapy aiming to replenish NTFs, or increase their signaling through addition of specific signaling molecules, might therefore serve as a disease-modifying therapy with potential to halt, or even reverse, the degeneration of neurons.

Figure 7 Different approaches that can be exploited in the search for novel therapies for neurodegenerative diseases.

2.4.1 SYMPTOMATIC GENE THERAPY
Symptomatic gene therapy aims at improving the clinical status of the patients without targeting the underlying pathology. This therapeutic approach is most straightforward in the treatment of PD, in which the characteristic motor symptoms originate from loss of DA
signaling in the nigrostriatal pathway (see paragraph 2.1.1). In addition, this strategy should be feasible in PD, since a relatively small (approximately 10%) increase in striatal DA levels is enough to mediate clinical improvement (Pavese et al., 2006).

**Parkinson’s disease: targeting the dopamine synthesis**

The adverse effects of oral L-DOPA treatment in PD are thought to be partly due to the continuous fluctuation in the L-DOPA level caused by oral drug administration (Obeso et al., 2000). This could be improved with continuous supply of L-DOPA or DA, e.g., by addition of enzymes required for their synthesis. DA is synthesized from tyrosine by the sequential action of TH and AADC (Figure 8) (Elsworth and Roth, 1997). For effective conversion of tyrosine to L-DOPA, TH requires the presence of its cofactor tetrahydrobiopterin (BH4). The rate-limiting step in the BH4 synthesis is in turn the conversion of guanosin triphosphate (GTP) to dihydroneopterin triphosphate by the enzyme GTP cyclohydrolase 1 (GCH1). When scrutinizing the DA synthesis pathway (Figure 8), the possible gene therapy alternatives for enzyme replacement in PD could therefore aim at 1) increasing only TH levels (with or without addition of GCH1), 2) introduction of TH (and GCH1) together with AADC, 3) addition of AADC alone (Elsworth and Roth, 1997; Björklund and Kordover, 2010).

![Figure 8 Dopamine (DA) synthesis and metabolism. DA is synthesized by the sequential action of tyrosine hydroxylase (TH; in combination with its cofactor tetrahydrobiopterin (BH4)), and L-amino acid decarboxylase (AADC). In the presynaptic terminal, DA is transported into storage vesicles by the vesicular monoamine transporter (VMAT). After release, DA undergoes re-uptake by the DA transporter (DAT) and is either recycled into storage vesicles, or metabolized by monoamine oxidase B (MAO-B). Alternatively, DA in the synaptic cleft can be internalized into other neurons or glia by an uptake 2 mechanism and undergo subsequent metabolism in these cell (Iversen et al., 2009). COMT, cathecol-O-methyltransferase; DOPAC, 3,4-dihydroxyphenylacetic acid; GCH, GTP cyclohydrolase; GTP, guanosin triphosphate; HVA, homovanillic acid; 3-MT, 3-methoxytyramine.](image)

**Tyrosine hydroxylase**

Introduction of human TH in the rat 6-OHDA model of PD using HSV (During et al., 1994) or AAV vectors (Kaplitt et al., 1994; Fan et al., 1998) has resulted in partial, although statistically significant, recovery of rotational asymmetry. Addition of only TH is however
generally considered as a rather ineffective treatment alternative due to the ongoing degeneration and loss of other enzymes and co-factors required for the DA synthesis (Björklund and Kordower, 2010).

**Tyrosine hydroxylase and GTP cyclohydrolase 1**

With the progressive degeneration of DA neurons, also the levels of BH4 are reduced, leading to a decrease in TH activity (Levine et al., 1981; Björklund et al., 2009; Cederfjäll et al., 2012). Indeed, in *in vivo* studies the production of L-DOPA following viral vector-mediated TH gene delivery is substantially increased after administration of exogenous BH4 in 6-OHDA-lesioned rats (Mandel et al., 1998; Corti et al., 1999). The BH4 levels can also be continuously increased by introduction of the *GCH1* gene. Several studies have shown that co-transduction with recombinant AAV-TH and AAV-GCH1 vectors results in marked improvement in motor behavior tests, as well as in a reduction of L-DOPA-induced dyskinesias in 6-OHDA-lesioned rats (Kirik et al., 2002a; Carlsson et al., 2005; Björklund et al., 2010). Because of the limited genome size of the AAV, all of these studies have used two AAV vectors to deliver the genes separately. This may affect the outcome since the same cells are not necessarily transduced by both vectors. Recently, Cederfjäll and co-workers (2012) reported the use of a recombinant pseudotyped AAV2/5 vector containing both *TH* and *GCH1* controlled as two different expression cassettes, ensuring the optimal *TH:* *GCH1* expression ratio of 5:1 (Björklund et al., 2009). Again, the treatment with this vector showed robust effect in the rat 6-OHDA model and resulted in recovery of motor function as well as increase in striatal DA levels (Cederfjäll et al., 2012).

**Tyrosine hydroxylase, GTP cyclohydrolase 1, and aromatic L-amino acid decarboxylase**

The introduction of only *TH* and *GCH1* transgenes relies on further decarboxylation of L-DOPA into DA in striatal serotonergic and spared DAergic fibers (Hökfelt et al., 1973; Björklund et al., 2010). In the rat dorsal striatum, 90% of the AADC activity seems to originate from the DAergic terminals and the AADC activity level is substantially reduced in 6-OHDA-lesioned rats (Cederfjäll et al., 2012). In addition, the effect of TH and GCH1 gene therapy is greatly enhanced in rats with partial DAergic lesions, as compared to more complete lesions, indicating that remaining DAergic axons in the striatum provide important functions in the synthesis and controlled storage and release of DA (Kirik et al., 2002a). Enzyme replacement aimed at increasing DA levels should therefore most likely also involve introduction of AADC. Indeed, triple transduction of striatal cells using recombinant AAV vectors containing AADC, *TH*, and *GCH1* proved to efficiently increase DA synthesis and reduced drug-induced rotational asymmetry in 6-OHDA-lesioned rats (Shen et al., 2000). When the same treatment was applied in MPTP-treated monkeys, the animals showed marked and long-lasting motoric recovery (Muramatsu et al., 2002).

In the first studies of triple gene therapy, the full-length TH was used. TH is known to be negatively regulated by catecholamines (Fujisawa and Okuno, 2005), and the accumulation of DA in the cytosol following co-transduction of the cells with *TH*, *GCH1*, and AADC can cause suppression of the TH activity and decrease in DA synthesis. The negative feedback of catecholamines on TH activity is mediated through the N-terminal end of the enzyme, and by removing this fragment, TH can be rendered constitutively active (Moffat et al.,
Azzouz and co-workers (2002) designed a LV vector derived from the equine infectious anemia virus (EIAV) that carried the gene for the N-truncated form of TH, together with GCH1 and AADC and tested this trisistronic vector in 6-OHDA-lesioned rats. The treatment resulted in partial recovery of drug-induced rotational behavior and striatal DA levels. Following further development of the EIAV vector, a similar therapeutic approach was tested in MPTP-treated macaques (Jarraya et al., 2009). Treatment of the MPTP-lesioned primates with the modified EIAV-TH-AADC-GCH1 vector significantly improved the motor performance and reduced L-DOPA-induced dyskinesia as well as normalized the striatal DA levels and restored dysfunctions in the basal ganglia circuitry. With these encouraging results, the EIAV vector under the brand name ProSavin® entered clinical trials. The results from the phase I/II clinical trial in patients with mid-to-late-stage PD have not yet been published, but Oxford Biomedica states in their homepage that the treatment has proven to be safe and led to improvement in motor function at six months post-injection (Oxford Biomedica, 2013).

Aromatic L-amino acid decarboxylase

A third strategy to increase the DA levels in PD would be to deliver AADC alone (Björklund and Kordower, 2010). This approach aims at replenishing the striatal tissue with AADC to improve the conversion of orally administered L-DOPA into DA in the striatum. Thus, the effect of the treatment is controlled by a peripherally given drug (L-DOPA) that, in case of safety considerations, can be removed. When recombinant AAV-AADC vectors were injected into the striatum of 6-OHDA-lesioned rats, an increase in striatal AADC activity and DA levels (after peripheral administration of L-DOPA) were reported (Leff et al., 1999; Sanchez-Pernaute et al., 2001). In addition, the L-DOPA-induced contralateral turning behavior was stronger in AAV-AADC-treated rats, also indicating a more efficient conversion of L-DOPA into DA in these rats (Sanchez-Pernaute et al., 2001). In MPTP-treated monkeys AAV-AADC treatment resulted in a normalization of AADC activity, measured as striatal uptake of 6-[18F]fluoro-L-m-tyrosine ([18F]FMT) detected with positron emission tomography (PET), and increase in striatal DA levels following administration of L-DOPA (Bankiewicz et al., 2000). Some of the monkeys in this study have been monitored for up to eight years, showing consistent normalization in striatal [18F]FMT uptake and improvement in a Clinical Rating Scale after administration of L-DOPA (Bankiewicz et al., 2006; Hadaczek et al., 2010).

To this date, one phase I study of AAV-AADC treatment in ten PD patients has been completed. The first reports from the clinical trial showed an increase in the striatal [18F]FMT uptake and improvement in the Unified Parkinson’s Disease Rating Scale (UPDRS) in both “on” and “off” medication stages up to 6 months post-operation (Eberling et al., 2008; Christine et al., 2009). In addition, the treatment was associated with a reduction in the “off” time, while the “on” time was correspondingly increased. In a follow-up study of the patients, the uptake of [18F]FMT was shown to remain elevated for up to four years, while the UPDRS scores, after the first 12 months of improvement, showed slow deterioration during the following three years (Mittermeyer et al., 2012). The authors concluded that the initial dramatic improvement in UPDRS scores could partly be due to a placebo effect which should be controlled in following studies. Moreover, they suggested...
that the transduced area of the human striatum was too limited, and state that this issue will be addressed in a follow-up phase I clinical trial (Mittermeyer et al., 2012).

Parkinson’s disease: modulating basal ganglia signaling with glutamic acid decarboxylase

As described in paragraph 2.1.1 (Figure 1), loss of DA neurons in PD results in dysregulation of the basal ganglia circuitry. In addition to the DA replacement therapy presented above, the dysfunction in the basal ganglia could be targeted by alternative strategies (Björklund and Kordower, 2010). One such approach is the delivery of the gene for glutamic acid decarboxylase (GAD) into the STN of PD patients. This treatment aims at increasing GABA production and thereby suppressing the STN activity with subsequent improvement in clinical symptoms.

Luo et al. (2002) showed that transduction of the excitatory glutamatergic neurons in the STN with a recombinant AAV-GAD vector resulted in inhibitory signaling and GABA release in the SNpr in rats. When the treatment was tested in 6-OHDA-lesioned rats, the overexpression of GAD was able to normalize motor abnormalities and partially protected DAergic neurons in the midbrain. When the same treatment was applied in unilaterally MPTP-lesioned macaques, improvements (although rather small) in the Clinical Rating Scale and increased activity in the ipsilateral motor cortex were observed (Emborg et al., 2007). The treatment also seemed to be safe, and on the basis of these results, 12 PD patients were enrolled in an open-label phase I clinical trial (Kaplitt et al., 2007).

In the clinical trial the PD patients received unilateral injections of three different doses of AAV-GAD into the STN (Kaplitt et al., 2007). Three months post-injection, the patients showed significant improvement in both “on” and “off” medication UPDRS motor scores, with more substantial benefits in the limbs on the contralateral side. The metabolic activity detected with $^{18}$F]fluorodeoxyglucose ($^{18}$F]FDG) PET was decreased in the ipsilateral STN, while an increase in activity was observed in the motor cortex (Kaplitt et al., 2007). The study of AAV-GAD continued as a phase II double-blind, sham surgery-controlled, randomized trial (LeWitt et al., 2011). The published results from this study cover the first six months post-operation, and show significant improvement in UPDRS "off" medication scores in AAV-GAD-treated patients, as compared to patients that underwent sham surgery. No serious adverse events that would have been attributed to the AAV-GAD treatment were observed in either of the clinical trials (Kaplitt et al., 2007; LeWitt et al., 2011).

Alzheimer’s disease: targeting acetylcholine synthesis and metabolism

In Alzheimer’s disease, dysfunction and degeneration of especially AChergic networks are thought to be the primary cause of the cognitive symptoms seen in the disease. In addition to targeting the ACh signaling using small molecular AChE inhibitors, the level of ACh can also be affected with gene therapy strategies.

One strategy to increase the level of ACh would be to down-regulate the expression of AChE. This approach was used by Fu and co-workers (Fu et al., 2005), who injected
liposomes carrying an AChE-ASO i.c.v. in wild-type and AD mice (i.c.v. injection of Aβ 11 days prior to treatment). The ASO caused a decrease in cortical AChE activity with a corresponding increase in ACh levels. In AD mice, the AChE-ASO was able to normalize Aβ-induced memory impairment. The observation time in the study was short, and it is unclear whether these effects would be maintained during longer periods of time.

The level of ACh can also be raised by increasing the synthesis, e.g., by supplying the tissue with choline acetyl transferase (ChAT). To achieve this, Park and co-workers (Park et al., 2012a; Park et al., 2012b) used ex vivo gene therapy and transferred an NSC line over-expressing ChAT by i.c.v. injections in two different rat models of AD (kainic acid and AF64A models). Following injection, the NSCs were found to be diffusely distributed in different brain regions (hippocampus, thalamus, hypothalamus, cortex and septum) and nine weeks post-injection, differentiation of these NCSs into astrocytes and neurons was observed (Park et al., 2012b). In both models, the treatment with ChAT-expressing cells resulted in restoration of ACh levels in the cerebrospinal fluid and normalization of learning and memory functions in the rats (Park et al., 2012a; Park et al., 2012b). Gene therapy aiming at modulating the ACh levels have not yet been tested clinically.

2.4.2 ELIMINATION OF PATHOGENIC PROTEIN

The idea that misfolded abnormal proteins would be a trigger of pathogenesis in neurodegenerative diseases would suggest that by removing these pathogenic proteins, neuroprotection could be achieved (for overview of aggregates typical to each disease, see Figure 9). The elimination or reduction of abnormal proteins can be done either by silencing the expression or by increasing the clearance of the proteins (San Sebastian et al., 2013).

Figure 9 Protein aggregation in late-onset neurodegenerative diseases.

Silencing of pathogenic protein

RNAi, the targeting of an mRNA for degradation with siRNAs or ASOs, can be used to silence the expression of specific proteins. The use of these techniques in neurodegenerative diseases has been most widely exploited in HD.
Silencing of huntingtin
Several rodent studies show that silencing of HTT with siRNAs delivered to the striatum with AAV vectors (Boudreau et al., 2009) or as cholesterol conjugates (DiFiglia et al., 2007) has a beneficial effect on both neuropathology and motor dysfunction in mouse models of HD. These studies used siRNAs for nonallele-specific silencing of HTT, affecting both wild-type and mutated protein. Since wild-type HTT is thought to execute important functions in the normal brain, it would be beneficial to avoid any excessive decrease in the levels of wild-type HTT. This can be achieved with siRNAs targeting specifically the mutated allele (Hu et al., 2009; Pfister et al., 2009). However, McBrinde et al. (McBride et al., 2011) did not observe any toxicity after nonallele-specific reduction of HTT by 45% in the striatum of intact rhesus macaques. Also, infusion of ASOs designed to target human HTT were tested in three different transgenic mouse models of HD (Kordasiewicz et al., 2012). I.c.v. infusion of HTT-ASOs into these mice resulted in suppression of HTT levels accompanied with marked changes in the pathologic phenotype, including delayed disease progression, behavioral improvement, and an increased lifespan. Kordasiewicz and co-workers (2012) continued their work by infusing an ASO complementary to both human and rhesus monkey HTT into the CSF of rhesus macaques, showing widespread reduction in HTT levels in the brain, with highest levels of suppression in the cortex and spinal cord.

Silencing of α-synuclein
There are a few reports on suppression of α-syn levels using RNAi in PD animal models. Sapru and colleagues (Sapru et al., 2006) showed that LV-mediated delivery of an shRNA specific for human α-syn was able to markedly reduce the levels of α-syn in rats with LV-induced striatal overexpression of α-syn. In another study, the silencing of human α-syn was tested in rats displaying motor dysfunctions and DAergic cell loss due to an intranigral injection of AAV-α-syn (Khodr et al., 2011). Although the silencing of human α-syn in these rats resulted in normalization of forelimb use, no protection of the nigral DAergic neurons was observed. In fact, the AAV-mediated delivery of shRNA specific for human α-syn to the nigra of healthy rats turned out to cause neuronal cell loss in the SNpc. Also injection of the control vectors caused reduction in TH-reactivity, although to a lesser extent than α-syn-shRNA vectors. The authors speculate that the toxic effects of their shRNA could be caused by unspecific silencing of rat α-syn or be a result of toxic shRNA effects caused by saturation of the RNAi processing in the cell (Khodr et al., 2011). Also Gorbatyuk and co-workers (2010) reported nigrostriatal neurodegeneration following silencing of rat endogenous α-syn. However, they found that the neurotoxicity of AAV-delivered shRNA was attributed to α-syn suppression, since none of the control vectors used in their study showed signs of neuronal cell loss. However, in a study in healthy squirrel monkeys, siRNA specific for α-syn was continuously infused into the SN showing a significant reduction of α-syn levels with no signs of toxicity (McCormack et al., 2010). These controversial results clearly point out the problems with silencing of the expression of proteins potentially important to the cells. We still do not know how big reduction in the normal protein expression can be tolerated in long-term, and using gene silencing as a therapeutic approach will still need to be thoroughly examined before the potential of this treatment alternative can be evaluated.
Silencing of SOD1
The expression of SOD1 has been suppressed both in vivo and clinically in ALS patients. In SOD1 transgenic mice, both intramuscular (i.m.) (Ralph et al., 2005) and intraspinal (Raoul et al., 2005) injections of LV-shRNA vectors, mediating silencing of human SOD1, were shown to delay the onset of disease, improve motor function and motoneuron survival, and prolonged the lifespan of the mice. Infusion of an SOD1-ASO into the CSF of transgenic rats resulted in wide distribution of the ASO and suppression of the levels of human SOD1 in the rats (Smith et al., 2006). Furthermore, the treatment was associated with significantly slower disease progression and prolonged survival. Recently, a report of the first clinical trial using SOD1-ASO in SOD1 familial ALS patients was published (Miller et al., 2013). This phase I randomized placebo-controlled trial was assigned to assess the safety of intrathecal infusions of the SOD1-ASO. The ASO was infused during 11.5 h, and the patients were followed up to 28 days post-infusion. The treatment was proven to be safe with some mild to moderate adverse events that were attributed to the infusion method and not to the treatment itself. The patients were also evaluated using an ALS functional rating scale, but SOD1-ASO treatment showed no clear benefit over placebo. When writing this text, no follow-up study has been announced.

Targeting the clearance of pathogenic protein
The Clearance of pathogenic proteins can be targeted, e.g., by enhancing the protein-specific metabolism, through the UPS or autophagy, or by immunization. This strategy to lower the levels of pathogenic protein has rendered special interest for the treatment of AD, since aggregation of Aβ in sporadic AD is thought to be due to a decrease in its clearance, and not an increase in production.

Targeting protein metabolism
The best example of increasing the expression or activity of metabolic enzymes to enhance the clearance of pathogenic protein comes from AD research. As already mentioned, Aβ can be enzymatically degraded by, e.g., neprilysin. Using transgenic mouse models expressing human mutated APP, injections of LV (Marr et al., 2003; Spencer et al., 2008) or AAV vectors (Iwata et al., 2004) encoding human neprilysin have been done into the frontal cortex and/or hippocampus. In these studies, overexpression of neprilysin has significantly reduced the levels of Aβ (Marr et al., 2003; Iwata et al., 2004; Spencer et al., 2008) and improved the performance in cognitive tasks (Spencer et al., 2008). Similar results have also been obtained with AAV-neprilysin treatment in APP + PSEN1 double-mutant mice (Carty et al., 2013) and with ex vivo gene therapy exploring the transplantation of neprilysin-expressing fibroblasts into the hippocampus of APP mice (Hemming et al., 2007).

Following unilateral i.c. delivery of neprilysin, a reduction of Aβ levels was also observed in the contralateral side (Iwata et al., 2004; Carty et al., 2013). In addition, peripheral (into the hind limb) delivery of AAV-neprilysin to transgenic mice expressing mutant human APP, PSEN1 and tau, reduced the levels of soluble Aβ and plaque load in the mouse brain (Liu et al., 2009). The same results were also achieved with intravenous (i.v.) transplantation of neprilysin-expressing leukocytes (Guan et al., 2009). Looking from a clinical perspective, the
Review of the literature

Peripheral route of administration has many advantages and further studies will show if this therapeutic alternative would be feasible in reducing the plaque load in AD patients.

Enhancing the ubiquitin-proteasome system and autophagy

Cells have mainly two different defense lines to cope with misfolded proteins: the UPS and autophagy-lysosome system (Ebrahimi-Fakhari et al., 2012). The degradation of soluble intracellular misfolded proteins is handled by the UPS and proceeds by several sequential steps. First, chains of small globular proteins, chaperons, are bound to the misfolded protein by the action of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). The chaperon chain targets the protein for degradation by the proteasome, which cleaves the proteins into smaller peptides that can further be degraded into amino acids by intracellular peptidases. The autophagy-lysosome system degrades cellular components in three different ways. 1) Expression of autophagy-related genes can induce de novo synthesis of double membrane vesicles that sequester part of the cytosol (macroautophagy). This autophagosome has no proteolytic activity, but needs to fuse with a lysosome before degradation of the sequestered material can take place. 2) Proteins can also be targeted for degradation in lysosomes by a ubiquitin-mediated pathway, or 3) simply by a process in which the lysosome 'swallows' the cytosol close to its surface by invagination of the its membrane. All of these autophagic pathways are constitutively active in the cells, but they can be further enhanced in situations of cellular stress.

The strategy to enhance protein degradation could be applied in all diseases with pathogenic protein misfolding and aggregation. In addition, dysfunction in the UPS has been implicated in neurodegenerative diseases, further supporting this approach as a therapeutic possibility. One way of enhancing the UPS and chaperon-mediated refolding of abnormal proteins would be to up-regulate the expression or activity of chaperons. Dong et al. (2005) induced expression of the chaperon heat shock protein 70 (Hsp70) with an AAV vector five weeks before the mice were treated with MPTP. Nigral overexpression of Hsp70 turned out to protect the DAergic cells from MPTP-induced toxicity and restored the DA levels in the striatum. However, in transgenic Hsp70-overexpressing mice there was no observed neuroprotection against MPTP (Gao et al., 2011), making this approach controversial. In addition, the enhancement of chaperon-mediated refolding and UPS may be a poor strategy to control proteins that are prone to aggregate. Instead, autophagy could be targeted by, e.g., expression or activation of autophagy-inducing pathways. Recently, the link between aggregation of α-syn and glucocerebrosidase (GBA) activity has received attention (Dehay et al., 2013). GBA activity is linked to lysosomal function, and a decrease in the enzyme activity seems to promote aggregation of α-syn. Aggregation of α-syn is in turn thought to interfere with the transport of GBA from ER to the lysosomes, leading to a further decrease in lysosomal GBA activity still excacerbating α-syn aggregation and neurotoxicity. There is a reduction in GBA activity in the SN of PD patients (Gegg et al., 2012) and mutations in the GBA gene have been linked to PD (Sidransky et al., 2009). One possible therapeutic approach could therefore be to increase the GBA activity by applying the enzyme using gene therapy. Indeed, delivery of the GBA gene with an AAV vector
reduced the levels of soluble α-syn in the A53T α-syn transgenic mouse (Sardi et al., 2013) suggesting therapeutic potential for this strategy.

Immunization against pathogenic protein
Proteins can be targeted for destruction by the immune system by immunization against the protein in question. Using gene therapy, transduced cells can be forced to continuously produce protein-specific antibodies, and this approach has been applied in AD research. AAV-mediated i.c.v. delivery of an anti-Aβ single-chain variable fragment (scFv) into neonatal APP transgenic mice resulted in reduced Aβ levels and plaque load (Levites et al., 2006). Also intrahippocampal injection of an AAV-Aβ-scFv into three month old transgenic AD mice led to a decrease in the levels of insoluble Aβ and was accompanied by improvement in cognitive function (Ryan et al., 2010). Furthermore, in a recent study also i.m. injection of an AAV encoding a monoclonal anti-Aβ antibody was shown to reduce the amount of Aβ as well as number and size of plaques in APP transgenic mice (Shimada et al., 2013).

Vector-mediated delivery of antibodies or antibody fragments offers several advantages as compared to more conventional methods. A clinical trial of active immunization, in which AD patients were treated with aggregated Aβ peptides in combination with an adjuvant, was stopped when part of the patients developed meningoencephalitis (Orgogozo et al., 2003). Passive immunization by infusion of monoclonal anti-Aβ antibodies can be a safer alternative, but requires repeated infusions of the antibody. Here, gene therapy is clearly beneficial in providing long-term and wide-spread expression of the antibody.

Since related to the neuronal defense system, it could also be mentioned that gene therapy with anti-inflammatory cytokines (IL-4 or IL-10) delivered with AAV vectors into the hippocampus has been shown to be beneficial in APP + PSEN1 double-mutant mice (Kiyota et al., 2010; Kiyota et al., 2012). When treated with these vectors, the mice show significant reduction in Aβ levels and neuroinflammation, together with enhanced neurogenesis and improvement in cognitive functions.

2.4.3 ADDITION OF THERAPEUTIC GENE
NTFs are important for the survival and maintenance of neurons and synapses (Bespalov and Saarma, 2007; Lindholm and Saarma, 2010). In the developing nervous system they control the number of neurons, and regulate migration, differentiation, and neurite outgrowth. They also promote neuronal survival, as well as regulate plasticity and regeneration of mature neurons. Strategies that aim to supply target areas with endogenous NTFs, or NTF mimetics, are therefore considered to be of importance in the search for disease-modifying therapies in neurodegenerative diseases. Several of the traditional NTFs have indeed been extensively studied in pre-clinical settings of neurodegenerative disorders, and some have even entered clinical trials (for summary of clinical trials with NTFs, see Table 5).
Table 5. Clinical trials with neurotrophic factors in neurodegenerative diseases.

<table>
<thead>
<tr>
<th>NTF</th>
<th>Disease</th>
<th>Administration</th>
<th>Status</th>
<th>Results; number of patients</th>
<th>Reference/Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF</td>
<td>AD</td>
<td>Infusion, i.c.v.</td>
<td>Completed</td>
<td>Increase in nicotine binding, no cognitive improvement, adverse effects; n=3.</td>
<td>Eriksdotter-Jönhagen et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ex vivo, basal forebrain</td>
<td>Completed</td>
<td>Decrease in cognitive decline rate, increase in cortical FDG uptake, treatment safe; n=8</td>
<td>Tuszyński et al., 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ex vivo, basal forebrain</td>
<td>Completed</td>
<td>Cognitive improvement and increase in nicotine binding in subset of patients, treatment safe and well tolerated; n=6</td>
<td>Eriksdotter-Jönhagen et al., 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAV2, basal forebrain</td>
<td>Ongoing</td>
<td>Phase I: completed but no results published; n=10 Phase II: ongoing; n=50</td>
<td>Ceregen Inc., San Diego, USA</td>
</tr>
<tr>
<td>BDNF</td>
<td>ALS</td>
<td>Infusion, s.c.</td>
<td>Completed</td>
<td>Phase I/II: slower decline in respiratory function and walking speed; n=283 Phase III: positive results not repeated, increase in survival in subset of patients; n=1135</td>
<td>Bradley, 1995 The BDNF Study Group, 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Infusion, intrathecal</td>
<td>Completed</td>
<td>No clinical benefits, dose-related adverse effects, n=25</td>
<td>Ochs et al., 2000; Beck et al., 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Infusion, i.c.v.</td>
<td>Completed</td>
<td>Phase I: treatment safe, clinical improvement and increased FDOPA uptake; n=5 Phase I/II: No clinical benefits, antibodies detected; n=34</td>
<td>Gill et al., 2003; Lang et al., 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAV2, putamen</td>
<td>Ongoing</td>
<td>No results published; n~100</td>
<td>NINDS, Bethesda, USA</td>
</tr>
<tr>
<td></td>
<td>PD</td>
<td>AAV2, putamen</td>
<td>Completed</td>
<td>Phase I: treatment safe, some clinical improvement, n=12 Phase II: small improvement only in secondary measurements, limited distribution of NTN; n=58</td>
<td>Marks et al., 2008, 2010; Huddleston et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAV2, putamen and SN</td>
<td>Ongoing</td>
<td>No results published; n~60</td>
<td>Ceregen Inc.</td>
</tr>
<tr>
<td></td>
<td>HD</td>
<td>Ex vivo, i.c.v.</td>
<td>Completed</td>
<td>Phase I: safe, no clinical benefits; n=6</td>
<td>Bloch et al., 2004</td>
</tr>
<tr>
<td></td>
<td>ALS</td>
<td>Infusion, s.c.</td>
<td>Completed</td>
<td>Phase II/III: no clinical benefits, adverse effects (cough, anorexia, asthenia); n=730</td>
<td>ALS CNTF Treatment Study Group, 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ex vivo, intrathecal</td>
<td>Completed</td>
<td>Phase I: treatment safe; n=6</td>
<td>Aebischer et al., 1996</td>
</tr>
<tr>
<td></td>
<td>ALS</td>
<td>Infusion, s.c.</td>
<td>Completed</td>
<td>Phase III: three studies with variable results, generally no significant clinical benefit; n=266/183/330</td>
<td>Lai et al., 1997; Borasio et al., 1998; Sorenson et al., 2008</td>
</tr>
<tr>
<td></td>
<td>ALS</td>
<td>Infusion, i.c.v.</td>
<td>Ongoing</td>
<td>No results published; n=18</td>
<td>NeuroNova, Stockholm, Sweden</td>
</tr>
</tbody>
</table>
**Neurotrophic factor families**

The classical NTFs can be divided into three families: neurotrophins, GDNF family ligands (GFL), and neurokines (Bespalov and Saarma, 2007). These proteins provide survival signals through receptor tyrosine kinases or via other intracellular kinases that interact with the NTF receptor (Bespalov and Saarma, 2007). In addition, CDNF and MANF have been suggested to constitute a novel evolutionary conserved family of NTFs based on their NTF-like properties (secretion, neuroprotection, neurorestoration, neurodevelopmental effect) (Lindholm and Saarma, 2010).

The first studies on NTFs were done on the neurotrophin nerve growth factor (NGF). According to the classical NTF hypothesis postulated by Rita Levi-Montalcini and co-workers, small amounts of NTFs are secreted from the target tissue of developing neural processes (Levi-Montalcini, 1987). Only a few of the neurons trying to innervate the target tissue will receive the NTF support needed for further outgrowth and establishment of synapses. Neurons that are devoid of the NTF signal will fail to form connections and are destined to undergo apoptosis. Even though still valid, this theory has later been proven to be a truth with modifications as additional functions and secretion patterns for the NTFs have been revealed.

**Neurotrophins**

The neurotrophins, NGF, BDNF, neurotrophin 3 (NT3) and neurotrophin 4 (NT4), are synthesized as pro-forms, which are processed intra- or extracellularly into mature proteins (Park and Poo, 2013). The mature neurotrophins bind to their specific tropomyosin-related tyrosine kinase receptor: NGF to TrkA, BDNF and NT4 to TrkB, and NT3 to TrkC. The binding of the homodimeric ligand initiates homodimerisation of two tyrosine kinase receptors, mediating trans-phosphorylation of intracellular tyrosine residues with subsequent activation of signal cascades resulting in neuronal differentiation, survival, and gene expression. In addition, all neurotrophins (both pro-forms and mature protein) have affinity to p75NTR, a member of the tumor necrosis factor (TNF) receptor superfamily. While p75 assists in the binding of neurotrophins to the respective tyrosine kinase receptor, binding of the pro-neurotrophins and subsequent activation of p75 can mediate cell death.

In accordance with the classical NTF hypothesis, NGF is produced and secreted by the target tissue of sympathetic and sensory neurons, and thereby stimulate the survival and maintenance of these neurons. In the brain, NGF is expressed in the cortex and hippocampus where it seems to be important for the maintenance and survival of, e.g., the basal forebrain cholinergic cells and projections (Hefti, 1986; Fischer et al., 1987; Koliatsos et al., 1990; Tuszynski et al., 1990). NGF has therefore been suggested to have therapeutic potential in AD. In a small clinical study, NGF was infused i.c.v. in three AD patients (Table
Although the patients showed an increase in \[^{11}C\]nicotine binding in several brain areas together with a reduction of slow-wave cortical activity, no clear cognitive improvements were observed. The NGF treatment was associated with weight loss and back pain, and the trial was stopped.

BDNF signaling is important for many neuron subpopulations and neuronal circuits, including sensory neurons, retinal ganglia, some cholinergic and DAergic neurons, and spinal motor neurons. BDNF can be secreted both in constitutive and regulated manners, and the production of BDNF in neurons is affected by neuronal activity and plays an important role in synaptic transmission, plasticity and growth (Nagahara et al., 2009; Nagahara and Tuszynski, 2011; Park and Poo, 2013). As showed in Figure 4, reduced levels and dysfunctional axonal transport of BDNF are thought to partly mediate the vulnerability and damage of striatal GABAergic medium spiny neurons in HD (Gauthier et al., 2004; Strand et al., 2007). Reduced levels of BDNF have also been reported in the AD brain (Connor et al., 1997), and this loss can experimentally be triggered with A\(\beta\) oligomers (Garzon and Fahnestock, 2007). In addition, the density of neurotrophin receptors in cholinergic neurons is reduced in the basal forebrain in AD. Due to its broad effects on different neuron populations, BDNF has been applied with various results in animal models of PD, AD, HD, and ALS (reviewed by Nagahara and Tuszynski, 2011), as well as tested clinically in ALS (Table 5) (The BDNF Study Group, 1999; Ochs et al., 2000; Beck et al., 2005).

GDNF has neurotrophic effect on a large variety of neurons, including, e.g., spinal and central motor neurons, and sympathetic, parasympathetic and enteric sensory neurons (reviewed by Airaksinen and Saarma, 2002). However, it is the strong survival-promoting effect of GDNF on DAergic neurons (Lin et al., 1993; Pascual et al., 2008) that has received most attention and led to numerous studies in animal models to elucidate its potential as a therapeutic agent for the treatment of PD. In a short summary, infusions of GDNF protein into the SN or striatum of 6-OHDA-lesioned rats have mediated neuroprotection, and in...
some cases even neurorestoration, of the midbrain DAergic neurons (Hoffer et al., 1994; Kearns and Gash, 1995; Sauer et al., 1995; Kearns et al., 1997; Aoi et al., 2000; Kirik et al., 2000a; Kirik et al., 2001). The effect of GDNF depends on the infusion site of both GDNF and 6-OHDA, and the most impressive neuroprotection has been achieved in studies where GDNF and 6-OHDA have been injected into the same site (striatum) with only a short time interval between the injections (Sauer et al., 1995; Kearns et al., 1997; Kirik et al., 2000a). In addition to rodent PD models, in vivo studies of GDNF injections in non-human primates have also rendered promising results. In MPTP-treated monkeys, a clear improvement in motor function was observed after i.c.v., intranigral or intraputaminal GDNF delivery, and the improvement could be sustained by repeated injections of GDNF (Gash et al., 1996; Zhang et al., 1997; Grondin et al., 2002).

The promising results from pre-clinical in vivo studies paved the way for clinical trials of GDNF protein delivery in PD patients (Table 5). A phase I trial of intraputaminal delivery of GDNF yielded positive behavioral results in PD patients (Gill et al., 2003; Patel et al., 2005), but these results were not replicated in the following phase I/II study (Lang et al., 2006). In addition, some patients developed antibodies against GDNF, and this, together with the failure of the clinical trials to show sustained improvement in the patients, led to discontinuation of the trials.

Also NTN supports the survival of midbrain DA neurons both in vitro and in vivo. Studies of intracerebral infusions of NTN in rodents (Horger et al., 1998; Akerud et al., 1999; Rosenblad et al., 1999; Oiwa et al., 2002; Li et al., 2003) and non-human primates (Li et al., 2003) have showed NTN-mediated protection of DAergic neurons in experimental PD models. Even though the protective effect of NTN on the DAergic neurons in the SN has been suggested to be similar to that of GDNF (Horger et al., 1998), unlike GDNF, neurturin does not seem to have the capability to induce axonal growth and hypertrophy (Akerud et al., 1999).

**Neuropoietic cytokines**

The cytokine family of NTFs includes ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and interleukin 6 (IL-6) (Ip and Yancopoulos, 1996; Gould and Oppenheim, 2011). They bind to heterodimeric receptor complexes consisting of the tyrosine kinase LIF receptor-β (LIFR-β) and glycoprotein130 (gp130), and through the janus kinase/signal transducer and activator of transcription-3 (JAK/STAT) and PI-3K pathways they activate different intracellular signaling cascades. Of the neurokines, CNTF is the most interesting member from the neurodegeneration point of view and the first NTF to enter clinical trials in HD (summarized below) (Bachoud-Levi et al., 2000; Bloch et al., 2004). CNTF promotes survival and regulate transmission of a broad range of neuronal populations in both the peripheral and central nervous system, including GABAergic neurons in the basal forebrain as a four-day infusion of CNTF into the striatum of rats prevented QA-induced loss of striatal neurons (Anderson et al., 1996). In addition, CNTF’s trophic effect on motor neurons has also been of special interest in the ALS research (reviewed by Gould and Oppenheim, 2011).
**CDNF/MANF family**

MANF and CDNF constitute a protein family with characteristics that differ from the traditional NTFs (Lindholm et al., 2007; Lindholm and Saarma, 2010). The MANF and CDNF proteins consist of two structural domains: an N-terminal saposin-like domain and an unstructured C-terminal domain containing a cysteine bridge (Mizobuchi et al., 2007; Parkash et al., 2009). According to their structure, it is hypothesized that MANF and CDNF could exert their action, e.g., by interacting with lipids (N-terminal domain), and/or by influencing protein folding during endoplasmic reticulum (ER) stress (Lindholm and Saarma, 2010). Indeed, several studies have provided evidence for the function of MANF in ER stress and the unfolded protein response. Endogenous MANF is localized to ER and the expression of MANF is up-regulated in response to both cardiac and cerebral ischemia as well as ER stress (Mizobuchi et al., 2007; Apostolou et al., 2008; Tadimalla et al., 2008; Shen et al., 2012; Henderson et al., 2013). Furthermore, MANF deprivation seems to sensitize cells to ischemia- and ER stress-induced cell death (Apostolou et al., 2008; Tadimalla et al., 2008). In line with this, addition of MANF promotes recovery and decreases tissue damage in models of myocardial infarction and ischemic brain injury (Tadimalla et al., 2008; Airavaara et al., 2009; Airavaara et al., 2010; Glembotski et al., 2012). In a recent publication, also CDNF was implied in the ER stress response since astrocytes over-expressing CDNF were less sensitive to ER stress-induced cell damage and showed decreased expression and secretion of pro-inflammatory cytokines (Cheng et al., 2013).

In addition to its intracellular actions, MANF is also secreted and its secretion is highly responsive to ER stress in several cell lines (Apostolou et al., 2008; Tadimalla et al., 2008; Glembotski et al., 2012; Henderson et al., 2013). Both the secretion and localization of MANF to the ER seems to be dependent on the four amino acid (RTDL) C-terminal end, and there are indications that the RTDL sequence also may mediate cell surface binding after secretion (Glembotski et al., 2012; Oh-Hashi et al., 2012; Henderson et al., 2013). How secreted or extracellularly applied MANF can affect target cells is, however, still not known, and no receptors or receptor-specific signaling pathways activated by MANF or CDNF have yet been clarified.

Both MANF and CDNF show wide expression in both central and peripheral tissue (Lindholm et al., 2007; Lindholm et al., 2008). The first indication that the MANF/CDNF family could serve as trophic factors for DAergic neurons came from a study showing that MANF mediates survival of DAergic neurons in vitro (Petrova et al., 2003). Later, the invertebrate analog to MANF was suggested to be essential for the development of Drosophila flies, with important functions for both the development and maintenance of DA neurites (Palgi et al., 2009). Importantly, human full-length MANF was able to rescue the DmMANF knockout phenotype, providing evidence for evolutionary conserved functions for the MANF protein (Palgi et al., 2009; Lindström et al., 2013). Also knockdown of manf in the zebrafish resulted in decrease in DA levels and TH expression, as well as decrease in a subset of TH-positive neurons – changes that could be partially reversed by delivery of manf mRNA to the zebrafish (Chen et al., 2012). Furthermore, intrastratal infusions of CDNF and MANF have proved to provide protection and promote recovery of the nigrostriatal DAergic pathway in the rat 6-OHDA and the mouse MPTP models of PD.
(Lindholm et al., 2007; Voutilainen et al., 2009; II; Airavaara et al., 2012). Taken together, these results indicate that the MANF/CDNF family of NTFs has therapeutic potential in neurodegenerative disorders, such as PD.

**Neurotrophic factor gene therapy in Parkinson's disease: GDNF and NTN**

Delivery of NTN or GDNF with viral vectors into the SN or striatum, or both, has been effective in several animal models of PD (reviewed by Deierborg et al., 2008; Björklund and Kordower, 2010), with the exception of the α-syn-overexpressing rat (Lo Bianco et al., 2004; Decressac et al., 2011). The most effective injection site has turned out to be the striatum, where induced overexpression of NTN or GDNF has resulted in protection of striatal fibers with a concurrent improvement in motor behavior, and protection of DA cell bodies in the SN (Bilang-Bleuel et al., 1997; Kirik et al., 2000b; Kordower et al., 2000; Eslamboli et al., 2003; Kordower et al., 2006; Gasmi et al., 2007a; Eberling et al., 2009). The capacity to protect cell bodies in the SN even though expressed in the striatum is probably due to the fact that both NTN and GDNF can be retrogradely transported from the striatum to the SNpc (Tomac et al., 1995; Oiwa et al., 2002). In contrast, while GDNF delivery in the SN provide efficient protection of DA cell bodies (Choi-Lundberg et al., 1997; Mandel et al., 1999b; Kirik et al., 2000b), no or very modest re-innervation of the striatal DAergic fibers is generally seen, partly explaining the lack of motor improvement in some of these studies.

**Figure 10** Midbrain dopaminergic pathways in the human brain (according to Cooper, Bloom, and Roth, 2002b). In Parkinson's disease (PD) dopaminergic cells in the substantia nigra (SN) are lost, causing dopamine depletion in the striatum. Clinical trials with glial cell-line derived neurotrophic factor and neurturin have aimed at rescuing and improving the function of the affected dopaminergic neurons applying the trophic therapy into the striatum alone or into both the striatum and the SN of PD patients.

Intrastriatal delivery of a recombinant AAV2-NTN vector has recently undergone phase I and II clinical trials (Figure 10; Table 5). The phase I clinical trial showed promising results (Marks et al., 2008), but in a double-blind randomized controlled phase II trial significant functional improvement in only a subset of patients was observed not until 18 months post-injections (Marks et al., 2010; Huddleston and Factor, 2011). Post-mortem analyses revealed very scarce amounts of NTN-positive cells in the SNpc without TH induction (Bartus et al., 2011). These results suggest that the lack of significant improvements could be due to the limited retrograde transport of NTN from the injection site to the SNpc. To overcome this, a new phase II study is ongoing using higher viral vector titers and multiple injection sites (striatum and SN) (Bartus et al., 2013), an approach supported by pre-clinical data in both non-human primates and rodents (Kordower et al., 2006; Herzog et al., 2013).
In addition to clinical trials on AAV2-NTN, recruitment of PD patients to an open-label clinical study on intrastriatal delivery of an AAV2-GDNF vector is now in progress and aims at assessing the safety and tolerability using four escalating doses of AAV2-GDNF (NCT01621581; U.S. National Institutes of Health, 2013).

### Neurotrophic factor gene therapy in other neurodegenerative diseases

#### Neurotrophic factor gene therapy in Alzheimer’s disease: NGF and BDNF

Gene therapy with the neurotrophins NGF and BDNF has been most extensively explored in AD. Fibroblasts or NSCs genetically modified to express NGF have been transplanted into the basal forebrain of both rodents (Rosenberg et al., 1988; Kawaja et al., 1992; Martinez-Serrano et al., 1996) and primates (Tuszynski et al., 1994; Smith et al., 1999). These studies show cholinergic re-innervation, axon regeneration and rescue of cholinergic neurons in response to NGF treatment following age-dependent or axotomy-induced cholinergic denervation. The *ex vivo* gene therapy approach has been translated into two phase I clinical trials in which AD patients received transplants of viral-vector transduced NGF-expressing fibroblasts (Tuszynski et al., 2005) or encapsulated retinal pigment epithelial cells (Eriksdotter-Jönhagen et al., 2012; Wahlberg et al., 2012) into the basal forebrain (Figure 11). No serious adverse effects attributable to the treatments were observed in either of the trials. In a small subset of patients, positive effects on cognitive functions and increased cortical activity measured with PET-FDG were detected (Table 5). There is still no information about possible follow-up studies or phase II clinical trials regarding the NGF *ex vivo* gene therapy concept.

Also AAV-vector mediated delivery of NGF has been processed into clinical trials by Ceregene Inc. (San Diego, CA, USA). A phase I study has been completed and a phase II randomized controlled trial is ongoing (NCT00087789 and NCT00876863; U.S. National Institutes of Health, 2010, 2012), but no results from these trials on AAV-NGF delivery into the basal forebrain have yet been published. In animal studies, AAV-NGF treatment has shown promising results. Injection of AAV-NGF into the medial septum of rats lead to an increase in both the survival and cell size of cholinergic neurons in a fibria-fornix lesion model of AD (Mandel et al., 1999a; Wu et al., 2003; Bishop et al., 2008). Similar outcomes were seen in aged rats when AAV-NGF was delivered into the basal nucleus of Meynert (Bishop et al., 2008). In intact young rodents (Bishop et al., 2008) and primates (Herzog et al., 2011), hypertrophy of cholinergic cells was observed after a single injection of AAV-NGF into the basal nucleus of Meynert, while no adverse effects could be detected in a two-year study (Herzog et al., 2011).

The effects of BDNF gene therapy in AD models were reported in an extensive study done by Nagahara and co-workers (Nagahara et al., 2009). They assessed the protective potential of LV-mediated overexpression of BDNF targeted to the entorhinal cortex in APP transgenic mice, and lesioned or aged rats and primates. In APP transgenic mice, BDNF gene therapy lead to an improvement in memory and learning, an increase in synaptic markers and normalization of APP-related changes in gene expression. The amyloid plaque density load seen in the model was not affected by the treatment. Anterograde transport of BDNF from the injection site to the hippocampus was observed with a concurrent
increase in ERK phosphorylation in the hippocampus. In lesioned rats and non-human primates, delivery of LV-BDNF in the entorhinal cortex prevented lesion-induced neuron loss, while in aged non-human primates treatment-dependent improvement in cognitive performance as well as an increase in entorhinal neuron size was observed (Nagahara et al., 2009). BDNF has not yet been applied in AD clinical trials.

**Figure 11** Main cholinergic innervations affected in Alzheimer’s disease, including cholinergic neurons in the medial septal nucleus (ms) and basal nucleus of Meynert (nb) (Cooper, Bloom, and Roth, 2002a). In clinical trials, neurotrophic factor therapies have been targeted to the basal forebrain in an attempt to increase the survival and function of the cholinergic cells and their projections.

**Neurotrophic factor gene therapy in Huntington’s disease: CNTF, BDNF, GDNF, NTN**

The effect of CNTF gene therapy in HD has been studied in both pre-clinical and clinical settings. Encapsulated CNTF-overexpressing baby hamster kidney (BHK) fibroblasts transplanted into the striatum of QA-lesioned rats provided significant protection of striatal neurons as well as normalized motor behavior (Emerich et al., 1996). Using the same method for CNTF delivery, the treatment was also tested in QA-lesioned monkeys, showing substantial reduction in lesion area, protection of striatal neurons and projections (GP and SNpr), as well as attenuation of QA-induced retrograde atrophy of the motor cortex (Emerich et al., 1997). In both studies performed by Emerich and co-workers, the CNTF-expressing cells were transplanted before the excitotoxic lesion was induced. Instead, Mittoux and co-workers (2000) used the 3-NP model and transplanted encapsulated BHK-CNTF cell into the striatum of monkeys two months after initiation of intoxication. Also in this model CNTF gene therapy protected the striatal neurons and restored the nigrostriatal function accompanied by improvements in cognitive and motor functions. Based on these promising results, *ex vivo* CNTF gene therapy was translated into clinical trials (Table 5) (Bachoud-Levi et al., 2000; Bloch et al., 2004). Encapsulated BHK-CNTF cells were implanted into the lateral ventricle of six HD patients and changed every sixth month over a two-year period. Even though the treatment turned out to be safe, no significant clinical benefits were observed. The authors speculate that this could be due to low CNTF release, since electrophysiological results were improved in a subset of patients that had received the highest amounts of CNTF (Bloch et al., 2004).

*In vivo* CNTF gene therapy has been studied using adenoviral, LV and AAV vectors. Delivery of CNTF with an adenoviral vector into the striatum of rats prior to 3-NP intoxication turned out to protect neurons in the striatum and striatal target areas. In addition, CNTF mediated significant improvement in motor behavior (Mittoux et al., 2002). The same results were achieved using recombinant LV vectors in the rat QA model of HD (Pereira de Almeida et al., 2001; Regulier et al., 2002). However, *in vivo* CNTF gene therapy using recombinant LV
Review of the literature

or AAV vectors in HTT transgenic mice pointed out potential problems with long-term CNTF overexpression showing CNTF-dependent loss of striatal neurons, striatal down-regulation of several markers including DARPP-35 (marker of DA signaling in GABAergic medium spiny neurons), weight loss, motor impairment and abnormal behavior in both wild-type and transgenic mice (Zala et al., 2004; Denovan-Wright et al., 2008).

The observations that dysfunction in the cortical expression and anterograde transport of BDNF is impaired in HD (Cattaneo et al., 2005) has laid the ground for the hypothesis that increasing the levels of BDNF in the cortico-striatal pathways may promote neuronal survival and slow down the disease progression. In QA-lesioned rats, delivery of BDNF into the striatum using recombinant adenoviral vectors (Bemelmans et al., 1999) or genetically engineered fibroblasts (Perez-Navarro et al., 2000b) protected the striatal neurons and reduced the lesion size. Also AAV vector-mediated BDNF gene therapy in the QA model provided striatal preservation and improvement in motor function (Kells et al., 2008). However, this study again demonstrated possible problems with uncontrolled long-term overexpression of NTFs since rats that had received high titer s of the AAV-BDNF vector developed seizure activity and experienced weight loss. These effects may have been caused by off-site delivery of BDNF to other brain regions due to anterograde and/or retrograde transport.

In addition to CNTF and BDNF, also GDNF and NTN have showed neuroprotective potential in animal models of HD. Both ex vivo (Perez-Navarro et al., 1999; Pineda et al., 2007) and in vivo (McBride et al., 2003) GDNF gene therapy has proven to normalize behavior and protect striatal neurons in the QA and 3-NP rat models of HD. In transgenic mice, AAV-GDNF delivery has resulted in inconsistent results. Whereas in one HTT transgenic mouse model (N171-82Q), AAV-GDNF was proven to be efficient in improving motor behavior as well as protecting striatal neurons and preventing neuronal atrophy (McBride et al., 2006), GDNF delivery with an LV vector showed no therapeutic benefits in the R6/2 transgenic mouse (Popovic et al., 2005). Regarding NTN, the same AAV vector used for clinical trials in PD patients has also been tested in rodent HD models. Intrastratial delivery of this vector provided partial protection of striatal neurons and ameliorated behavioral deficits in both 3-NP-treated rats (Ramaswamy et al., 2007a) and HTT transgenic mice (N171-82Q) (Ramaswamy et al., 2009). Interestingly, there are indications that the neuroprotective effect of intrastratial GDNF and NTN in animal HD models may differ regarding specificity for neuronal circuits (Perez-Navarro et al., 1999; Perez-Navarro et al., 2000a). While ex vivo GDNF gene therapy was capable of protecting GABAergic neurons only in the direct circuit (projections to the SN) together with cholinergic interneurons, NTN preserved GABAergic projection neurons also in the indirect circuit, without effect on interneuron survival in the QA-lesion rat.

Neurotrophic factor gene therapy in amyotrophic lateral sclerosis: GDNF, IGF-1, VEGF, CNTF, BDNF

Several NTFs, including GDNF, BDNF, NT-3, and CNTF, affect the development and survival of motor neurons (reviewed by Gould and Oppenheim, 2011). Even though in vitro studies have provided evidence for strong positive effects of GDNF in models of motor neuron
degeneration (Henderson et al., 1994; Corse et al., 1999), *in vivo* benefits from GDNF treatment have been more inconsistent in animal SOD1 models of ALS. When administered centrally into the spinal cord, GDNF gene therapy generally protects the motor neurons, but does not affect the motor neuron innervation of the muscle, and, hence, no significant improvement of motor behavior has been observed (Suzuki et al., 2007). Since GDNF can be retrogradely transported, delivery of GDNF with viral vectors or GDNF overexpressing cells into muscles targeted by the degenerating motor neurons has been explored. Contrary to central administration, delivery of GDNF into the muscle mediates preservation of muscle innervation with concomitant improvement in motor function (Mohajeri et al., 1999; Acsadi et al., 2002; Wang et al., 2002; Suzuki et al., 2008). In addition, the retrograde transport of GDNF also allows some protection of the motor neurons in the ventral horn of the spinal cord. However, GDNF gene therapy has generally resulted in rather modest changes in disease onset or survival time in the SOD1 rodent models of ALS.

More robust changes in disease progression and survival parameters were obtained with i.m. viral vector-based gene delivery of insulin-like growth factor-1 (IGF-1) (Kaspar et al., 2003) or vascular endothelial growth factor (VEGF) (Azzouz et al., 2004), two growth factors that in addition to their other effects on peripheral tissues and other neuronal populations, also provide motor neurons with direct trophic support and/or enhance neuronal survival through angiogenesis (Gould and Oppenheim, 2011). Despite the positive effects of IGF-1 in animal models of ALS, a phase III clinical trials of s.c. administration of IGF-1 in ALS patients showed no benefits regarding motor performance or overall survival (Table 5) (Sorenson et al., 2008). The same disappointing results were obtained in a phase II-III clinical trial of subcutaneous injections of CNTF (ALS CNTF Treatment Study Group, 1996), an NTF that is known for its motor neuron regenerative effect both *in vitro* (Sendtner et al., 1990) and *in vivo* (Mitsumoto et al., 1994). In addition, systemic treatment with CNTF was associated with adverse events, such as cough, anorexia and asthenia (ALS CNTF Treatment Study Group, 1996), side effects that were overcome in a phase I clinical trial applying *ex vivo* CNTF gene therapy intrathecally in ALS patients (Aebischer et al., 1996). Also BDNF has been investigated in ALS clinical trials given as s.c. (Bradley, 1995; The BDNF Study Group, 1999) or intrathecal infusions (Ochs et al., 2000; Beck et al., 2005), but again, the results have been controversial. The status of clinical trials regarding BDNF, CNTF, or IGF-1 treatments in ALS patients is not known. The Swedish company NeuroNova is currently evaluating the safety and tolerability of i.c.v. infusion of VEGF in ALS patients, but no results from the study are yet available (NCT01384162; U.S. National Institutes of Health, 2011).
3 AIMS OF THE STUDY

Despite promising results in non-clinical study settings, the clinical trials conducted on NTFs in neurodegenerative diseases have this far failed to show robust clinical benefits. There is therefore still a need for testing novel neurotrophic agents with potentially better therapeutic characteristics together with improving techniques for delivery of the NTFs. The main purpose of this study was to examine the therapeutic potential of long-term i.c. delivery of the NTF CDNF in the rat partial lesion model of Parkinson’s disease.

The specific aims of this study were:

1) To characterize the rat partial 6-OHDA PD models used in our studies and to validate the use of in vivo single-photon emission computed tomography/computed tomography (SPECT/CT) in this model.

2) To study whether a two-week infusion of CDNF or MANF is neuroprotective in the rat 6-OHDA model.

3) To determine the AAV vector-induced expression of CDNF and examine the neuroprotective effect of intrastriatal CDNF gene therapy in the rat 6-OHDA model.
4 MATERIAL AND METHODS

4.1 DRUGS, TOXINS, AND NEUROTROPHIC FACTORS

The following drugs and toxins were used for the in vivo experiments: amphetamine hydrochloride (Faculty of Pharmacy, University of Helsinki, Helsinki, Finland), 2β-carbomethoxy-3β-(4-[123]I]iodophenyl)tropane ([123]I-B-CIT, MAP Medical Technologies Oy, Tikkakoski, Finland), desipramine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA), 6-OHDA hydrochloride (Sigma-Aldrich), pentobarbital (Mebunat®, Orion Oyj, Espoo, Finland), tramadol (Tramal®, Orion Oyj).

In study II, the following NTF proteins were used: CDNF (Biovian, Turku, Finland) (Lindholm et al., 2007), MANF (Lindholm et al., 2008), GDNF (Amgen Inc., Thousand Oaks, CA, USA; ProSpec-Tany TechnoGene Ltd, Rehovot, Israel).

For study III, AAV2 vectors carrying the genes for human CDNF (hCDNF, for plasmid map, see study III), GDNF (Lonka-Nevalaita et al., 2010), and green fluorescent protein (GFP) were produced in the National Virus Vector Laboratory (University of Eastern Finland, Kuopio, Finland). The AAV2 vectors were made using the Stratagene AAV Helper-Free System according to the manufacturer’s instructions (Stratagene, La Jolla, CA, USA). The titers of the viral vectors were determined by quantitative PCR.

4.2 ANIMALS

Wistar male rats (Harlan, Netherlands) were used in all in vivo experiments. Rats were normally housed in groups of 3-4 individuals in 12h/12h light/dark cycle (light 6.00-18.00, dark 18.00-6.00). All behavioral experiments were conducted during the light period. Rats had access to conventional laboratory animal food (Harlan) and water ad libitum. All animal experiments were reviewed and approved by the National Animal Experiment Board (ESLH-2007-06679/Ym-23, ESLH-2009-05234/Ym-23, ESAVI/4706/04.10.03/2011) and carried out in accordance with the European Communities Council Directive 86/609/EEC.

4.3 STEREOTAXIS

All i.c. injections were done under isoflurane anesthesia using a stereotaxic frame (Stoelting, Wood Dale, IL, USA), automatized injector (Stoelting) and 10 µl 26G syringes (Hamilton, Bonaduz, Switzerland). All coordinates were determined according to the rat brain atlas of Paxinos and Watson (1997). During the operation, rats received an injection of tramadol (1 mg/kg, s.c.) for post-operative pain. The animals were allowed to recover in single cages overnight.
Material and methods

4.3.1 RETROGRADE LABELING OF DOPAMINE NEURONS WITH FLUORO-GOLD
For labeling rat nigral DA neurons in vivo, the retrograde neuronal tracer FluoroGold (FG) (4% in sterile saline) (Fluorochrome, LLC, Denver, CO, USA) was injected bilaterally into the striatum (A/P +1.0; L/M ±2.7; D/V -5.0; 0.2 µl, 0.05 µl/min) 7-8 days before injection of 6-OHDA (Schmued and Fallon, 1986; Divac and Mogensen, 1990).

4.3.2 6-OHDA INJECTIONS
6-OHDA was diluted in 0.02% ascorbic acid solution and 4 µl were injected at 1 µl/min (Ungerstedt, 1968; Sauer and Oertel, 1994). Single injections (8 µg or 20 µg 6-OHDA) were done into A/P +1.0, L/M ±2.7, D/V -5.0, and two 6-OHDA injections (2x10 µg) into A/P +1.6, L/M ±2.2, D/V -5.0 and A/P -0.4, L/M ±4.0, D/V -5.0. For protection of the noradrenergic neurons, rats received an injection of desipramine (15 mg/kg, i.p.) at least 30 min before the 6-OHDA injection (Luthman et al., 1989).

4.3.3 TREATMENT WITH NEUROTROPHIC FACTORS
Neurotrophic factors CDN, MANF, or GDNF were delivered into the rat brain as single protein injections, two-week infusions, or gene therapy with recombinant AAV serotype 2 vectors (treatment groups and study design in Table 6 and Figure 12).

Single protein injections were done into the rat striatum with the equipment described above.

For two-week infusions of the neurotrophic factors, osmotic pumps were used (Alzet®, Durect Corporation, Cupertino, CA, USA). The pumps were filled with NTF solution under aseptic conditions, and allowed to reach the steady-state pumping rate by incubation in 37°C over night. A brain cannula (Alzet® brain infusion kit) attached to the pump was inserted into the rat striatum in a stereotaxic operation, and the cannula was fixed to the skull with two screws and dental cement. The osmotic pump was placed under the skin on the rat's neck, and the incision was closed by suturing. Infusion of phosphate-buffered saline (PBS, vehicle) was used as a negative control.

Gene therapy with neurotrophic factors was accomplished using recombinant AAV2-CDNF, and AAV2-GDNF vectors. Viral vector stocks were, if necessary, diluted in PBS and injected into the rat striatum with the equipment described above. Controls used in the study consisted of PBS or AAV2-GFP injections.
Materials and methods

Table 6. Study design and treatment groups for neurotrophic factor (NTF) studies (II, III). The proteins or recombinant viral vectors were infused into the coordinates A/P +1.0, L/M +2.7, D/V -5.0. The 6-OHDA dose was 2x10 µg in both studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Delivery method</th>
<th>Infusion</th>
<th>Treatment groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>Neurorestoration: infusion of NTFs for two weeks starting two weeks post-lesion.</td>
<td>Osmotic pump (Alzet®, model 2002)</td>
<td>0.5 µl/h, striatum</td>
<td>CDNF 1.5 µg/24h CDNF 4.5 µg/24h MANF 3.0 µg/24h MANF 4.5 µg/24h GDNF 3.0 µg/24h PBS (vehicle)</td>
</tr>
<tr>
<td>II</td>
<td>Effect on intact brain: infusion of NTFs for two weeks.</td>
<td>Osmotic pump (Alzet ®, model 2002)</td>
<td>0.5 µl/h, striatum</td>
<td>CDNF 3.0 µg/24h MANF 3.0 µg/24h GDNF 3.0 µg/24h</td>
</tr>
<tr>
<td>II</td>
<td>Diffusion of NTFs: infusion of NTFs for 3, 5, 7, or 14 days.</td>
<td>Osmotic pump (Alzet ®, models 2002 and 1007D)</td>
<td>0.5 µl/h, striatum</td>
<td>CDNF 3.0 µg/24h MANF 3.0 µg/24h GDNF 3.0 µg/24h</td>
</tr>
<tr>
<td>II</td>
<td>NTF transportation: injection of 125I-labeled NTFs.</td>
<td>Protein injection</td>
<td>1 µl/min, 6 µl, striatum</td>
<td>125I-CDNF 125I-GDNF</td>
</tr>
<tr>
<td>III</td>
<td>Neuroprotection: injection of viral vectors two weeks before 6-OHDA injections. Analysis of expression 1, 2, 4, 8, and 12 weeks post-injection. Effect of CDNF gene therapy on dopamine levels in lesioned and intact rat striatum</td>
<td>AAV2 vector</td>
<td>1 µl/min, 5 µl, striatum</td>
<td>AAV2-CDNF 4x10⁷ vg AAV2-CDNF 2x10⁸ vg AAV2-CDNF 1x10⁹ vg AAV2-GDNF 1x10⁶ vg AAV2-GFP 2x10⁶ vg PBS</td>
</tr>
</tbody>
</table>

4.4 BEHAVIORAL TESTS

4.4.1 DRUG-INDUCED ROTATIONAL BEHAVIOR
Asymmetric rotational behavior was induced in unilaterally 6-OHDA-lesioned rats with amphetamine 2.5 mg/kg i.p. (Ungerstedt and Arbuthnott, 1970). The amount of full 360° drug-induced turns was detected for 120 min using a rotometer (Rotorat, Med Associates, Inc., Georgia, VT, USA).

4.4.2 FORELIMB AKINESIA TEST
The forelimb akinesia test was done according to Olsson et al. (1995). The rats were tested for three consecutive days, with two tests each day. The first forelimb akinesia test was preceded by pre-training for three days to allow the rats to get used to the handling. All tests were made by the same person at the same time of the day (12.00-17.00).

The rat was held firmly with both hindlimbs and one forelimb fixed. With one free forelimb touching the surface of the table, the rat was moved sidewise for a distance of 90 cm in 5 s.
All the rats were tested in the same sequence: first with the left forepaw in the forehand and backhand direction, and then with the right forepaw in the forehand and backhand direction. The rats were videotaped and the amount of correcting steps taken in each set of the test was counted. Results are given as the average of six measurements (two trials every day for three consecutive days).

4.4.3 CYLINDER TEST
Limb use asymmetry of unilaterally lesioned rats was measured using the cylinder test (Schallert et al., 2000). Rats were put in a transparent cylinder (diameter 24 cm, height 30 cm) and videotaped for 5 min. The amount of wall touches made with the ipsilateral and contralateral paw during a rear was detected. Results are given as ipsilateral paw use as percentage of total paw use (ipsi- and contralateral).

Figure 12. Study design for the experiments presented in this thesis. Characterization of the partial 6-OHDA lesion in rats (A; study I), evaluation of a two-week infusion of NTFs (B; study II) and delivery of CDNF with a recombinant AAV2 vector (C; study III) in the rat partial 6-OHDA lesion model. AAV, adeno-associated virus; CDNF, cerebral dopamine neurotrophic factor; DA, dopamine; IHC, immunohistochemistry; NTF, neurotrophic factor; 6-OHDA, 6-hydroxydopamine; SPECT/CT, single-photon emission computed tomography/computed tomography.
4.5 IMMUNOLOGICAL DETECTION

4.5.1 IMMUNOHISTOCHEMISTRY
For immunohistochemical staining, rats were anesthetized with a lethal dose of pentobarbital and intracardially perfused with PBS and 4% paraformaldehyde. Following post-fixation in paraformaldehyde, the brains were stored in 20% sucrose in +4 °C until freezing. Frozen brains were cut on a microtome into 40 µm sections and stored in a cryoprotective solution in -20 °C.

Immunohistochemical staining was done on free floating brain sections with primary and secondary antibody combinations shown in Table 7. Briefly, after washing the brain sections in buffer, immunoreactivity was improved by heat-treating the samples in 80 °C in citrate buffer, pH 6.0 (all immunohistochemical stainings except TH). For staining with biotinylated secondary antibodies, the sections were incubated in hydrogen peroxide to block endogenous peroxidases. To decrease the amount of background staining, the sections were blocked with appropriate serum, and then incubated in primary antibody. Following wash and incubation in biotinylated secondary antibody, the staining was reinforced using avidin-biotin complex (ABC-kit, Vector labs) and visualized with diaminobenzidine (DAB). When using secondary antibodies labeled with fluorescent marker, sections were washed after incubation in the secondary antibody and mounted on glass slides using Vectashield mounting medium (Vector labs).

4.5.2 ENZYME-Linked IMMUNOSORBENT ASSAY
The CDNF sandwich enzyme-linked immunosorbent assay (ELISA) was done as described in III. Briefly, homogenized and acid-treated samples from rat striatum and SN were incubated on a 96-well plate coated with an anti-CDNF antibody. Following incubation with a detection anti-CDNF antibody, a secondary horse-radish peroxidase (HRP)-conjugated antibody was applied and using 3,3',5,5'-tetramethylbenzidine (TMB) substrate (DuoSetELISA Development System, R&D Systems) the CDNF protein concentration in the samples was estimated. The CDNF-ELISA was performed in the Institute of Biotechnology, University of Helsinki.
Table 7. Antibodies used for immunohistochemical stainings.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibody</strong></td>
<td><strong>Product</strong></td>
</tr>
<tr>
<td>Rabbit polyclonal anti-CDNF</td>
<td>#4343, ProSci, Inc., Poway, CA, USA</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-MANF</td>
<td>Institute of Biotechnology, Helsinki, Finland</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-Iba1</td>
<td>#019-19741, Wako Pure Chemical Industries, Tokyo, Japan</td>
</tr>
<tr>
<td>Goat polyclonal anti-GDNF</td>
<td>#AF-212-NA, R&amp;D Systems</td>
</tr>
<tr>
<td>Rat monoclonal anti-DAT</td>
<td>#MAB369, Millipore</td>
</tr>
<tr>
<td>Mouse monoclonal anti-TH</td>
<td>#MAB318, Millipore</td>
</tr>
</tbody>
</table>

RT, room temperature
4.6 MORPHOMETRIC ANALYSES

All morphometric analyses were done on every sixth brain section (40 µm sections).

4.6.1 CELL COUNTS
TH-immunoreactive (DAB as a chromogen) or FG-positive (fluorescent) cells in rat SNpc were estimated according to the optical fractionator method combined with the dissector principle with unbiased counting rules using the Stereo Investigator platform (MicroBrightField, Williston, VT, USA) (West et al., 1991). Cells in the SNpc were counted bilaterally in either three or six sections from each brain. Results are given as percentage of cells in the lesioned rat SNpc as compared to the intact SNpc.

Estimation of the total number and number of activated microglia in the dorsal striatum was done in three Iba1-stained sections using the same stereological counting procedure as described above. Resting and activated microglia were separated based on their morphology (Kreutzberg, 1996). Resting microglia were identified as Iba1-reactive cells with a small cell body and fine processes extending from a few long thin prolongations. Round, intensely Iba1-stained cells with enlarged, ameobic-like cell bodies with or without short, thick processes were labeled as activated microglia.

4.6.2 FIBER DENSITY MEASUREMENT
For estimation of the TH-reactive fiber density in the striatum, pictures of immunohistochemically stained coronal striatal sections were acquired with a digital camera (Nikon Corporation, Tokyo, Japan) attached to a stereomicroscope. The fiber density was assessed by measuring the density along a line drawn across the dorsal striatum (II, III), or by encircling the dorsal part of the striatum (I) using Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA) (Deumens et al., 2002). All density values from the striatum were corrected for the background staining from corpus callosum, an area devoid of neuronal somas. Three (II, III) or five (I) coronal sections from the striatum of each rat brain were analyzed and the results are given as percentage of the lesioned striatum as compared to the intact striatum.

4.6.3 ESTIMATION OF BLOOD-BRAIN BARRIER INTEGRITY
To evaluate the condition of the BBB, the rat endothelial barrier antigen (EBA) was used as a marker for the competent BBB. After immunohistochemical staining, digital pictures from three striatal sections were taken and the amount of EBA-reactive blood vessels (area ≥300 dpi) in the dorsal striatum was estimated with Image-Pro Plus software (Media Cybernetics) (Piltonen et al., 2011).
Material and methods

4.6.4 DIFFUSION VOLUME
The diffusion volume of CDNF, MANF, and GDNF was assessed using immunohistochemically NTF-stained brain sections (Voutilainen et al., 2009; II). The area showing immunoreactivity in each section was estimated and the diffusion volume was assessed from all analyzed sequential sections with the Cavalier Estimator function on the Stereo Investigator platform (MicroBrightField).

4.6.5 SPROUTING
Using microscopy, the presence of sprouting was defined as an accumulation of intensely TH-stained fibers in areas that in the negative controls were devoid of such changes, or in areas that normally display little or no dopaminergic innervation (Georgievska et al., 2002a).

4.7 BRAIN DOPAMINE AND DOPAMINE METABOLITES
Estimation of DA and DA metabolite levels in the rat brain tissue was done according to Airavaara et al. (2006). Briefly, rat brains were snap frozen in cold isopentane and stored in -80 °C until dissection. Frozen brains were dissected in a cryostat and tissue samples from the striatum (3 mm punch, approximately 2 mm in A/P direction) and SN (2 mm punch, approximately 1 mm A/P) were collected and stored in -80°C. Tissue samples were homogenized in 0.2 M HClO₄-antioxidant solution (oxalic acid, acetic acid, L-cysteine) using a sonicator (Rinco Ultrasonics, Romanshorn, Switzerland). The homogenates were centrifuged (4°C, 14 000 rpm, 35 min) and the supernatant was moved into Vivaspin® sample concentrators (Vivascience AG, Hannover, Germany) and centrifuged at 9 000 rpm for 35 min, 4°C. The concentration of monoamines (DA, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA)) in the filtered samples was analyzed with high performance liquid chromatography (HPLC) using electrochemical detection. Eighty microliters of the filtrates were injected into the HPLC system with an autosampler (Shimadzu, Kyoto, Japan). The column (Kinetex 2.6 µm, 50x4.60 mm²; Phenomenex, Inc., Torrance, CA, USA) was heated to 45°C with a column heater (Croco-Cil, Bordeaux, France). The flow rate of the mobile phase was set to 1 ml/min with a pump (ESA Model 582 Solvent Delivery Module; ESA, Chelmsford, MA, USA). Monoamines in the samples were detected using ESA CoulArray Electrode Array Detector (ESA). Results are given as ng/mg wet weight of the tissue.

4.8 TRANSPORTATION OF NEUROTROPHIC FACTORS
To trace i.c. injected CDNF and GDNF, the NTFs were labeled with ¹²⁵I, and injections of ¹²⁵I-NTF (1-1.5ng/6 µl) alone or together with an excess (100x to 20 000x) of “cold” unlabeled NTF were done into the striatum of naïve rats (Tomac et al., 1995; Voutilainen et al., 2009; II). After perfusion and fixation of the rat brains (24 h post-injection), samples from the frontal cortex, striatum, hippocampus and nigra were gathered, and the activity in the
Materials and methods

samples was analyzed using a gammacounter (Wizard 3”, 1480 Automatic Gamma Counter, Wallac, Finland). For autoradiography measurements, fixed coronal paraffin sections (7 μm) were made from a different set of rats and put against an autoradiography film (Kodak Biomax MS) for four weeks.

4.9 SPECT/CT

Rat brains were scanned with a nanoSPECT/CT (Bioscan Inc., Washington, DC, USA) under isoflurane anesthesia four hours after an i.v. injection of $[^{123}]$-CIT (Neumeyer et al., 1991; Scherfler et al., 2002; III). The mean striatal $[^{123}]$-CIT activity was estimated, and corrected for by the activity measured in the cerebellum.

4.10 STATISTICS

All results are given as the mean ± standard error of the mean (SEM) for the treatment group. The statistical analyses were done using Pasw Statistics 10 (SPSS, Inc., Chicago, IL, USA). For pair-wise comparisons, the t-test was used. In the case of group numbers ≥ 3, group-wise comparisons were done using one-way analysis of variances (ANOVA), or repeated measures ANOVA, followed by Tukey HSD post-hoc test for normally distributed data. If Levene’s test for homogeneity showed statistical significance, Games-Howell post-hoc test was used for group-wise comparisons. For correlation analyses, the Pearson correlation test was applied. The results were considered statistically significant if $P<0.05$. 
5 RESULTS

5.1 CHARACTERIZATION OF THE PARTIAL 6-OHDA LESION

The experimental setup for characterization of the rat partial 6-OHDA lesion can be found in Figure 12A. Briefly, one week after retrograde labeling of nigral DAergic neurons with an intrastriatal injection of FG, three different doses of 6-OHDA were injected into the rat striatum. The progression of the lesion was evaluated using behavioral tests (8 weeks post-lesion), immunohistochemistry (1, 2, 4, and 8 weeks post-lesion), and SPECT/CT (2 and 4 weeks post-lesion).

5.1.1 MOTOR IMPAIRMENT INDUCED BY INTRASTRIATAL 6-OHDA (UNPUBLISHED RESULTS)

The dose-dependent effect on motor behavior following unilateral intrastriatal injections of 6-OHDA was estimated at eight weeks post-lesion using amphetamine-induced rotational test, cylinder test and forelimb akinesia test.

![Figure 13](image)

Figure 13 Asymmetric motor performance in unilaterally 6-hydroxydopamine (6-OHDA)-lesioned rats. Eight weeks post-lesion, rats lesioned with intrastriatal injections of 8, 20, or 2x10 µg 6-OHDA were tested for amphetamine-induced rotational behavior (2.5 mg/kg amphetamine i.p.) (A), limb use asymmetry in the cylinder test (B), and their ability to make adjusting steps when moved in the forward (C) and backward (D) side-wise direction. **P<0.01 as compared to the ipsilateral side (paired samples t-test), #P<0.05 as compared to 8 µg group (one-way ANOVA and Tukey post-hoc test) n=6/group.

Rats lesioned with only 8 µg showed no turning preference (Figure 13A), limb use asymmetry (Figure 13B) or forelimb akinesia (Figure 13C, 13D). While the dysfunction in
motor behavior was not significant following a single injection of 20 µg in any of the test, dividing the dose into two injection sites (2x10 µg) resulted in a trend towards imbalance between the contra- and ipsilateral paw use in the cylinder test (Figure 13B; \( P = 0.077 \), one-way ANOVA \( P = 0.091, F_{2,17} = 2.818 \) and Tukey post-hoc test). The 2x10 µg lesion group showed also significant impairment in the ability to make adjusting steps with the contralateral forepaw both as compared to the ipsilateral side (Figure 13C; \( P = 0.001 \), paired-samples t-test), as well as compared to the results for the 8 µg group \( (P<0.05, F_{2,17} = 4.809) \) and Tukey post-hoc test). No changes in the ability to make adjusting steps in the backward direction was observed in any of the groups (Figure 13D).

5.1.2 6-OHDA-INDUCED CHANGES IN THE NIGROSTRIATAL DOPAMINERGIC PATHWAY

The degeneration of the midbrain DAergic neurons following intrastriatal administration of 8, 20, or 2x10 µg 6-OHDA was assessed by counting TH-reactive and FG-positive neurons in the SNpc, by estimating densities of TH- and DAT-reactive fibers in striatal sections, and by evaluating striatal DAT function in vivo with \( ^{123} \text{I} \)β-CIT SPECT/CT (I). The results are presented in Figure 14 and in study I.

As expected, the decrease in TH-reactive cells in the SNpc showed to some degree time- and dose-dependence (Figure 14A, unpublished results). One week after the 6-OHDA injection, there were 72 ± 6% and 71 ± 6% TH-reactive cells remaining in the lesioned SNpc in the 20 µg and 2x10 µg 6-OHDA groups, respectively. In these groups, the most prominent loss of TH-reactive cells happened between weeks two and four, and at week four there was a statistical tendency towards a difference in the TH-reactive cell loss between rats treated with 8 µg versus 2x10 µg 6-OHDA (73 ± 4% and 45 ±5% cells remaining, respectively; \( P = 0.057 \), one-way ANOVA \( P = 0.063, F_{2,17} = 3.336 \) and Tukey post-hoc test). No clear progression of the lesion was observed in the 8 µg 6-OHDA group. Starting from week two, the cell loss in the group treated with 2x10 µg was consistently more pronounced that in the group that had received one single 20 µg injection. At eight weeks post-lesion, the results from TH-reactive cell counts implied a dose-related cell loss with 65 ± 10%, 51 ± 11%, and 38 ± 6% cells remaining in the SNpc in the 8, 20, 2x10 µg 6-OHDA groups, respectively (difference not significant, one-way ANOVA: \( P = 0.140, F_{2,17} = 2.245 \)).

The expected dose-dependent 6-OHDA-induced degeneration of DA neurons was not obvious when analyzing the amount of FG-positive cells in the SNpc (Figure 14B, unpublished results). At four weeks post-lesion, the remaining cell numbers were estimated to 39 ± 6%, 24 ± 5%, and 35 ± 8% as compared to the intact side in the 8, 20, 2x10 µg 6-OHDA groups, respectively. After this time point, the cell loss progress appeared to stop in the 8 µg and 20 µg lesion groups, whereas the loss of FG-positive neurons continued until week eight in the 2x10 µg lesion group, with only 23 ± 6% of the FG-positive neurons remaining at this time point. There was no significant decrease in the amount of FG-positive cells in the contralateral side (Figure 14C, unpublished results).
The loss of TH-reactive fibers in the striatum had reached its maximum level in all groups already one week post-lesion (Figure 14D, unpublished results). At both one and four weeks post-lesion, the damage to the striatal DAergic fibers were significantly more pronounced in the 20 µg group as compared to the 8 µg group (week one: $P<0.05$, one-way ANOVA ($P=0.016$, $F_{2,17}=3.336$), and week four: $P<0.01$, one-way ANOVA ($P=0.002$, $F_{2,17}=9.964$), followed by Tukey post-hoc test). The absence of progressive degeneration in the striatum was also observed when analyzing striatal DAT-reactive fiber densities (study I, Figure 2).

**Figure 14** Evaluation of the decrease in dopaminergic markers in 6-hydroxydopamine (6-OHDA)-treated rats. Rats were sacrificed 1, 2, 4, or 8 weeks after intrastriatal injections of 8, 20, or 2x10 µg 6-OHDA and their brains were processed for tyrosine hydroxylase (TH) immunohistochemistry. One week prior to the 6-OHDA injections, the nigral dopaminergic neurons were retrogradely labeled with an intrastriatal injection of FluoroGold (FG). TH-reactive (A) and FG-positive (B) cells in the ipsilateral substantia nigra pars compacta (SNpc) were counted and compared to the contralateral side. TH-reactive cell numbers in the contralateral SNpc can be seen in (C). The 6-OHDA-dependent loss of TH-reactive fiber densities in the ipsilateral rat striatum are shown in (D). The dopamine transporter (DAT) density was estimated with in vivo $[^{123}I]$$\beta$-CIT SPECT/CT two and four weeks post-lesion (E). Representative coronal sections from $[^{123}I]$$\beta$-CIT SPECT/CT can be seen in (F) with color code (blue-red-white) according to the measured activity. #P<0.05, ##P<0.01, ###P<0.001 as compared to the 8 µg 6-OHDA group, ***P<0.001 as compared to intact rats (one-way ANOVA and Tukey post-hoc test), n=6 (A-D), n=4-5 (E).
When comparing the results from morphologic assessment of TH-reactive striatal fibers and nigral cells to results from the behavioral test (Figure 15), we found that a decrease in TH-reactive cells in the SNpc >45% and striatal TH-reactive fiber loss >60% induced motor deficits in approximately 50% of the rats. However, a robust impairment in the forelimb akinesia and cylinder tests was observed only in the four rats that each showed a cell loss >70% and fiber loss ≥80% (encircled individuals in Figure 15).

**Figure 15** Comparison of the degree of dopaminergic innervation and response in motor performance tests. The 6-OHDA-induced loss of tyrosine hydroxylase (TH) –reactive cells in the substantia nigra pars compacta (SNpc) and TH-reactive fiber density in the striatum was compared to results from the forelimb akinesia test (left y axis) and the cylinder test (right y axis) eight weeks post-lesion. Encircled individuals denote rats showing consistent response in all parameters measured. The line shows 50% use of the ipsilateral limb in the cylinder test, indicating the level for no limb use asymmetry.

In line with the results from the striatal density measurements, no lesion progression was observed in estimates of striatal DAT density with [123I]β-CIT SPECT/CT (Figure 14E). However, there were statistically significant differences in striatal [123I]β-CIT binding between intact rats, and rats lesioned with 8 µg and 2x10 µg 6-OHDA (Figure 14E, F). In addition, individual results from [123I]β-CIT SPECT/CT and TH and DAT immunohistochemistry showed a high degree of correlation at both two and four weeks post-lesion (for correlation coefficients and P-values, see study I, Figure 3 and Table 1).
5.1.3 THE EFFECT OF 6-OHDA DOSE ON MICROGLIAL ACTIVATION AND BLOOD-BRAIN BARRIER INTEGRITY (UNPUBLISHED RESULTS)

To study some aspects of the unspecific actions of 6-OHDA, fixed striatal sections were immunolabeled with antibodies against Iba1 (microglial marker) and EBA (rat-specific marker for competent BBB) to examine whether there are 6-OHDA dose-dependent influences on microglia activation and/or BBB permeability.

One week after the 6-OHDA injections, a robust activation of striatal microglia was observed in the ipsilateral striatum. There was a tendency towards a difference between the 8 µg and 20 µg lesion groups in both the total number of Iba1-reactive microglial cells (one-way ANOVA \( P=0.051, F_{2,17}=3.663 \)) and microglia with activated morphology (one-way ANOVA \( P=0.047, F_{2,17}=3.789 \), significance not detected with post-hoc test \( P=0.51 \) between 8 µg and 20 µg groups, Tukey post-hoc test)) (Figure 16A). Also in the contralateral side a clear microglial response could be seen around the FG injection tract, but the total amount of Iba1-reactive cells was significantly less pronounced in all lesion groups as compared to the contralateral side (8 µg: \( P=0.006 \), 20 µg: \( P=0.041 \), 2x10 µg: \( P=0.002 \), paired samples t-test). There was also a significant difference in Iba1-reactive cells displaying activated morphology in the 8 µg (\( P=0.014 \)) and 2x10 µg (\( P<0.001 \)) 6-OHDA groups, while only a trend was seen in rats lesioned with 20 µg 6-OHDA (\( P=0.064 \)).

**Figure 16** The impact of striatal 6-OHDA injections on the amount of microglia and blood-brain barrier (BBB) competence. One week post-lesion, striatal sections from rat brains injected with 8, 20, or 2x10 µg 6-OHDA were immunohistochemically stained for Iba1 and rat endothelial barrier antigen (EBA). The total amount of Iba1-reactive cells (white (complete) bars) and proportion of Iba1-reactive cells with activated morphology (black bars) were determined as stereologic estimates of the amount of cells counted from three striatal sections (A). L denotes left (ipsilateral) side and R right (contralateral) side. The contralateral side (R) had received an injection of FluoroGold two weeks prior to the analysis, and is thereby not intact. The number of EBA-reactive blood vessels in the striatum presented in (B) represents the average amount of EBA-reactive blood vessels estimated from three striatal sections. *\( P<0.05 \), **\( P<0.01 \), ***\( P<0.001 \) as compared to the ipsilateral (L) side (paired samples t-test), n=6.

At one week post-lesion, the amount of EBA-stained blood vessels in the dorsal striatum did not significantly differ between the 6-OHDA doses 8, 20, and 2x10 µg (Figure 16B). As compared to the contralateral side (that had received an injection of FG one week before the lesion, and was, thus, exposed to mechanical damage), the percentage of EBA-reactive
blood vessels in the lesioned side was 101±3%, 103±6 %, and 93±4% in the 8, 20, and 2x10 µg 6-OHDA groups, respectively (differences not statistically significant).

5.2 DELIVERY OF NEUROTROPHIC FACTOR IN INTACT RAT BRAIN

Long-term delivery of NTFs was carried out using osmotic pumps delivering the NTF into the striatum via a cannula, or by intrastriatal injections of recombinant AAV2 vectors carrying the NTF gene of interest.

5.2.1 INFUSION OF NEUROTROPHIC FACTORS

Following a three-day infusion of NTFs (CDNF, MANF, GDNF) into the striatum of naïve rats, immunohistochemical analysis of the striatal diffusion volume of the infused proteins showed that MANF had spread to a statistically significantly larger area than GDNF (Figure 17A). After two weeks of NTF infusion, no difference between the treatment groups could be seen. A two-week infusion of NTFs into the striatum of naïve rats did not cause any statistically significant changes in the nigral TH-reactive cell counts, although infusion of GDNF showed a trend for reducing the TH-reactive cells as compared to the CDNF group (P=0.068, one-way ANOVA (P=0.073, F_{2,13}=3.353) and Tukey post-hoc test) (Figure 17B).

5.2.2 NEUROTROPHIC FACTOR GENE DELIVERY

A single intrastriatal injection of AAV2-CDNF resulted in long-term overexpression of hCDNF in the rat striatum that had reached its maximum between four and eight weeks post-injection (Figure 18A). The total striatal amounts of hCDNF were 0.17 ± 0.07 ng, 0.54 ±
Results

0.08 ng, 0.78 ± 0.12 ng, 1.85 ± 0.30 ng, and 1.35 ± 0.52 ng at 1, 2, 4, 8, and 12 weeks post-injection, respectively. At later time points our CDNF-ELISA also detected hCDNF in the rat SN (Figure 18A).

5.2.3 TRANSPORT OF $^{125}$I-CDNF AND $^{125}$I-GDNF

The transport of striatally administered CDNF to the SN was further studied in naïve rats using $^{125}$I-labeled CDNF. The presence of $^{125}$I-CDNF in the SN 24 h after an intrastriatal injection (1-1.5 ng) was confirmed by autoradiography (study II, Figure 8) and gamma counter measurements (Figure 18B). Furthermore, the signal from $^{125}$I-CDNF in the SN could be blocked in a dose-dependent fashion by addition of increasing amounts of unlabeled "cold" CDNF. Intrastriatally injected $^{125}$I-labeled GDNF was detected in the rat SN, and the presence of $^{125}$I-GDNF in the nigra was almost completely blocked by simultaneous injection of a 100x molar excess of unlabeled GDNF (Figure 18B).

Figure 18 Detection of CDNF in the substantia nigra (SN) following intrastriatal delivery of AAV2-CDNF and $^{125}$I-CDNF. The level of CDNF protein in dissected tissue from the striatum and SN 1, 2, 4, 8, and 12 weeks after an intrastriatal injection of 1x10^9 vg AAV2-CDNF was determined with a CDNF-ELISA assay (A). Twenty-four hours after an intrastriatal injection of $^{125}$I-labeled CDNF or GDNF (1-1.5 ng protein) with or without an excess of unlabeled "cold" neurotrophic factor, brains were dissected and the proportion of radiolabeled NTF in the SN was estimated using gamma counter measurements (B).

5.3 EFFECT OF LONG-TERM DELIVERY OF CDNF ON ROTATIONAL ASYMMETRY IN 6-OHDA LESIONED RATS

To evaluate the progression of the lesion and estimate the efficacy of long-term delivery of NTFs in normalizing neural circuits controlling movement after 6-OHDA lesioning (2x10 μg), rats were tested using amphetamine-induced rotational test. Treatment with CDNF, both as two-week continuous infusions (starting two weeks post-lesion) and as AAV-mediated
gene therapy (AAV2-CDNF injection two weeks pre-lesion), resulted in significant decrease in ipsilaterial rotations (Figure 19). In the protein infusion study, infusion of CDNF 3 µg/24h for two weeks provided optimal effect, while either smaller or larger doses did not affect the turning asymmetry (Figure 19A). When delivering CDNF with an AAV2 vector, the larger titers (2x10^8 and 1x10^9 vg) were able to normalize the drug-induced turning behavior, while the smallest titer (4x10^7 vg) showed no effect (Figure 19B). For more exact P-values, see study II and III.

In the protein infusion study, neither GDNF nor MANF showed sufficient neurotrophic potential to significantly reverse the ipsilateral turning asymmetry (Figure 19A and study II, Figure 3C, 3D). However, when delivered with an AAV2 vector, GDNF proved to efficiently reduce the amount of amphetamine-induced turns as compared to rats that had received the control treatments (Figure 19B).

![Figure 19](image)

**Figure 19** The effect of CDNF treatment on amphetamine-induced turning asymmetry in 6-hydroxydopamine (6-OHDA) -lesioned rats (6-OHDA 2x10 µg intrastriatally). CDNF or GDNF was administered intrastriatally as two-week protein infusions starting two weeks post-lesion (A), or as adeno-associated virus (AAV) vector-mediated gene delivery with the AAV vector injection two weeks prior the the lesion (B). Every second week, rats received an i.p. injection of amphetamine 2.5 mg/kg, and the amount of full ipsi- and contralateral turns were detected for 120 min. Statistics presented in (A): *P<0.05, CDNF 3.0 µg/24h as compared to vehicle (repeated measures ANOVA followed by Tukey post-hoc test). Statistics presented in (B): *P<0.05, **P<0.01, AAV-CDNF compared to control (AAV2-CDNF 2x10^8 vg at week two and four, AAV2-CDNF 1x10^7 vg at week six and ten); #P<0.05, ##P<0.01, AAV-GDNF compared to control (one-way ANOVA followed by Tukey post-hoc test).
5.4 EFFECTS ON THE MIDBRAIN DOPAMINERGIC SYSTEM FOLLOWING LONG-TERM DELIVERY OF CDNФ IN 6-OHDA LESIONED RATS

To study the degree of DAergic cell loss and NTF-induced protection and/or recovery of DAergic cells in 6-OHDA-lesioned rats, fixed rat brain sections were immunohistochemically stained with TH and number of TH-reactive cells in the SNpc and TH-reactive fiber densities in the striatum were estimated.

As compared to the control groups, a two-week delivery of CDNФ protein as well as CDNФ gene therapy in the striatum resulted in partial protection of TH-reactive cells in the rat SNpc (Figure 20 A, B). In agreement with the results from amphetamine-induced rotational behavior, the most effective protection was achieved with a protein infusion dose of 3 µg/24h or with injection of AAV2-CDNF 2x10⁸ to 1x10⁹ virus genomes (vg). The CDNФ-mediated preservation of nigral TH-reactive cells was higher in the protein infusion study (approximately 67%) as compared to the gene therapy study (approximately 49%). The degree of cell loss in the vehicle-treated groups was similar in both studies with

Figure 20 The effect of CDNФ treatment on dopaminergic cell survival in the rat partial 6-hydroxydopamine (6-OHDA) (2x10 µg) lesion model. CDNФ or GDNФ was delivered as two-week protein infusions starting two weeks post-lesion (A and C), or as adeno-associated viral (AAV) vector-mediated gene therapy initiated two weeks before the 6-OHDA injections (B and D). Fourteen (protein infusion) or ten (gene therapy) weeks post-lesion, rat brains were fixed and stained for tyrosine hydroxylase (TH) - immunoreactivity. The number of TH-reactive cells in the ipsilateral SNpc (A and B) and TH-reactive fiber density in the ipsilateral striatum (C and D) were determined and compared to the measures in the contralateral side. *P<0.05, **P<0.01 as compared to the vehicle/control group (one-way ANOVA and Tukey post-hoc test).
approximately 25% and 27% cells remaining in the protein infusion and gene therapy study, respectively.

Note that the results in Figure 20B represent the survival of TH-reactive cells in three nigral sections, ranging from approximately 5.0 to 5.5 mm posterior to bregma, while corresponding figure in the original study II presents results from six sections. The re-analysiz was done to be able to compare the results from the two separate studies. When analyzing six sections, ranging from approximately 4.5 to 6.0 mm posterior to bregma, we found variation in the degree of TH-reactive cell protection in the AAV2-CDNF-treated groups depending on the level (rostral, central, caudal) of the SNpc, with the most pronounced protection in the two central brain sections (III). On the contrary, the amount of surviving TH-reactive cells following AAV2-GDNF treatment was more consistent throughout all three levels.

Intrastriatal infusion of CDNF 3 μg/24h resulted in a significant restoration of TH-reactive fiber density (P<0.05, one-way ANOVA (P=0.006, F_{4,31}=4.551) and Tukey post-hoc test) (Figure 20C). This increase in density was accompanied by signs of a sprouting-like phenomenon (network of intensely TH-stained fibers) at the site of infusion (study III, Figure 6). However, no sprouting was observed in the lesioned striata treated with AAV2-CDNF, even though the treatment showed a strong trend towards protection of the DAergic fibers (Figure 20D). Despite sprouting of TH-reactive fibers at the GDNF protein infusion site, the density was not significantly increased by the GDNF treatment (Figure 20C). However, in the gene therapy study, intrastriatal delivery of AAV2-GDNF did result in significant protection of the striatal TH-reactive fibers (Figure 20D). A two-week protein infusion of MANF had no significant effect on the number of nigral TH-reactive cells or striatal TH-reactive fiber density (II). Note that the results from fiber density measurements in the protein infusion study (Figure 20C) were re-analyzed according to the protocol used for density measurement in the gene therapy study (correction for background staining) and therefore differ from data presented in the original study II. This was done to allow comparison of the results from study II and III.

To study the effect of CDNF gene therapy on midbrain DA levels, tissue samples from non-lesioned or 6-OHDA-lesioned (2x10 μg 6-OHDA) rats injected with either AAV2-GFP or AAV2-CDNF 1x10^7 vg were collected four weeks after the AAV2 injection (two weeks after the 6-OHDA injection in lesioned rats) (Figure 12C). Analysis of the levels of DA, DOPAC, HVA, 5-HT, and 5-HIAA in the striatum of non-lesioned and lesioned animals revealed no statistically significant differences between AAV2-GFP and AAV2-CDNF treatment (Table 8, unpublished data). In 6-OHDA-lesioned rats, the striatal DA level was approximately 42% higher in the AAV2-CDNF-treated group as compared to the AAV2-GFP group (19.4 ± 5.7% versus 12.9 ± 6.8% of the right intact side DA levels), but this difference did not reach statistical significance. In the 6-OHDA-treated control group a 25.2 ± 8.6% loss of striatal 5-HT levels was observed, while the corresponding loss was only 1.3 ± 10.8% in the AAV-CDNF-treated striatum (difference not statistically significant).
Table 8  DA, DOPAC, HVA, 5-HT, and 5-HIAA concentrations (ng/mg wet weight of tissue) in the rat striatum four weeks after AAV2-GFP (control) or AAV2-CDNF injection in non-lesioned and lesioned animals (2x10 µg 6-OHDA two weeks after AAV injection; n=6/group). The virus vector and 6-OHDA injections were done in the left striatum while the right hemisphere was left intact.

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<tr>
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<th>Non-lesioned</th>
<th>Lesioned</th>
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<tr>
<td></td>
<td>Left (AAV)</td>
<td>Right (naive)</td>
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<tr>
<td><strong>DA</strong></td>
<td></td>
<td></td>
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<tr>
<td>AAV2-GFP</td>
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<td>14.14 ± 0.60</td>
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<td><strong>DOPAC</strong></td>
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<tr>
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<td>0.97 ± 0.06</td>
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<tr>
<td>AAV2-CDNF</td>
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<td>0.83 ± 0.02</td>
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<tr>
<td><strong>HVA</strong></td>
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<tr>
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<tr>
<td><strong>5-HT</strong></td>
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<tr>
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<tr>
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AAV, adeno-associated virus; CDNF, cerebral dopamine neurotrophic factor; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; GDNF, glial cell line-derived neurotrophic factor; GFP, green fluorescent protein; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; HVA, homovanillic acid; 6-OHDA, 6-hydroxydopamine
6 DISCUSSION

The purpose of this work was to evaluate the neuroprotective potential of CDNF in the rat partial 6-OHDA model of PD. In addition, we wanted to study the partial 6-OHDA models used in our present and previous works to be able to better estimate treatment outcomes and more efficiently time the treatment initiation. Our main results show that CDNF, given as two-week protein infusions or delivered with a recombinant AAV vector, provides functional improvement seen as normalization of the amphetamine-induced ipsilateral turning bias. CDNF treatment was also accompanied by partial preservation of TH-reactive neurons in the SNpc together with a restoration of TH-reactive fiber density in the striatum that was dependent on the way CDNF was delivered.

6.1 RAT 6-OHDA LESION MODELS OF PARKINSON’S DISEASE

The use of 6-OHDA to induce degeneration of DA neurons in rats was first described by Dr. Urban Ungerstedt in 1968 (Ungerstedt, 1968). Since then the model and its modifications has become a widely used tool in PD research. The mechanisms of 6-OHDA toxicity is still not completely understood, but induction of oxidative stress through auto-oxidation and the generation of reactive oxygen species (ROS) is believed to be the main cause of the deleterious effects (Mazzio et al., 2004; Duty and Jenner, 2011). In addition, 6-OHDA has been shown to interact with and inhibit the mitochondrial respiratory chain, further exacerbating the oxidative stress (Glinka et al., 1996), as well as induce neuroinflammation through microglial activation (Cicchetti et al., 2002; Depino et al., 2003).

The midbrain DAergic pathway can be targeted for degeneration by injection of 6-OHDA in the rat SN, in the medial forebrain bundle (MFB; tract in which the nigrostriatal DAergic fibers ascend), or in the striatum (Deumens et al., 2002; Duty and Jenner, 2011). While infusion of 6-OHDA in the rat SN or MFB creates a rapid neuron degeneration that is almost complete in less than one week (Jeon et al., 1995; Zuch et al., 2000), a more slowly progressive degeneration can be achieved with intrastratial injections of 6-OHDA (Berger et al., 1991; Ichitani et al., 1991; Sauer and Oertel, 1994; Przedborski et al., 1995). The progressive nature of this partial 6-OHDA lesion model makes it suitable for monitoring the efficacy of neuroprotective agents and has been used in our NTF studies. In these studies, we have generally applied three different partial lesions: single injections of 8 µg or 20 µg, or two injections á 10 µg 6-OHDA (Lindholm et al., 2007; Voutilainen et al., 2009; II; III). To be able to time the initiation of treatment and predict the clinical relevancy of the therapeutic interventions, we wanted to determine the degree and progression of the lesions induced by these three different 6-OHDA lesion paradigms.
6.1.1 PROGRESSION OF 6-OHDA LESION: DOSE-DEPENDENCE

The TH-reactive fiber loss in the striatum was complete already at one week post-lesion in all lesion groups. This conclusion is supported by published data showing that when intrastriatally administered, 6-OHDA causes rapid damage to the dopaminergic terminals at the injection site (Sauer and Oertel, 1994; Przedborski et al., 1995). The extent of fiber loss was dependent on the 6-OHDA dose. Logically, injection of 1x20 µg or 2x10 µg 6-OHDA created a larger striatal lesion than 8 µg 6-OHDA. The reason why 1x20 µg and 2x10 µg 6-OHDA resulted in a similar decrease in TH-reactive fiber density may be due to the fact that only three coronal sections from the striatum were analyzed. In study I, the density was estimated from five striatal sections showing a decrease in fiber density of approximately 90% two weeks after injection of 2x10 µg 6-OHDA. When analyzed from three striatal sections, the corresponding fiber density loss was estimated to approximately 78%. This was in line with the CDNF protein infusion (II) and gene therapy (III) studies in which also only three striatal sections were analyzed showing a TH-reactive fiber loss of approximately 73% and 78% in the control groups, respectively.

Intrastriatal 6-OHDA injections cause a progressive loss of DAergic cells in the SNpc (Sauer and Oertel, 1994; Przedborski et al., 1995). Stereologic counting of FG-positive (DAergic) neurons in the SNpc indicated that, even though all three lesion paradigms were associated with progressive cell loss, the progressiveness differed between the groups. The cell loss following injection of 8 µg was rapid until two weeks. After this, the progression seemed to stop and the amount of cells remained stable until the end of the study. A single injection of 20 µg 6-OHDA mediated a rapid progression of the lesion until week four, where after the cell loss reached a plateau. Instead, the loss of cells in the group treated with 2x10 µg was characterized by a slower ongoing progressive cell loss that continued until the end of the experiment (eight weeks post-lesion). The observation that a single injection of 6-OHDA led to a more rapid FG-positive cell loss than the two-site lesion could have been influenced by the fact that in the single 6-OHDA injection groups the FG and 6-OHDA injections were made in the same site. This would allow the same cells with projections in a close proximity to the FG injection site to also be most influenced by the toxic effects of 6-OHDA. On the other hand, in the 2x10 µg 6-OHDA group, the injection site for FG was different from that of 6-OHDA. However, also when counting TH-reactive cell, this group showed a slightly more evenly progressive cell loss throughout the study.

Initially, the loss of TH-reactive cells was more pronounced than the loss of FG-positive cells. This has also been shown in previous studies, and is suggested to indicate an initial 6-OHDA-induced loss of TH phenotype that precedes the actual cell degeneration (Sauer and Oertel, 1994; Bowenkamp et al., 1996; Cohen et al., 2011). Starting from two weeks post-lesion, the loss of FG-positive cells exceeded the loss of TH-reactive cells. The explanation for this difference may be technical. Striatal injection of FG retrogradely labeled mainly cells located medially in the SNpc and constituted about 50-70% of the TH-reactive cells in the contralateral side. As mentioned above, the shared injection site could render the FG-positive cells more susceptible to the 6-OHDA-induced damage. A more marked decrease in FG-positive cells could also be due to eventual toxicity of FG. However, FG has previously
been reported to have no toxic effects on its retrogradely labeled neurons (Schmued and Fallon, 1986; Divac and Mogensen, 1990).

The FG-positive cells in the contralateral side showed generally DA neuron morphology (medium-sized with polygonal shape and/or at least one long dendrite) (Fallon and Loughlin, 1995). In addition, mainly in the ipsilateral, but also in the contralateral side, glia-like cells (small, star-like cells with fine processes) were detected, probably due to ingestion of FG-containing debris. In the ipsilateral side, variable amounts of small intensely fluorescent shrunken remnants of DAergic cells were observed. These were not counted as DA neurons. Even though we observed a small continuous decrease in the number of FG-reactive cells in the contralateral side of animals that had received a single injection of 20 µg 6-OHDA, this decrease was not statistically significant. In earlier studies, a striatal injection of 20 µg (Sauer and Oertel, 1994) or 40 µg 6-OHDA (Berger et al., 1991) has caused a statistically significant, although rather mild, loss of contralateral nigral FG-positive or [3H]mazindol-labeled DA neurons.

Which lesion would serve the best purpose for pre-clinical research and at what time point should the experimental neuroprotective treatments be initiated? Parkinson’s disease is a slowly progressive disorder and it is estimated that the motor symptoms start to appear when approximately 30% of the DAergic cells in the SNpc are lost (Fearnley and Lees, 1991; Cheng et al., 2010). This limit is exceeded in all 6-OHDA groups (8, 20, 2x10 µg) by two weeks post-lesion, and therefore, if clinical relevance is the goal, experimental treatments should not be initiated before this time point. While the neurodegeneration in the rats lesioned with 8 µg 6-OHDA halted after two weeks post-lesion, a slowly progressive neurodegeneration that continued until the end of the study was seen especially in the 2x10 µg group. Compared to clinical PD, this is a desirable characteristic.

At the time of motor symptom onset, it is estimated that there is up to a 50-70% loss of striatal DAergic terminals together with a 68-82% age-dependent decrease in striatal DA levels (Cheng et al., 2010). Following striatal injection of 2x10 µg 6-OHDA, the striatal tissue DA level was decreased by approximately 87% at two weeks post-lesion, being slightly more pronounced than at symptom onset in clinical PD. At the same time point, there was a corresponding 88% loss of striatal TH-reactive fiber density. The loss of striatal DA terminals was also estimated with [123I]β-CIT SPECT/CT, showing a much milder, approximately 30% decrease in striatal DAT density. Since the results from DAT-reactive fiber density measurements (approximately 90% loss, I) was similar to the results obtained with TH immunohistochemistry, the discrepancy is most probably due to methodological issues, such as area analyzed and correction for background signal, and not dependent on the marker (DAT or TH). Finally, at eight weeks post-lesion, the DA cell loss in the 2x10 µg group was approximately 77%, corresponding to the situation at 10 to 15 years of symptomatic PD (Fearnley and Lees, 1991; Cheng et al., 2010).
6.1.2 SPECT/CT PROVIDES RELIABLE ESTIMATE OF DOPAMINE NEURON DEGENERATION IN THE RAT PARTIAL 6-OHDA MODEL

The DAergic system can be studied in vivo with, e.g., SPECT and PET detection of radiotracers with affinity to structural components of the DAergic transmission system (DAT, VMAT2, DA receptors) (Nikolaus et al., 2011). These types of imaging studies have mainly been conducted on rats that have received a 6-OHDA injection into the MFB or SN. For example, injections of 8 µg 6-OHDA into the rat MFB have produced a wide variety of unilateral decrease in striatal binding of DAT markers ([123I]β-CIT, [11C]CFT, [11C]PE2I), ranging from 29% to 85% (Chen et al., 1997; Scherfler et al., 2002; Inaji et al., 2005; Pellegrino et al., 2007; Zhu et al., 2007a). A few of these studies have tried to correlate the imaging results to nigral DAergic cell counts (Scherfler et al., 2002) or to behavioral data (Inaji et al., 2005; Pellegrino et al., 2007) and have found significant correlations between the measures.

The intrastriatal partial 6-OHDA model is generally thought to be better suited for preclinical studies of neuroprotective agents than the more complete SN or MFB lesion models (Duty and Jenner, 2011). However, the partial lesion model has been scarcely studied with in vivo imaging methods. A PET study with the DAT marker [11C]CFT (2-β-carbomethoxy-3β-(4-fluorophenyl)tropane) estimated the decrease in ligand binding to 65% following intrastriatal injection of 4x6 µg 6-OHDA (Cicchetti et al., 2002). This decrease was accompanied by loss of TH-reactive nigral neurons, but the correlation between these two measures was not determined. We chose the DAT ligand [123I]β-CIT to evaluate the neurodegeneration in the partial 6-OHDA model with our small animal nano-SPECT/CT system. In addition to DAT, β-CIT has also affinity to the serotonin transporter (SERT), and is, thus, not as specific as CFT (Bergström et al., 1997; Booij et al., 1998). The [123I]β-CIT binding to SERT can be a contributing factor for the seemingly small decrease in striatal activity (approximately 32% in 2x10 µg 6-OHDA group) as compared to the study done by Cicchetti and colleagues. Unspecific background activity is suggested to be further exacerbated in the 6-OHDA lesioned striatum (Sossi et al., 2012). The serotonergic system is known to be affected in PD (Jellinger, 1991). In 6-OHDA-lesioned rats, results have been inconsistent with reports showing both increases and decreases in serotonergic innervation and serotonin levels (Zhou et al. 1991; Ichitani et al., 1994; Maeda et al., 2003; Lindgren et al., 2010). We observed an approximately 25% decrease (not statistically significant) in the serotonin levels in the striatum of 6-OHDA-lesioned rats (treated with control vector AAV-GFP). It is unclear whether this is due to a loss of serotonergic innervation or decrease in serotonin production. The proportional decrease in DAT density is, however, more extensive than a possible decrease in SERT density and this shift may affect the results. The background activity due to [123I]β-CIT binding to SERT could have been at least partly avoided by administration of a selective serotonin reuptake inhibitor (e.g., paroxetine) (Booij et al., 2007) prior to the [123I]β-CIT injection. However, it is obvious that there is generally rather big variation in results from SPECT and PET studies which makes the numeric results from these studies difficult to compare.

Our results show, that the striatal DAT binding detected with [123I]β-CIT SPECT/CT is highly correlated to both TH-reactive cell loss in the SNpc, as well as TH- and DAT-reactive fiber
density loss in the striatum both two and four weeks post-lesion (study I, Figure 3). We therefore conclude that the method can reliably be used to estimate the degree of degeneration of the nigrostriatal DAergic pathway in the partially 6-OHDA-lesioned rat. With our $^{123}$I-β-CIT SPECT/CT protocol we were able to distinguish partial lesions induced by two different amounts of 6-OHDA (8 µg versus 2x10 µg). In addition, there was a highly significant decrease in $^{123}$I-β-CIT binding in rats that had received 8 µg 6-OHDA as compared to the DAT binding in intact rats. This difference was also detected in measurements of striatal fiber densities, but not in TH-reactive cell counts. On the other hand, as for $^{123}$I-β-CIT SPECT/CT, there was a significant difference in the loss of TH-reactive cells between the two lesion groups, while this difference again was not detected with TH- or DAT-reactive fiber density measurements. Hence, when comparing these three methods ($^{123}$I-β-CIT SPECT/CT, TH-reactive nigral cell counts, and TH-reactive striatal fiber density measurement), $^{123}$I-β-CIT SPECT/CT seems to be the most sensitive and selective method, showing significant differences both between intact rats and rats with mild lesions, as well as between rats with different degree of lesion. Our results indicate that compared to more conventional methods, such as immunohistochemistry, in vivo imaging of partially 6-OHDA-lesioned rats with SPECT offers a considerable potential for reliable monitoring of changes in the midbrain DAergic pathway allowing non-invasive longitudinal studies in living animals.

6.1.3 BEHAVIORAL EVALUATION OF THE 6-OHDA LESION

Since bilateral injections of 6-OHDA result in severe aphagia, 6-OHDA is generally applied unilaterally (Ungerstedt, 1971; Duty and Jenner, 2011). The imbalance between the ipsi- and contralateral side is manifested as an asymmetry in motor performance that can be monitored with specific drug-induced or spontaneous behavioral tests that allow evaluation of the lesion (reviewed by Meredith and Kang, 2006).

The most used behavioral test in unilaterally 6-OHDA-lesioned rats is the drug-induced rotational test originally described by Ungerstedt and Arbuthnott (Ungerstedt and Arbuthnott, 1970). The test is based on the observation that rats with an imbalance in DA concentration between the hemispheres show a characteristic turning behavior in response to drugs acting on the DAergic system. The use of amphetamine to induce rotations usually detects already a 40-50% decrease in the striatal DA level, and 30-50% loss of cell bodies in the SN (Przedborski et al., 1995; Lee et al., 1996; Kirik et al., 1998). In our study, 8 µg 6-OHDA, causing an approximately 35% loss of TH-reactive and 59% loss of FG-positive cells, did not cause an amphetamine-induced ipsilateral rotational response at eight weeks post-lesion. In our previous studies, on the other hand, the same dose has given rise to clear rotational asymmetry (Lindholm et al., 2007; Voutilainen et al., 2009). In these studies, the amphetamine-induced rotations were detected two and four weeks post-lesion. Especially mild partial lesions are associated with compensatory mechanisms in the lesion side, such as changes in DA re-uptake and turnover, and axonal sprouting (Zigmond et al., 1990; Schwarting and Huston, 1996). It could be that these compensatory mechanisms are not yet fully developed at earlier time points, while at eight weeks post-lesion they are able to silence the rotational bias. In the two groups injected with a total of
20 µg 6-OHDA, there was a clear ipsilateral rotation bias, but due to big inter-individual variations, the amount of netipsilateral turns did not differ significantly from the 8 µg 6-OHDA group.

Even though the assumption is that the amount of amphetamine-induced rotations reflects the loss of DA innervation (i.e., loss of striatal DA and post-synaptic receptor hypersensitization), the correlation between the number of rotations and actual denervation has often proven to be rather poor and non-linear, showing same magnitude of rotations for a wide range of DA cell loss (Lee et al., 1996; Klirik et al., 1998; Chang et al., 1999). In addition, statements that the test is not relevant to PD (the need for drug to provoke a behavior not related to clinical PD) have been raised, questioning the use of the test in pre-clinical PD research (Meredith and Kang, 2006). Two other tests that have been suggested to provide good measures of unilateral lesions are the cylinder test, measuring limb use asymmetry (Schallert et al., 2000), and forelimb akinesia test, measuring the rat’s ability to initiate movement (Olsson et al., 1995).

The forelimb akinesia test has earlier been reported to show an all-or-nothing response to a decrease in striatal DA (Chang et al., 1999). Hence, an 80% depletion of DA induced a reduction in forelimb adjusting steps with no additional impairment due to further decrease in DA. In our hands, positive responses in both the forelimb akinesia and cylinder tests were detected in approximately 50% of the animals with cell loss ranging between 45% and 70%. However, a DA cell loss of >70% and DA fiber loss >80% were required for robust response in both tests. Of the four rats that showed clear impairment in all analyses, two were from the 20 µg and two from the 2x10 µg 6-OHDA lesion group. The same rats showed variable response in the amphetamine-induced rotational test, with netipsilateral rotations ranging from 133 to 1647 (960 ± 312).

All three behavioral tests used in the study show a response to L-DOPA (Olsson et al., 1995; Lundblad et al., 2002). However, while L-DOPA, like in the clinical situation, improves the performance in the forelimb akinesia and cylinder tests, it exacerbates the abnormal rotational asymmetry in rat with lesion-induced sensitized DA receptors. Yet another advantage of the forelimb akinesia and cylinder tests is that they are based on normal behavior without being dependent on drug administration, learning skills or motivation. However, drug-induced rotational test provides an easy and objective way of assessing DA system impairments in the unilateral 6-OHDA lesioned rat. In addition, the test is rather sensitive, while both forelimb akinesia and cylinder test require more robust changes in the nigrostriatal DAergic system to induced changes measured behavior (Table 9). A combined used of all three tests is therefore probably the optimal solution, allowing detection of a wider range of lesion degree together with assessment of both clinically relevant physiologic behavior as well as treatment-induced enhancement in function and/or density of striatal DAergic terminals.
Table 9 Estimation of the loss of nigral tyrosine hydroxylase (TH) -reactive cells and striatal TH-reactive fiber density needed to induce asymmetric motor behavior in the rats. The estimates are based on the results presented in 5.1.1 with average values for the 6-OHDA lesion groups that showed response in the tests.

<table>
<thead>
<tr>
<th>Test</th>
<th>Drug-induced or spontaneous</th>
<th>Loss of TH-reactive cells in the SNpc</th>
<th>Loss of TH-reactive fiber density in the striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotational asymmetry</td>
<td>Amphetamine-induced</td>
<td>&gt;35-50%</td>
<td>&gt;61-67%</td>
</tr>
<tr>
<td>Forelimb akinesia test</td>
<td>Spontaneous</td>
<td>&gt;49-62%</td>
<td>&gt;67-72%</td>
</tr>
<tr>
<td>Cylinder test</td>
<td>Spontaneous</td>
<td>&gt;62%</td>
<td>&gt;72%</td>
</tr>
</tbody>
</table>

6.1.4 ASPECTS OF NEUROINFLAMMATION IN THE 6-OHDA MODEL

When injected into the brain, 6-OHDA is taken up by monoamine transporters (DAT and NET), and its toxicity is therefore considered to be rather specific for DA and noradrenaline neurons (Duty and Jenner, 2011; Bove and Perier, 2012). However, it is now recognized that in addition to the specific effects on monoaminergic neurons, 6-OHDA treatment can also mediate unspecific cellular damage by extracellular actions (Blum et al., 2000; Hanrott et al., 2006). In addition, the 6-OHDA-induced degeneration of DA neurons seems to be accompanied by neuroinflammation and microglial activation (Cicchetti et al., 2002; Depino et al., 2003; Maia et al., 2012). The actual role of microglial activation in the 6-OHDA model is still debated. Some studies suggest that it constitutes an important mechanism of action along with the production of ROS and inhibition of mitochondrial function (Rodriguez-Pallares et al., 2007; Marinova-Mutafchieva et al., 2009; Pabon et al., 2011). Our results show, that there was a trend towards a 6-OHDA dose-dependent increase in both the total amount of Iba1-stained microglia as well as in the amount of microglia with morphology indicative of activation one week post-lesion. The dose-dependency would suggest that 6-OHDA partly acts through microglial activation, but the result could also merely be due to the more extensive tissue damage caused by increasing amounts of 6-OHDA.

Microglial activation and subsequent neuroinflammation has been shown to cause BBB disruption (Lynch et al., 2004), and 6-OHDA has earlier been reported to induce an increase in BBB permeability (Carvey et al., 2005). However, we found no significant differences in the amount of EBA-expressing blood vessels in the striatum between the lesion groups one week post-lesion. In addition, the amount of EBA-reactive blood vessels in the ipsilateral side did not differ from that measured in the contralateral hemisphere. In the contralateral side there was a loss of EBA-reactive blood vessel together with a clear microglial response surrounding the FG injection tract (injection made two weeks prior to staining) indicating that the mechanical damage and/or possible FG toxicity at the site of injection is enough to induce clearly visible and measurable changes in both parameters.
6.1.5 ANIMAL MODELS OF PARKINSON’S DISEASE: PROBLEMS AND ALTERNATIVES

The toxic mechanisms of 6-OHDA, i.e., the increase in oxidative stress due to increase in ROS and mitochondrial dysfunction, as well as (primary or secondary) microglial activation (Duty and Jenner, 2011), are mechanisms also implicated in the pathogenesis of clinical PD (Figure 2) (Bossy-Wetzel et al., 2004). Moreover, the 6-OHDA-induced physiological changes, including DA cell loss, decrease in striatal DA and TH levels, dysfunction in the basal ganglia signaling, and alterations in levels of striatal transmitters other than DA (encephalin, substance P, dynorphin, ACh), are neurochemical features also present in PD (Schwarting and Huston, 1996). In addition, the rat 6-OHDA model has successfully been used in the search for novel symptomatic agents and shows responsiveness to L-DOPA, to a wide range of DA agonists, to MAO-B inhibitors, and to some anticholinergic drugs (Duty and Jenner, 2011). Taken together, this suggests that the rat 6-OHDA model is characterized by construct validity, face validity, and predictive validity, which would support the use of this model in pre-clinical PD research.

However, the 6-OHDA model is associated with some concerns. First, toxin models of neurodegenerative diseases cause acute cell death. This is very different from the clinical situation in which the degeneration generally is an ongoing progress that takes years to develop with initial pathological processes suggested to start in some cases already early in life (Braak and Del Tredici, 2012). Even though the partial 6-OHDA lesion model is characterized by some progressiveness, the same degree of cell loss that is seen only after more than a decade of PD symptoms was in our hands completed already eight weeks after the striatal 6-OHDA injection. Second, Lewy bodies, representing a pathological hallmark in PD, are absent in the 6-OHDA model (Duty and Jenner, 2011). In contrast to PD, there are also no pathological changes in other brain areas. One could therefore argue that the model is merely a model of DA neuron degeneration, and not actually a model of PD. Finally, the unilaterality of the model may induce compensatory mechanisms in the contralateral side, affecting the outcome of the studies (Zigmond et al., 1990; Schwarting and Huston, 1996).

In an attempt to overcome the problems associated with toxin-induced models, known familiar genetic mutations have been used to create transgenic animal models of neurodegenerative diseases. However, these models have generally been rather disappointing showing no or very mild histopathological and/or behavioral changes. How come a genetic alteration with clear connection to a certain neurodegenerative disease does not create disease-specific changes in the animal? The reasons could be many, including species-specific functions of the gene, age-dependent processes, and other, yet unidentified, genes or secondary mechanisms needed to initiate the neurodegenerative process. As for toxin models, there seems to be rather big variations in the model outcome between different laboratories reflecting differences in, e.g., level of transgene expression and type of promoter used (for transgenic PD models, see review by Crabtree and Zhang, 2012). In addition, genetic alterations induced in the embryonic stage may be associated with compensatory mechanisms that serve to make up for the genetic deficit. To tackle this, the genetic alterations could be induced in adult animals. For example, in the AAV-α-
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syn model, overexpression of α-syn in the adult rat SN results in intracellular aggregates of α-syn combined with progressive loss of nigral DA neurons of slower magnitude than the partial 6-OHDA model. In addition to providing important information about α-syn-induced DA neuron dysfunction in vivo, the PD-like features of the model may be of importance in drug discovery research.

The usefulness of current animal models in the evaluation of neuroprotective and -restorative agents still needs to be proved, since numerous disappointing clinical trials show that positive treatment effects in animal disease models do not necessarily translate into the clinical situation. In addition to species-specific characteristics that can affect the outcome, the pathways of neuron loss in neurodegenerative diseases are still not completely understood. Moreover, the high degree of heterogeneity, reflecting the multitude of underlying pathogenic genetic and molecular interactions (Figure 2), makes these diseases very difficult to model. Often the model mimics only one distinct characteristic of the disease (e.g., Aβ toxicity in transgenic AD models, SOD1 in transgenic ALS models) while overlooking other features. The evaluation of these models is also often challenging, and the question is whether we are looking at the appropriate features and if measurements used are actually relevant to the clinical disease. Still adding to the disease complexity, but also providing therapeutic opportunities, there seems to be an overlap between different neurodegenerative diseases (e.g., ALS and frontotemporal dementia, ataxia, and PD (Al-Chalabi et al., 2012), that suggests the existence of susceptibility genes and neurodegenerative mechanisms in common for the diseases.

6.2 NEUROTROPHIC POTENTIAL OF LONG-TERM DELIVERY OF CDNF

6.2.1 CDNF PROVIDES PROTECTION IN THE RAT PARTIAL 6-OHDA LESION MODEL

Earlier studies show, that MANF and CDNF provide protection of the nigrostriatal DAergic system when given as single protein injections in the rat partial 6-OHDA and mouse MPTP models of PD (Lindholm et al., 2007; Voutilainen et al., 2009; Airavaara et al., 2012). To evaluate the in vivo effect of long-term delivery of NTFs on the rat midbrain DAergic pathway, rats lesioned with 2x10 µg 6-OHDA were treated with NTFs given as protein infusions or gene therapy. While vehicle-treated rats showed a strong amphetamine-induced ipsilateral turning behavior throughout the studies, CDNF was able to normalize the rotational bias. The significant decrease in rotations in the CDNF-treated groups was obviously due to the CDNF treatment, since the lowest CDNF dose (1.5 µg / 24h) had no effect on the rotational behavior. Moreover, in agreement with the very low levels of striatal CDNF in rats injected with AAV2-CDNF 4x10^7 vg (III), this titer did not affect the 6-OHDA-induced rotational bias. In addition to the lowest infusion dose of CDNF, neither the highest dose (4.5 µg / 24h) altered the behavior. Thus, the effect of CDNF in this model seems to be characterized by a U-shaped dose-response curve. The same type of non-linear dose-response curve has previously also been reported for GDNF (Hou et al., 1996; Shults
et al., 1996) and MANF (Voutilainen et al., 2009). The U-shaped dose-response curve for
CDNF was further translated to the TH-reactive cells and fibers analyses where the CDNF
dose 3.0 µg / 24h provided the strongest neuroprotection.

In line with the reduction in ipsilateral rotations, there were signs of sprouting together
with a significant increase in TH-reactive fiber density in the striatum following infusion of
CDNF protein. Supported by earlier observations (Kirik et al., 2000b; Kordower et al., 2000;
Georgievskia et al., 2002a; Georgievskia et al., 2002b), sprouting of TH-reactive fibers not
only in the striatum, but also in the GPe and SNpr, was also detected following AAV-GDNF
delivery. However, although treatment with AAV2-CDNF led to an almost significant
increase in TH-fiber density together with behavioral improvement, no sign of sprouting
around the injection site was observed. The increase in fiber density due to AAV2-CDNF
treatment was accompanied with a minor increase in striatal DA level as measured two
weeks post-lesion (four weeks after viral vector injection). Although this increase was very
small (approximately 42% as compared to the negative control treatment and 5% of the DA
levels in the intact striatum), this may still be the explanation for the observed reduction in
amphetamine-induced turning asymmetry (Rioux et al., 1991).

6.2.2 NEUROTROPHIC MECHANISMS OF CDNF
The function of CDNF is still not known, but based on structural analyses it is hypothesized
that the protein may be bifunctional. Thus, both interaction with lipids (the N-terminal
domain, mediating, e.g., interaction with transmembrane proteins) and reduction of ER
stress and blocking apoptosis (the C-terminal domain) could be involved (Parkash et al.,
2009; Hellman et al., 2011; Cheng et al., 2013). If this holds true, it can be speculated that
sprouting can be seen only after infusion of CDNF protein, reflecting N-terminal domain
effect, while intracellular expression of CDNF after AAV2-CDNF reflects mainly C-terminal
domain effect. The CDNF signal in immunohistochemically stained AAV2-CDNF-injected
striata was mainly located intracellularly. When intrastriatally injected, AAV2 is known to
transduce striatal GABAergic neurons that project to the SNpr (Paterna et al., 2004). These
neurons are part of the extrapyramidal system controlling locomotor activity. It would be
tempting to propose that expression of CDNF alters the function of GABAergic neurons
normalizing the lesioned nigrostriatal circuitry without acting directly on the DA neurons.
However, this suggested mechanism does not explain the restoration of the DA phenotype
seen in the protein infusion study, in which the treatment was initiated not until two weeks
post-lesion.

Alternatively, based on the results showing that MANF secretion is increased in response to
ER stress (Apostolou et al., 2008; Tadimalla et al., 2008; Glembotski et al., 2012), could also
6-OHDA induce a transient increase in CDNF secretion from AAV2-CDNF-transduced striatal
neurons? And could this boost of secreted CDNF be responsible for the partial
neuroprotection? In our study, the AAV2 vector was delivered two weeks pre-lesion. At the
time of the 6-OHDA injection, the viral vector-induced CDNF expression had not yet
reached its maximum level and the prevailing total amount of CDNF in the rat striatum was
approximately 0.54 ng (0.04 ng/mg tissue wet weight). Compared to the earlier studies,
showing that 10 µg CDNF gives optimal neuroprotection (Lindholm et al., 2007), the 20,000 times smaller dose at the time of the 6-OHDA injection could explain the modest results. However, the same amount of GDNF (0.04 ng/mg tissue) in the putamen of AAV-GDNF-injected marmosets did provide significant protection (both behavioral and morphological) from 6-OHDA-induced DAergic degeneration (Eslamboli et al., 2005). In addition to a hypothetical direct effect on the DAergic neurons, CDNF was in a recent study suggested to act by suppressing neuroinflammation (Zhao et al., 2013). This theory still needs to be verified, but regarding the accompanying neuroinflammation in 6-OHDA-treated rats, this could be of importance for the neuroprotective effect of CDNF in this model.

Further clues to the mechanisms of action of MANF and CDNF could be obtained from studying the intracerebral transportation profiles of the NTFs. While MANF has been shown to favor transportation to cortical areas (Voutilainen et al., 2009), we found that 24 h after a striatal injection of $^{125}$I-CDNF, the signal from $^{125}$I was detected not only at the injection site, but also in the frontal cortex, hippocampus and SN. Importantly, the transport of CDNF to SN could be blocked in a dose-dependent fashion by adding increasing levels of unlabeled CDNF. However, compared to GDNF, many-fold higher excess of unlabeled CDNF was needed to block the CDNF signal from the SN. Reasons for this could, e.g., be a substantially higher dissociation constant and/or higher abundance of a hypothetical pathway for CDNF transportation. In the studies of CDNF transportation using radiolabeled NTF, the whole SN was analyzed without separation of the SNpc and SNpr. The same is true for the CDNF-ELISA analysis that detected CDNF in the SN following striatal AAV2-CDNF injection. It is therefore impossible to say whether the nigral CDNF originated mainly from retrograde or anterograde transport. The presence of CDNF in the SNpc following intrastratial delivery with our AAV2-CDNF vector was confirmed with immunohistochemistry, favoring a retrograde transportation mechanism. However, it is still unclear if the nigral CDNF-reactivity was due to retrograde transport of striatally expressed CDNF, or whether in fact the signal was due to transportation of the AAV2 vector (Paterna et al., 2004).

6.2.3 CDNF AND MANF VERSUS GDNF

Treatment with CDNF showed similar neuroprotective potential as GDNF in both the protein infusion and gene therapy studies. The most striking difference between the NTFs was seen in the gene therapy experiment, where GDNF caused aberrant sprouting of TH-reactive fibers in the basal ganglia while this phenomenon was absent in the AAV2-CDNF-treated animals. In addition, protein infusion of GDNF for two weeks into the intact rat striatum resulted in an approximately 21% decrease in TH-reactive neurons in the SN. Instead, CDNF protein infusion caused an approximately 12% increase in cell numbers. GDNF has previously been reported to induce TH down-regulation in intact rat brain (Rosenblad et al., 2003; Salvatore et al., 2004; Salvatore et al., 2009) and our results suggest that this is not a property of CDNF. This is in agreement with a recent study, showing that AAV vector-mediated striatal overexpression of CDNF did not alter the striatal TH levels (Ren et al., 2013). In addition, in our own gene therapy study we found no CDNF-induced differences in the striatal DA levels in intact rat brain four weeks after an AAV-
CDNF injection. On the contrary, GDNF has been reported to cause an increase in tissue DA and DA turnover (Hudson et al., 1995; Beck et al., 1996; Salvatore et al., 2004).

Protein infusion of MANF did not improve the rotation asymmetry nor did it increase the cell survival in the SN. This was surprising since single injections of MANF were earlier shown to promote recovery of 6-OHDA-lesioned rats both when given six hours before and two weeks after the 6-OHDA injection (Voutilainen et al., 2009). In the previous studies, MANF was given into the same site as 6-OHDA. This differs from the present study design in which the protein was infused between the 6-OHDA injection sites. One could speculate that this difference in setup was the reason for lack of effect. However, when the diffusion of the NTFs was measured after a three-day infusion, MANF showed extensive spreading throughout the striatal tissue with a significantly larger diffusion volume than GDNF. After a two-week infusion, no differences in diffusion volumes between MANF, CDNF, and GDNF were observed. It seems, though, that the spreading of MANF reaches its steady-state faster than GDNF. This may be due to differences in biochemical properties, such as isoelectric point and interactions with components of the extracellular matrix, e.g., GDNF binding to heparan sulphates (Piltonen et al., 2009). The difference between diffusion volumes could also merely be a technical issue and reflect properties of the antibodies used. Because of the seemingly effective spreading of MANF, it appears unlikely that the injection site would be the obstacle in our study. An alternative explanation for the lack of effect of MANF could simply be the obvious difference in lesion severity in the vehicle-treated group between the two studies (MANF versus CDNF), which may mask the effect of MANF.

GDNF did not provide statistically significant protection or restoration of nigral TH-reactive cells in neither the protein infusion nor the gene therapy study, even though there was an increase in surviving cells as compared to the vehicle-treated rats (56% versus 25% in the protein infusion study and 45% versus 27% in gene therapy study) (Table 10). Our results from the protein infusion study are in line with results from Kirik and colleagues (2001), who showed that a similar GDNF infusion gave very minor preservation of nigral cells and only transient functional improvement in the rat partial 6-OHDA model. The lack of significant effect of GDNF gene therapy is, however, in contrast to earlier studies, in which the treatment has generally led to significant neuroprotection (Bilang-Bleuel et al., 1997; Choi-Lundberg et al., 1998; Kirik et al., 2000b; Georgievsk et al., 2002b). This lack of significant effect on the nigral DA cell level is probably attributed to the rather low expression levels after a single injection of our AAV2-GDNF vector.

Overall, these results propose that the neuroprotective mechanism of CDNF is different from that of GDNF. However, the possible mechanisms discussed above are only speculations and no further conclusions can be made until the actions of CDNF and MANF on neuronal survival and neurotransmission have been studied in more detail. The neuron selectivity of the neurotrophic effect of CDNF and MANF is also still unsolved. We found a normalization of the striatal serotonin levels in 6-OHDA-lesioned rats after AAV-CDNF treatment, which would indicate that, in addition to the dopaminergic pathway, CDNF also

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Discussion
affects the serotonergic system. This could be of importance for the treatment of PD, since also the serotonergic neurons are subjected to degeneration in the disease.

Table 10 Summary of the neuroprotective effects of GDNF and CDNF on nigrostriatal tyrosine hydroylase (TH) -reactive dopaminergic neurons in the rat partial 6-hydroxydopamine (6-OHDA) model. The neurotrophic factors were given as protein infusions or gene therapy and their effects were compared to the negative control (denoted vehicle (PBS) or control (AAV-GFP or PBS)). The results are for protein infusion doses of 3 µg/24 h and AAV2 vector titers of 1x10^9 vg and represent the mean ± SEM for ipsilateral values as percentage of contralateral values.

<table>
<thead>
<tr>
<th>Protein infusion</th>
<th>Gene therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEHICLE</td>
<td>GDNF</td>
</tr>
<tr>
<td>TH-reactive cells (SNpc)</td>
<td>25.1 ± 3.5</td>
</tr>
<tr>
<td>TH-reactive fiber density (striatum)</td>
<td>26.7 ± 3.6</td>
</tr>
</tbody>
</table>

AAV, adeno-associated virus; CDNF, cerebral dopamine neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; GFP, green fluorescent protein; PBS, phosphate-buffered saline; SNpc, substantia nigra pars compacta. *P<0.05, **P<0.01, one-way ANOVA and Tukey post-hoc test.

6.2.4 GENE THERAPY VERSUS INFUSION

When comparing the neuroprotective potential of the two different ways of administration, CDNF protein infusion seems to be superior to delivery of CDNF with an AAV2 vector (67% versus 46% of TH-reactive cells remaining, and 56% versus 37% of TH-reactive fiber density preserved) (Table 10). This was surprising, since the gene therapy was initiated already two weeks before the 6-OHDA injections, while the protein infusion was not started until two weeks post-lesion. This could be explained by the rather limited transduction of the striatal neurons with subsequent low levels of CDNF together with the seemingly poor secretion of CDNF from the transduced cells. The spreading and transduction capacity of the AAV2 vector could be improved by increasing the titer, by doing multiple simultaneous injections (Kirik et al., 2000b) or by changing the AAV serotype (Burger et al., 2004; Asokan et al., 2012).

In a recent study (Ren et al., 2013), injection of AAV-CDNF into the rat striatum six weeks after a striatal injection of 18 µg 6-OHDA mediated substantial restoration of the nigrostriatal DAergic system, with significant increase in DA cell survival, TH and DAT expression, as well as striatal DAT binding of [11C]CFT. These changes were accompanied by an almost complete reduction of amphetamine-induced rotations up to 54 weeks post-lesion (Ren et al., 2013). In this study, the same AAV serotype (serotype 2) and promoter (CMV) as in our study was used. Compared to our study, the total titer injected per animal was only approximately two to three times bigger and divided into two injections 1 mm apart in the D/V direction. Serotype 2 AAV was also used for delivery of NTN in the rat striatum, and in the 6-OHDA model an approximately six times smaller titer (as compared
to the highest titer used in our study) of this vector was enough to provide significant protection of the DAergic system (Gasmì et al., 2007a). A four-time higher titer of this AAV2-NTN vector led to an approximately ten-fold higher protein expression in the striatum, as compared to titers and protein levels measured by us (Gasmì et al., 2007b). The serotype of our AAV2-CDNF vector is therefore probably not the main cause of the minor improvements seen in our study, even though there are clear indications that alternative serotypes would provide better transduction efficacy and spread than serotype 2 AAV (Burger et al., 2004; McFarland et al., 2009; Asokan et al., 2012). The low transduction and expression could also be due to other viral vector properties. One potential technical problem is that the estimated titer used may have differed from the actual titer. The different titers were prepared by dilution of the viral stock solution with PBS. Since AAV vectors have a tendency to aggregate (Huang et al., 2000; Wright et al., 2003), this can result in titers that are not directly related to the dilution coefficient. To overcome this problem in the future, the titers of the viral vector solutions should be assessed after the dilution.

No pathology or disease has been linked to AAV, and even though a large proportion of humans have been naturally infected with AAV, the immunogenicity seems to be rather low (Chtarto et al., 2013b). This makes AAV a good candidate for gene delivery in humans. In addition to pathogenicity and immunogenicity, one concern regarding all viral vectors is the uncontrolled expression of the introduced gene following viral vector transduction. Several studies in animals have pointed at potential problems with continuous delivery of high levels of NTFS (Georgievskà et al., 2002a; Zala et al., 2004; Hovland et al., 2007; Denovan-Wright et al., 2008; Kells et al., 2008). One way to try to solve this problem is by regulation of transgene expression with inducible (or repressible) promoters together with transactivators able to interact with the promoter and an inducer (or repressor) (Chtarto et al., 2013b). Tetracyclin-responsive elements are the most widely used system for drug-dependent regulation of viral vectors. However, continuous high doses of antibiotics are associated with adverse effects (e.g., phototoxicity, accumulation in bone, disturbances in bowel flora) and risk for selection of tetracyclin-resistant bacteria, which limits the clinical use of these vectors. In the search for more clinically relevant regulated vectors, Chtarto and co-workers (Chtarto et al., 2013a) reported the use of a recombinant AAV vector regulated by inflammation-induced increase in NF-κB activation in the brain. This type of disease-dependent expression opens up an exciting possibility to express the transgene specifically in diseased cells.

6.2.5 NOVEL THERAPEUTIC STRATEGIES FOR NEURODEGENERATIVE DISORDERS: GENERAL DISCUSSION

In my thesis I have reviewed novel therapeutic interventions that take advantage of gene therapy in the attempt to provide symptomatic improvements or even a cure for neurodegenerative diseases such as PD, AD, HD, and ALS. Although not covered here, in addition to gene therapy approaches, transplantation of cells in an attempt to replace degenerating neuron populations constitutes another exciting and promising novel
treatment alternative under development (Lunn et al., 2011). The gene therapy strategies, divided in the literature review into symptomatic therapy, elimination of pathogenic protein, and addition of therapeutic protein (Figure 7), have all different characteristics and are each associated with certain advantages and problems.

The symptomatic gene therapy, e.g., the delivery of enzymes or proteins needed for neurotransmitter production and function, is disease-specific and serves to enhance certain signaling pathways in the diseased brain. The effect should be seen soon after treatment initiation, but as the disease progresses, the effect may diminish. Potential additional problems associated with this treatment alternative is the unregulated synthesis, storage and release of neurotransmitter due to transduction of cells not involved in the endogenous signaling pathway of the transmitter in question.

The second therapeutic strategy, the elimination of pathogenic protein, can be considered as a potentially curative treatment alternative. However, even if this strategy may theoretically have the ability to halt the progression of the degeneration, it is probably not enough alone to reverse the pathology and induce restoration of the impaired neuronal signaling pathways. In addition, the excessive reduction of proteins that may be important for the normal cell function could be harmful, as seen in the silencing of α-syn in the rat SN (discussed in 2.4.2). This could be avoided by specific silencing of only the mutated allele (e.g., SOD1 in ALS) or by targeting of protein degradation instead of expression. There is also an ongoing discussion about the importance of the protein aggregates seen in PD, AD, HD, and ALS. It is still debated whether the aggregates cause the cell damage, or if they are results of the cells’ attempt to protect themselves from toxic misfolded protein. The toxicity could also be mediated by sequestration of other proteins or genetic material important to the cell into the aggregates. These issues need to be clarified before this treatment strategy can be aimed at the right target in the process.

The third strategy, the use of NTFs, could be considered as an unspecific and indirect approach offering in some cases similar survival-promoting and regenerative effects in different neurodegenerative disorders (e.g., GDNF in PD, HD, and ALS). Treatment with NTFs can be labeled as disease-modifying with the potential to halt or even reverse the neurodegeneration. The possible problems with this treatment alternative are, e.g., unwanted effects due to off-site administration or abnormal changes in protein expression caused by high levels of NTF. In addition, it would be important to re-establish meaningful connections instead of just inducing uncontrolled axonal growth/regeneration following NTF delivery. NTFs are also subjected to anterograde and/or retrograde transport, bringing additional challenges to the delivery of NTFs.

Even if the third strategy hypothetically could by itself be enough to reverse and treat neurodegenerative diseases, the subsequent use of all three alternatives appears as the optimal way of treating these patients. While giving fast symptomatic relief with an enzyme replacement therapy, the underlying pathogenic process of protein misfolding/aggregation could be stopped by, e.g., RNA interference, and at the same time the degenerating neurons could be stimulated by NTFs to boost survival and restoration of the impaired
neuronal circuits. Especially for the two latter strategies, timing is most probably of great importance. When does neuronal dysfunction turn into neuronal cell death, and at what time point is the disease still reversible? In PD, even if 70-80% of the striatal DA connections are lost by the time of symptom onset, the actual nigral cell loss is much less (30%), providing an opportunity to rescue and revitalize the remaining cells if neuroprotective or -restorative treatments were available. With the aim of starting treatments as soon as possible, three very exciting ongoing trials are testing the potential of anti-amyloid treatments in people that have not yet developed clinical symptoms of AD (Miller, 2012). Two of these trials (Alzheimer’s Prevention Initiative (API) and Dominantly Inherited Alzheimer Network (DIAN)) focus on patients with mutations associated with familial early-onset AD. The third trial (Anti-Amyloid Treatment of Asymptomatic Alzheimer’s (A4)) seeks to start anti-amyloid therapy in healthy individuals who show developing amyloid plaques in brain scans. The results from these trials will be of great importance for determining the future direction of neurodegenerative disease research.
7 CONCLUSIONS

The main objective for this work was the assessment of the neuroprotective potential of long-term delivery of CDNF in the rat partial 6-OHDA model of PD. Generally, the results show that CDNF, administered as protein infusions or gene therapy, provides protection of the nigrostriatal DAergic system both on a functional and a histological level.

The following principle findings have been presented in this thesis:

1) The partial lesion model used in the neuroprotection studies (2x10 µg 6-OHDA) show more beneficial characteristics than injections of 6-OHDA into a single site. The cell loss was progressive during the study (eight weeks) with a magnitude at two weeks post-lesion corresponding to the cell loss seen in PD at symptom onset. In addition, the 2x10 µg 6-OHDA lesion showed response in the two spontaneous motor behavior tests used. Evaluation of the dopaminergic system in partially 6-OHDA-lesioned rats with $^{[123]}$I-β-CIT SPECT/CT showed high correlation to immunohistochemical findings suggesting that the method could reliably be used for in vivo detection of lesion progression and treatment effects.

2) Using a restoration paradigm, two-week protein infusions of CDNF 3 µg/24 h attenuated the amphetamine-induced ipsilateral rotational behavior. The treatment was accompanied with significant protection of the midbrain DAergic cells with signs of sprouting of TH-reactive fibers at the site of infusion. Treatment with MANF did not provide restoration of the midbrain DAergic system.

3) While CDNF gene therapy normalized the amphetamine-induced rotational bias in 6-OHDA-lesioned rats, the effect on DA neuron survival was rather modest. In addition, the small and non-significant increase in striatal DA fiber density seen in the CDNF-treated striatum was not accompanied with signs of sprouting. Results from the CDNF protein infusion and gene therapy studies suggest a retrograde transport system for CDNF. Delivery of CDNF did however not induce changes in the intact rat DAergic system.

These results confirm the therapeutic potential of CDNF in PD. In the rat 6-OHDA model, the effect of intrastriatal CDNF protein infusion seems to be more beneficial than that of CDNF gene therapy. This may be due to differences in extra- versus intracellular effects of CDNF, or caused by low expression levels of CDNF following AAV vector-mediated delivery.
8 ACKNOWLEDGEMENTS

This work was done at the Division of Pharmacology and Toxicology (now Division of Pharmacology and Pharmacotherapy), Faculty of Pharmacy, University of Helsinki during the years 2008 to 2014.

The work was supported by the Academy of Finland (Era-Net Neuron), TEKES, and Michael J. Fox Foundation for Parkinson’s Research. Personal financial support from the following foundations and societies is gratefully acknowledged: The Finnish Parkinson Foundation, The Finnish Cultural Foundation, Svensk-Österbottniska Samfundet, Finnish Pharmaceutical Society, Oscar Öflund Foundation, and The Finnish Pharmacological Society.

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

Professor Raimo K. Tuominen, the Head of the Division of Pharmacology and Pharmacotherapy, for all the guidance and support during these years. Your profound pharmacological knowledge, guidance in scientific writing and thinking, and optimism have been invaluable for the completion of this work.

Professor emeritus Pekka T. Männistö, for all the advices and encouragement. Your expertise in neuropharmacology and experience in scientific writing have been of great importance, and I am grateful for having had the opportunity to work under your supervision.

Professor Atso Raasmaja, for guidance in both technical and scientific issues. I sincerely appreciate your enthusiasm for science and that you have always had time for discussions. I also wish to thank you for valuable comments and advices during the process of writing this thesis.

The reviewers of my thesis, Professor Seppo Kaakkola and Docent Pekka Rauhala, for constructive comments and suggestions that substantially improved the thesis manuscript. Docent Markus Forsberg, PhD Vootele Võikar, and PhD Ilkka Reenilä are sincerely acknowledged for their valuable remarks on my research plan.

MD, PhD Barry Hoffer, for agreeing to act as my opponent in the public defense of this thesis.

My co-authors, especially PhD Merja Voutilainen, who introduced me to the division and to the research area of neurotrophic factors and Parkinson’s disease. I have learned so much from you and it has been a pleasure to work together with you. I am also very grateful for the close collaboration with Professor Mart Saarma, PhD Johan Peränen, PhD Päivi Pulkkila, MSc Emilia Galli, and PhD Liina Lonka-Nevalaita at the Institute of Biotechnology, University
of Helsinki. From the former Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, PhD Kim Bergström and PhD Mari Raki are gratefully acknowledged for their expertise in in vivo SPECT/CT imaging.

The present and former technicians in the division, Anna Niemi, Liisa Lappalainen, and Marjo Vaha, for excellent technical assistance. Especially I would like to thank Kati Rautio whose help has been invaluable, and without whom this work would have taken another year. I also wish to thank Docent Mikko Airavaara for valuable advices and instructive collaboration, as well as for reviewing and commenting on my thesis manuscript. Docent Petteri Piepponen is sincerely acknowledged for his guidance concerning statistical analyses.

The Master thesis students who have been involved in this work: MSc Helinä Minkkinen, MSc Juho-Matti Renko, MSc Tuulia Tamminen, and MSc Johanna Toivonen. Your contributions are gratefully acknowledged.

All my present and former colleagues. There are so many of you that I would like to acknowledge by name and that not only have been my colleagues during these years, but that also have become my friends. Especially I would like to thank lida Peltonen, Marjo Piltonen, Nadia Schendzielorz, Milla Summanen, Reeta Talka, Virpi Talman, Timo Myöhänen, and Bernardino Ossola for all scientific and not-so-scientific discussions, lunches, after-works, parties, and dinners. Thanks to you, it has been a great pleasure to go to work every morning.

My dear friends outside the university, Lena Frost, Anna Mügge, and Ann-Mari Snickars, for all rememberable moments we have shared. Even though we are nowadays spread across three countries, when we meet, it always feels like we have never been apart. And to Kenneth, for your love and encouragement and for all the fun we have together. Thanks to you I have realized that there is actually a life outside the lab.

Finally, I owe my deepest gratitude to my dear parents Helena and Rainer, for all your support throughout the seemingly endless years of education, and for raising me with the belief that nothing is impossible or undoable. And to my sister Anette with family (Jonas, Ida, and “lillasyster”) and my brother Roger for all the good times spent together.

Helsinki, January 2014

Susanne
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